Universidade Estadual de Londrina

THALITA EVANI SILVA DE OLIVEIRA

DOENÇA RESPIRATÓRIA BOVINA NO BRASIL: PADRÕES HISTOPATOLÓGICOS E IDENTIFICAÇÃO IMUNO-HISTOQUÍMICA DE AGENTES INFECCIOSOS

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Orientador: Prof. Dr. Selwyn Arlington Headley

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BANCA EXAMINADORA

Prof. Orientador: Prof. Dr. Selwyn Arlington Headley
Universidade Estadual de Londrina – UEL

Pro Dr. Amauri Alcindo Alfieri
Universidade Estadual de Londrina – UEL

Prof. Dr. Eduardo Furtado Flores
Universidade Federal de Santa Maria – UFSM

Prof. Dr. Júlio Augusto Naylor Lisbôa
Universidade Estadual de Londrina – UEL

Prof. Dr. Paulo Henrique Jorge da Cunha
Universidade Federal de Goiás – UFG

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OLIVEIRA, Thalita Evani Silva. **Doença respiratória bovina no Brasil: padrões histopatológicos e identificação imuno-histoquímica de agentes infecciosos**. 2022. 175f. Tese (Doutorado em Ciência Animal) – Universidade Estadual de Londrina, Londrina, 2023.

RESUMO

Complexo de doenças respiratórias dos bovinos (DRB) está associado como uma das principais causas de mortes em bovinos confinados no mundo. Todavia, os aspectos histopatológicos e imuno-histoquímicos (IHQ) associados ao diagnóstico da DRB são pouco descritos. Dessa forma, nesta tese estão incluídos quatro artigos abordando esse tema. O primeiro estudo foi uma breve revisão que aborda dados epidemiológicos, agentes infecciosos e as manifestações clínicas e patológicas associadas à DRB. Esta é uma enfermidade multifatorial e multietiológica descrita em todas as regiões do Brasil, relacionada ao alfaherpesvírus bovino tipo 1 (BoAHV1), vírus da diarreia viral bovina (BVDV), vírus respiratório sincicial bovino (BRSV) e *Mycoplasma bovi*s (MB), entre outros agentes. Gammaherpesvírus-ovino 2 (OvGHV2) e HoBi-like pestivírus foram associados ao desenvolvimento de pneumonia em bovinos adultos e bezerros. O segundo artigo descreveu os padrões histopatológicos e achados de IHQ associados aos agentes MB, BoAHV1, vírus da parainfluenza tipo 3 (BPIV-3), BRSV e BVDV nos pulmões de 35 vacas holandesas, do estado do Paraná. Pneumonia foi diagnosticada em 91,4% das vacas (32/35), sendo pneumonia intersticial (*n*=15), broncopneumonia necrosupurativa (*n*=9) e broncopneumonia supurativa (*n*=6). Dentre os agentes, constatou-se BVDV (*n*=18), MB (*n*=16), BoAHV1 (*n*=14) e BRSV (*n*=11). O terceiro artigo descreveu a associação de um vírus do grupo do vírus da febre catarral maligna (MCFV) e MB, com desenvolvimento da DRB em 145 bovinos de corte e leite, bezerros e adultos, dos estados do Paraná, Goiás, São Paulo e Minas Gerais. Os principais padrões de lesão pulmonar foram pneumonia intersticial (72/120;60%) e broncopneumonia supurativa (31/120;25,8%). Antígenos intralesionais de MCFV (64/120) foram os mais frequentes, seguidos por MB (57/120), BVDV (51/120), BoAHV1 (34/120), BRSV (29/120) e BPIV-3 (10/120). Sobre os antígenos de MCFV identificados acredita-se que sejam do OvHV-2, pois é o único MCFV descrito no Brasil até o presente O quarto artigo descreve a presença de agentes infecciosos associados com a DRB em 37 pulmões de fetos bovinos abortados, dos estados do Paraná e Minas Gerais, para determinar se esses patógenos estavam associados à pneumonia e/ou outras alterações pulmonares. IHQ foi realizada para os mesmos agentes descrito anteriormente. Para o painel de doenças reprodutivas, PCR foram realizadas para BoAHV1, BVDV, *Listeria monocytogenes*, *Histophilus somni*, *Neospora caninum*, *Leptospira* spp., *Brucella abortus* e gammaherpesvirus bovino 6 (BoGHV6). Pneumonia intersticial foi observada em 29,7% dos fetos (11/37) e esteve associada a antígenos virais BRSV, BoAHV1 (*n*=3,cada), BVDV e DNA BoGHV6 (*n*=2,cada). Dentre os patógenos reprodutivos detectou-se *Leptospira* spp. (*n*=3), BVDV, *Neospora caninum* e *Brucella abortus* (*n*=2, cada). Abortos não relacionados aos agentes infecciosos testados foram observados em 59,5% (22/37) dos pulmões examinados. Conclui-se com esses estudos que a imunorreatividade positiva dos anticorpos utilizados são intracitoplasmáticas. Pneumonia intersticial e broncopneumonias supurativa e

necrosupurativa foram os principais tipos de pneumonia no Brasil. BPIV-3 demonstrou baixa ocorrência nestes rebanhos e quando presente esteve associado a outro agente infeccioso. Finalmente, a amplificação de ácidos nucléicos do BoGHV6, BRSV e BPIV-3 dos pulmões fetais com pneumonia intersticial, sugere que esses patógenos podem ser considerados como potenciais agentes de fetopatia em bovinos, derivada de infecção intrauterina/transplacentária.

Palavras-chave: complexo respiratório bovino. Diagnósticos imuno-histoquímicos. Pneumonia. Patógenos respiratórios bovinos.

OLIVEIRA, Thalita Evani Silva. **Bovine respiratory disease in Brazil: histopathological patterns and immunohistochemical identification of infectious agents**. 2022. 175p. Thesis (Doctorate degree in Animal Health Science) – Universidade Estadual de Londrina, Londrina, 2023.

ABSTRACT

Bovine respiratory disease complex (BRD) is associated as one of the main causes of death in confined cattle in the world. However, the histopathological and immunohistochemical (IHC) aspects associated with the diagnosis of BRD are rarely described. Therefore, this thesis includes four articles addressing this topic. The first paper was a brief review that addresses epidemiological data, infectious agents and the clinical and pathological manifestations associated with BRD. This is a multifactorial and multietiological disease described in all regions of Brazil, associated to bovine alphaherpesvirus type 1 (BoAHV1), bovine viral diarrhea virus (BVDV), bovine respiratory syncytial virus (BRSV) and *Mycoplasma bovis* (MB), among other agents. Ovine gammaherpesvirus type 2 (OvGHV2) and HoBi-like pestiviruses have been associated with the development of pneumonia in cattle and calves. The second article described the histopathological patterns and IHC findings associated with MB agents, BoAHV1, parainfluenza virus type 3 (BPIV-3), BRSV and BVDV in the lungs of 35 Holstein cows from the state of Paraná. Pneumonia was diagnosed in 91.4% of the cows (32/35), being interstitial pneumonia (n=15), necrosuppurative bronchopneumonia (n=9) and suppurative bronchopneumonia (n=6), associated with BVDV (n=18), MB (n=16), BoAHV1 (n=14) and BRSV (n=11). The third article described the association of a virus from Malignant Catarrhal Fever virus group (MCFV) and MB, with the development of BRD in 145 calves, beef, and dairy cattle from the states of Paraná, Goiás, São Paulo and Minas General. The main patterns of lung injury were interstitial pneumonia (72/120;60%) and suppurative bronchopneumonia (31/120;25.8%). MCFV intralesional antigens (64/120) were the most frequent, followed by MB (57/120), BVDV (51/120), BoAHV1 (34/120), BRSV (29/120) and BPIV-3 (10/120). The identified MCFV antigens are believed to be from OvHV-2, as it is the only MCFV described in Brazil to date. The fourth article describes the presence of infectious agents associated with BRD in 37 lungs of aborted bovine fetuses, from the states from Paraná and Minas Gerais, to determine whether these pathogens were associated with pneumonia and/or other pulmonary disorders. IHC was performed for the same agents described above. For the reproductive diseases panel PCR was performed for BoAHV1, BVDV, *Listeria monocytogenes, Histophilus somni, Neospora caninum, Leptospira spp., Brucella abortus* and bovine gammaherpesvirus 6 (BoGHV6). Interstitial pneumonia was observed in 29.7% of fetuses (11/37) and was associated with viral antigens BRSV, BoAHV1 (n=3,each), BVDV, and DNA BoGHV6 (n=2,each). Among the reproductive pathogens *Leptospira spp* (n=3), BVDV, *Neospora caninum* and *Brucella abortus* (n=2,each). Abortions unrelated to the infectious agents tested were observed in 59.5% (22/37) of the examined lungs. It is concluded from these studies that the positive immunoreactivity of the antibodies used are intracytoplasmic. Interstitial pneumonia and suppurative and necrosuppurative bronchopneumonia were the main types of pneumonia in Brazil. BPIV-3 showed low occurrence in these herds, and, when present, was associated

with another infectious agent. Finally, the amplification of nucleic acids of BoGHV6, BRSV and BPIV-3 from fetal lungs with interstitial pneumonia, suggests that these pathogens can be considered as potential agents of foetopathy in cattle, derived from intrauterine/transplacental infection.

Key words: bovine respiratory complex. Immunohistochemical diagnoses. Pneumonia. Bovine respiratory pathogens.

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LISTA DE ABREVIATURAS E SIGLAS

BALT	bronchus-associated lymphoid tissue hyperplasia
BoAHV1	bovine alphaherpesvirus 1
BoGHV6	bovine gammaherpesvirus 6
BRD	bovine respiratory disease
BRSV	bovine respiratory syncytial virus
BPIV-3	parainfluenza virus type 3
BVDV	bovine viral diarrhea virus
EDTA	ethylenediaminetetraacetic acid
FFPE	formalin-fixed paraffin-embedded
H&E	Hematoxylin and eosin
IHC	Immunohistochemistry
ISH	*in situ* hybridization
Mab	monoclonal antibody
M. bovis	*Mycoplasma bovis*
MCFV	malignant catarrhal fever virus
OvGHV2	ovine gammaherpesvirus 2
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
TRIS	tris (hydroxymethyl) aminomethane

SUMÁRIO

1 INTRODUÇÃO

O complexo de doenças respiratórias bovina (DRB) é uma doença cosmopolita, onerosa e comumente descrita em bovinos de leite e corte, nas Américas do Norte (FULTON et al., 2009; GRIFFIN et al., 2010; TAYLOR et al., 2010; HAY et al., 2016) e Sul (FULTON et al., 2009; GRIFFIN et al., 2010; FRANCOZ et al., 2015), Europa (FRANCOZ et al., 2015), Oceania (CUSACK et al., 2003; HAY et al., 2016), Ásia (CERIBASI et al., 2014; ÇOMAKLI et al., 2019; GUO et al., 2021) e África (OLA et al., 2020).

Dentre os principais patógenos bacterianos que danificam o epitélio respiratório bovino tem-se *Mannheimia haemolytica* (BOOKER et al., 2008; GRIFFIN et al., 2010), *Pasteurella multocida* (YAMAN et al., 2018), *Histophilus somni* (GERSHWIN et al., 2015; HEADLEY et al., 2017) e *Mycoplasma bovis* (HAINES et al., 2001; HERMEYER et al., 2012) e sua patogenicidade pode ser potencializada por infecções virais (GRIFFIN et al., 2010). Dentre os principais patógenos virais mais frequentemente associados à DRB destacam-se o vírus respiratório sincicial bovino (BRSV), alfaherpesvírus bovino 1 (BoAHV1), vírus da diarreia viral bovina (BVDV) e vírus da parainfluenza bovina 3 (BPIV-3) (FULTON, 2009; FULTON et al., 2009; FULTON, 2013).

Em 2021, o rebanho brasileiro foi estimado em 196,47 milhões (196.468.110) de cabeças (ABIEC, 2022), sendo Mato Grosso (27.800.728; 14,15%), Minas Gerais (22.261.360; 11,33%) e Mato Grosso do Sul (22.023.409; 11,21%), os estados com maior rebanho (ABIEC, 2022).

Embora o Brasil seja o segundo maior produtor de bovinos do mundo (ABIEC, 2022), as informações relacionadas ao desenvolvimento de BRD no país são escassas (BAPTISTA et al., 2017). Estimou-se que os prejuízos econômicos associados à DRB no Brasil cheguem a $14.334,00 e $16.315,40 USD/10.000 cabeças de bovinos, associadas à mortalidade e morbidade, respectivamente (BAPTISTA et al., 2017). Comparando esses custos com o produto interno bruto (PIB) brasileiro de 2017 (R$ 6,56 trilhões), a pecuária representa 6,5% (R$0,43 trilhões) do faturamento nacional, sendo 31% dos rendimentos obtidos pelo produto interno bruto (PIB) do setor agropecuário (R$1,42 trilhões) (ABIEC, 2018). Deste modo, existe a necessidade de investigar aspectos relacionados à epidemiologia, agentes infecciosos e prejuízos econômicos relacionados à DRB em bovinos de corte e de leite no Brasil.

1.1 Referências

ABIEC. Perfil da pecuária no Brasil. **Relatório anual 2018**. Associação Brasileira das Indústrias Exportadoras de Carne (ABIEC). São Paulo, SP: Brazilian Beef Exporters Association: 49p. 2018. Disponível em: https://edisciplinas.usp.br/pluginfile.php/4392168/mod_resource/content/1/Sum%C3%A1rio%20ABIEC%202017.pdf

ABIEC. Perfil da pecuária no Brasil. **Relatório anual 2022**. Associação Brasileira das Indústrias Exportadoras de Carne (ABIEC). São Paulo, SP: Brazilian Beef Exporters Association: 72 p. 2022. Disponível em: https://www.abiec.com.br/publicacoes/beef-report-2022/

BAPTISTA, A. L.; REZENDE, A. L.; FONSECA, P. D. A.; NOGUEIRA, G. M.; HEADLEY, S. A.; MENEZES, G. L.; ALFIERI, A. A.; SAUT, J. P. E. Bovine respiratory disease complex associated mortality and morbidity rates in feedlot cattle from southeastern Brazil. **Journal of Infection in Developing Countries**, v. 11, n. 10, p. 791-799, 2017.

BOOKER, C. W.; ABUTARBUSH, S. M.; MORLEY, P. S.; JIM, G. K.; PITTMAN, T. J.; SCHUNICHT, O. C.; PERRETT, T.; WILDMAN, B. K.; FENTON, R. K.; GUICHON, P. T.; JANZEN, E. D. Microbiological and histopathological findings in cases of fatal bovine respiratory disease of feedlot cattle in Western Canada. **The Canadian Veterinary Journal**, v. 49, n. 5, p. 473-481, 2008.

CERIBASI, A. O.; OZKARACA, M.; CERIBASI, S.; OZER, H. Histopathologic, immunoperoxidase and immunofluorescent examinations on natural cattle pneumonia originated from parainfluenza type 3, respiratory syncytial virus, adenovirus type 3 and herpesvirus type 1. **Revue de Médecine Vétérinaire**, v. 165, n. 1, p. 201-212, 2014.

ÇOMAKLI, S.; SAĞLAM, Y.; TIMURKAN, M. O. Comparative detection of bovine herpesvirus-1 using antigen ELISA, immunohistochemistry and immunofluorescence methods in cattle with pneumonia. **Turkish Journal of Veterinary and Animal Sciences**, v. 43, p. 314-322, 2019.

CUSACK, P.; MCMENIMAN, N.; LEAN, I. The medicine and epidemiology of bovine respiratory disease in feedlots. **Australian Veterinary Journal**, v. 81, n. 8, p. 480-487, 2003.

FRANCOZ, D.; BUCZINSKI, S.; BELANGER, A. M.; FORTE, G.; LABRECQUE, O.; TREMBLAY, D.; WELLEMANS, V.; DUBUC, J. Respiratory pathogens in Quebec dairy calves and their relationship with clinical status, lung consolidation, and average daily gain. **Journal of Veterinary Internal Medicine**, v. 29, n. 1, p. 381-387, 2015.

FULTON, R. W. Chapter 42 - Viral diseases of the bovine respiratory tract. *In*: ANDERSON, D. E. e RINGS, D. M. (Ed.). Food Animal Practice 5th. Saint Louis: W.B. Saunders. https://doi.org/10.1016/B978-141603591-6.10042-9, 2009. cap. 42, p.171-191.

FULTON, R. W. Host response to bovine viral diarrhea virus and interactions with infectious agents in the feedlot and breeding herd. **Biologicals: Journal of the**

International Association of Biological Standardization, v. 41, n. 1, p. 31-38, 2013.

FULTON, R. W.; BLOOD, K. S.; PANCIERA, R. J.; PAYTON, M. E.; RIDPATH, J. F.; CONFER, A. W.; SALIKI, J. T.; BURGE, L. T.; WELSH, R. D.; JOHNSON, B. J.; RECK, A. Lung pathology and infectious agents in fatal feedlot pneumonias and relationship with mortality, disease onset, and treatments. **Journal of Veterinary Diagnostic Investigation**, v. 21, n. 4, p. 464-477, 2009.

GERSHWIN, L. J.; VAN EENENNAAM, A. L.; ANDERSON, M. L.; MCELIGOT, H. A.; SHAO, M. X.; TOAFF-ROSENSTEIN, R.; TAYLOR, J. F.; NEIBERGS, H. L.; WOMACK, J. Single pathogen challenge with agents of the bovine respiratory disease complex. **PLoS One**, v. 10, n. 11, p. e0142479, 2015.

GRIFFIN, D.; CHENGAPPA, M. M.; KUSZAK, J.; MCVEY, D. S. Bacterial pathogens of the bovine respiratory disease complex. **The Veterinary Clinics of North America Food Animal Practice**, v. 26, n. 2, p. 381-394, 2010.

GUO, T.; ZHANG, J.; CHEN, X.; WEI, X.; WU, C.; CUI, Q.; HAO, Y. Investigation of viral pathogens in cattle with bovine respiratory disease complex in Inner Mongolia, China. **Microbial Pathogenesis**, v. 153, p. 104594, 2021.

HAINES, D. M.; MARTIN, K. M.; CLARK, E. G.; JIM, G. K.; JANZEN, E. D. The immunohistochemical detection of *Mycoplasma bovis* and bovine viral diarrhea virus in tissues of feedlot cattle with chronic, unresponsive respiratory disease and/or arthritis. **The Canadian Veterinary Journal**. 42: 857-860 p. 2001.

HAY, K. E.; MORTON, J. M.; MAHONY, T. J.; CLEMENTS, A. C. A.; BARNES, T. S. Associations between animal characteristic and environmental risk factors and bovine respiratory disease in Australian feedlot cattle. **Preventive Veterinary Medicine**, v. 125, p. 66-74, 2016.

HEADLEY, S. A.; BALBO, L. C.; ALFIERI, A. F.; SAUT, J. P. E.; BAPTISTA, A. L.; ALFIERI, A. A. Bovine respiratory disease associated with *Histophilus somni* and bovine respiratory syncytial virus in a beef cattle feedlot from Southeastern Brazil. **Semina: Ciências Agrárias**, v. 38, p. 283-294, 2017.

HERMEYER, K.; BUCHENAU, I.; THOMASMEYER, A.; BAUM, B.; SPERGSER, J.; ROSENGARTEN, R.; HEWICKER-TRAUTWEIN, M. Chronic pneumonia in calves after experimental infection with *Mycoplasma bovis* strain 1067: characterization of lung pathology, persistence of variable surface protein antigens and local immune response. **Acta Veterinaria Scandinavica**, v. 54, n. 1, p. 9, 2012.

OLA, O. O.; ADENIRAN, G. A.; OPOKU-AGYEMANG, T.; BEDIAKO, E.; ISHOLA, O.; JARIKRE, T. A.; EMIKPE, B. O. Pathology and immunohistochemical evaluation of lungs of cattle slaughtered at metropolitan abattoirs in Nigeria and Ghana. **Journal of Immunoassay & Immunochemistry**, v. 10.1080/15321819.2020.1730889, p. 1-14, 2020.

OLIVEIRA, T. E. S.; PELAQUIM, I. F.; FLORES, E. F.; MASSI, R. P.; VALDIVIEZO, M. J. J.; PRETTO-GIORDANO, L. G.; ALFIERI, A. A.; SAUT, J. P. E.; HEADLEY, S. A. *Mycoplasma bovis* and viral agents associated with the development of bovine

116 respiratory disease in adult dairy cows. **Transboundary Emerging Diseases**, v. 67
117 Suppl 2, n. Suppl 2, p. 82-93, 2020.

118 OLIVEIRA, T. E. S.; SCUISATO, G. S.; PELAQUIM, I. F.; CUNHA, C. W.; CUNHA, L.
119 S.; FLORES, E. F.; PRETTO-GIORDANO, L. G.; LISBÔA, J. A. N.; ALFIERI, A. A.;
120 SAUT, J. P. E.; JORGE DA CUNHA, P. H.; HEADLEY, S. A. The participation of a
121 malignant catarrhal fever virus and *Mycoplasma bovis* in the development of single and
122 mixed infections in beef and dairy cattle with bovine respiratory disease. **Frontiers**
123 **Veterinary Science**, v. 8, p. 691448, 2021.

124 OLIVEIRA, T. E. S.; SCUISATO, G. S.; FRITZEN, J. T. T.; SILVA, D. C.; MASSI, R. P.;
125 PELAQUIM, I. F.; SILVA, L. E.; FLORES, E. F.; LIMA SANTOS, R.; PRETTO-
126 GIORDANO, L. G.; LISBÔA, J. A. N.; ALFIERI, A. A.; HEADLEY, S. A. Infectious
127 disease agents associated with pulmonary alterations in aborted bovine fetuses.
128 **Animals**, v. 12, n. 13, p. 1596, 2022.

129 TAYLOR, J. D.; FULTON, R. W.; LEHENBAUER, T. W.; STEP, D. L.; CONFER, A. W.
130 The epidemiology of bovine respiratory disease: What is the evidence for predisposing
131 factors? **The Canadian Veterinary Journal**. 51: 1095-1102 p. 2010.

132 YAMAN, T.; BUYUKBAYRAM, H.; OZYILDIZ, Z.; TERZI, F.; UYAR, A.; KELES, O. F.;
133 OZSOY, S. Y.; YENER, Z. Detection of bovine respiratory syncytial virus, *Pasteurella*
134 *multocida*, and *Mannheimia haemolytica* by immunohistochemical method in naturally-
135 infected cattle. **Journal of Veterinary Research**, v. 62, n. 4, p. 439-445, 2018.

2 JUSTIFICATIVA

Embora o Brasil seja o segundo maior produtor efetivo de bovinos do mundo e a DRB seja uma das principais doenças de bovinos, as informações relacionadas ao desenvolvimento de DRB no país são escassas, principalmente quando se compara a disponibilidade e a quantidade de informações estabelecidas em rebanhos bovinos na América do Norte, Europa e Oceania. Consequentemente, há necessidade de investigar os aspectos relacionados à ocorrência de DRB em rebanhos nas principais regiões geográficas de pecuária bovina no Brasil. As informações obtidas a partir dessa investigação serão fundamentais para conhecer os principais agentes infecciosos associados ao desenvolvimento da DRB em nosso país. Consequentemente, esses resultados e conhecimento poderão ser utilizados como base para desenvolver estratégias nacionais eficientes de diagnóstico, controle e profilaxia da DRB na pecuária brasileira.

3 HIPÓTESES

A DRB é uma situação clínica multietiológica e multifatorial dependente da presença e da intensidade de fatores de risco específicos. Pode apresentar taxas variáveis de morbidade e mortalidade, em rebanhos bovinos das mais distintas regiões geográficas brasileiras. Deste modo queremos definir:

Verificar e categorizar as diferenças da DRB em infecções singulares e mistas dentre os agentes etiológicos estudados, em rebanhos bovinos de corte e leite.

Associar as lesões histopatológicas com agente etiológico específico, permitindo auxiliar em pesquisas futuras, na área de sanidade e epidemiologia

4 OBJETIVOS

4.1.1 Objetivo geral

Realizar estudos histopatológicos e imuno-histoquímicos (IHQ) para determinar a incidência e distribuição de agentes infecciosos associados doença respiratória bovina (DRB) nas principais regiões produtoras de bovinos no Brasil.

4.1.2 Objetivos específicos

- Padronizar protocolos de IHQ para os diferentes tipos de agentes infecciosos investigados.
- Correlacionar as características do diagnóstico morfológico microscópico para cada agente infeccioso identificado na IHQ.
- Determinar a ocorrência de agentes infecciosos induzindo lesão única e/ou mista de pneumonia em bovinos
- Detectar e descrever as interações entres os agentes infecciosos.
- Determinar a incidência dos agentes etiológicos infecciosos associados com DRB no Brasil.
- Associar os achados histopatológicos com a imunorreatividade positiva aos agentes infecciosos.

5 ARTIGO 1 - BOVINE RESPIRATORY DISEASE IN BRAZIL: A SHORT REVIEW[1]

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5.1 ABSTRACT

The bovine respiratory disease (BRD) complex is a multifactorial and multietiological disease entity described in all geographic regions of Brazil. This brief review discusses aspects related to epidemiology, etiologic agents, clinical and pathological manifestations, and challenges in the diagnosis of BRD in Brazil. The main infectious agents associated with respiratory outbreaks in cattle from Brazil are bovine alphaherpesvirus type 1, bovine viral diarrhea virus, bovine respiratory syncytial virus, and *Mycoplasma bovis*. Ovine gammaherpesvirus-2 and HoBi-like pestivirus have been associated with the development of pneumonia in adult cattle and calves, respectively in Brazil, and should be considered as possible causes of BRD. Additionally, studies using epidemiological data, histopathological and molecular associations with morbidity and mortality should be carried out in Brazil, to demonstrate the real impacts of BRD on livestock.

Key words: Epidemiology. Cattle disease. Diagnosis. Respiratory pathogens.

5.2 INTRODUCTION

The bovine respiratory disease (BRD) complex is a consequence of the interaction of multiple factors related mainly to host immunity, the pathogenicity of respiratory microorganisms, and environmental characteristics that alter the probability of exposure to pathogens (Fulton, 2009). BRD occurs in beef and dairy cattle, and it has been suggested that cattle reared in feedlots or

[1] Part of the PhD thesis of MMC and TESO.

semi-confined conditions can increase the occurrence and mortality related to this disease (Baptista, Rezende, Fonseca, Nogueira, & Headley, 2017). Additionally, BRD can be considered as a syndrome caused by the interaction of several viral and bacterial infections that result in clinical and pulmonary alterations (Fulton, 2009; Gershwin, Van Eenennaam, Anderson, McEligot, & Shao, 2015) with a high economic loss to the farmer (Baptista et al., 2017).

The clinical signs manifested in cattle with BRD may be non-specific, which often impede the clinical diagnosis (Cooper & Brodersen, 2010) some signs frequently encountered include dyspnea, tachypnea, cough, nasal and ocular discharge, depression, anorexia, fever, and pulmonary rales (Fulton, 2009; Griffin, Chengappa, Kuszak, & McVey, 2010; Timsit, Hallewell, Booker, Tison, & Amat, 2017). Since BRD is a syndrome that has multiple signs and is associated with several infectious disease agents; the specific pathogens involved in individual cases or outbreaks are often undiagnosed. Consequently, assessment of the animal response and risk factors associated with each pathogen can result in a better understanding of this disease (Grissett, White, & Larson, 2015).

This brief review discusses the data of the respiratory disease complex in cattle from Brazil, considering the epidemiological data, the infectious agents, and the clinical and pathological manifestations associated with its development, based on studies published in scientific journals. Additionally, this review has a great significance in terms of its timing, since currently no study was focused on the collective evaluation of the manifestations and agents related to BRD in Brazil.

5.3 EPIDEMIOLOGICAL DATA

BRD is a frequent disease that causes mortality in calves (Brazil et al., 2013; USDA, 2017; Dubrovsky et al., 2019), and dairy cattle (Francoz et al., 2015; Richter et al, 2017; Yarnall & Thrusfield, 2017), and is the leading cause of losses in feedlot cattle (Fulton et al., 2009; Hay et al., 2016; Baptista et al., 2017), resulting in substantial losses to livestock (Blakebrough- Hall et al., 2020).

Comprehensive clinical-epidemiological data collected to assess the prevalence and degree of losses induced by the BRD complex are still scarce

in Brazil (Oliveira, Pelaquim, et al., 2020), especially when compared to data from North America (Loneragan, Dargatz, Morley, & Smith, 2001; Gagea, Bateman, van Dreumel, McEwen, & Carman, 2006; Hessman, Fulton, Sjeklocha, Murphy, & Ridpath, 2009; Taylor, Fulton, Lehenbauer, Step, & Confer, 2010) and Australia (Moore, O'Dea, Perkins, & O'Hara, 2015; Hay et al., 2016; Schibrowski, Gibson, Hay, Mahony, & Barnes, 2018). Published data show that most studies done in Brazil regarding BRD were conducted in the southern and southeastern regions of the country (Table 1).

Table 1 – Microorganisms frequently associated with bovine respiratory disease in Brazil

PATHOGENIC RESPIRATORY AGENTS	GEOGRAPHICAL REGION	REFERENCES
Bovine alphaherpesvirus-1, BoAHV1	Paraná São Paulo	(Dias, Médici, Alexandrino, Medeiros, & Alfieri, 2010; Headley, Alfieri, Oliveira, Beuttemmuller, & Alfieri, 2014; Hoppe, Souza- Pollo, Medeiros, Samara, & Carvalho, 2019; Oliveira, Pelaquim, et al., 2020)
Bovine coronavirus, BCoV	Paraná São Paulo	(Beuttemmuller, Alfieri, Headley, & Alfieri, 2017; Oliveira, Dall Agnol, et al., 2020)
Bovine parainfluenza virus-3, BPIV-3	Paraná Rio Grande do Sul São Paulo	(Cole, 1971; Gonçalves, Spilki, Chiminazzo, Oliveira, & Franco, 2003; Vaucher, Dezen, Simonetti, Spilki, & Roehe, 2011; Oliveira, & Pelaquim, et al. ,2020; Oliveira, Dall Agnol, et al., 2020)
Bovine respiratory syncytial virus, BRSV	Alagoas Paraná Rio Grande do Sul São Paulo	(Gonçalves, Simanke, Jost, Hôtzel, & Soglio, 1993; Driemeier, Gomes, Moojen, Arns, & Vogg, 1997; Flores, Weiblen, Medeiros, Botton, & Irigoyen, 2000; Oliveira, Pelaquim, et al., 2020; Oliveira, Dall Agnol, et al., 2020)

continue...

continuation...

PATHOGENIC RESPIRATORY AGENTS	GEOGRAPHICAL REGION	REFERENCES
Bovine viral diarrhea virus, BVDV	Rio Grande do Sul Paraná	(Flores, Weiblen, Vogel, Roehe, & Alfieri, 2005; Hoppe et al., 2019; Oliveira, Pelaquim, et al., 2020; Oliveira, Dall Agnol, et al., 2020)
Histophilus somni	Minas Gerais Paraná	(Headley, Oliveira, Figueira, Bronkhorst, & Alfieri, 2013; Headley et al., 2014; Headley, Balbo, Alfieri, Saut, & Baptista, 2017; Oliveira, Dall Agnol, et al., 2020)
Mannheimia haemolytica	Minas Gerais Paraná São Paulo	(Coutinho, Oliveira, Silva, Oliveira, & Marcondes, 2009; Baptista et al., 2017; Magalhães, Baptista, Fonseca, Menezes, Nogueira, 2017)
Mycoplasma bovis	Bahia Minas Gerais Paraná São Paulo	(Pretto, Müller, Freitas, Mettifogo, &, Buzinhanr, 2001; Marques, Buzinhani, Oliveira, Yamaguti, & Ferreira, 2007; Tortorelli, Carrillo Gaeta, Mendonça Ribeiro, Miranda Marques, & Timenetsky, 2017; Gaeta, Ribeiro, Alemán, Yoshihara, & Nassar, 2018; Oliveira and Pelaquim, et al., 2020; Oliveira, & Dall Agnol, et al., 2020)
Pasteurella multocida	Minas Gerais Paraná São Paulo	(Coutinho et al., 2009; Baptista et al., 2017)

In a 12-year retrospective study (Brasil et al., 2013) done in Rio Grande do Sul with 306 cattle, respiratory infections were responsible for 15% of 33 calves necropsied (Brasil et al., 2013). Age ranged from 2 days to 12 months, with calves up to 3 months old (51.5%; 17/33) being the most affected (Brasil et al., 2013). In Brazil, statistical data collected over a period of two years from beef cattle feedlots revealed morbidity caused by BRD as 6.13% (11,577/188,862) and mortality as 0.21% (397/188,862) (Baptista et al., 2017).

The impact of BRD can be estimated by the sum of direct costs, which include mortality, morbidity, reduced animal performance and carcass

quality, and indirect labor and infrastructure costs (Jim, 2009). The economic loss caused by morbidity and mortality linked to the BRD complex is extensive and is mainly related to the costs of treating and preventing the disease (Apley, 2006; Griffin et al., 2010; Timsit et al., 2017).

In Brazil, the expenses due to mortality and morbidity associated with BRD were estimated at US$ 14,334.00 and US$ 16,315.40 for every 10,000 cattle, respectively (Baptista et al., 2017). Additionally, it was estimated that the economic effects due to morbidity associated with BRD in Brazil were US$ 6.31 million/year, while mortality losses were US$ 5.54 million, totaling an annual loss of US$ 11.85 million (Baptista et al., 2017).

Data from a feedlot in the state of Goiás revealed that BRD was the biggest health problem encountered, accounting for 46.7% of all herd diseases (Malafaia, Granato, Costa, Souza, & Costa, 2016). Additionally, these authors indicated that treatment costs were high on average, from 7% to 15% of live weight to be gained during the finishing period of the traditional system.

Australian researchers evaluated the economic effects of BRD and observed morbidity rates of 18% and mortality of 2.1%, with an average net loss of Australian dollar (AUD $) 1,647.53 (US$ 1,1738) per mortality (Blakebrough-Hall et al., 2020). This study demonstrated that cattle that were treated three times or more for BRD had lighter carcasses averaging 39.6 kg and yielded revenue of around AUD $ 384.9 (US$ 274.2) less compared to untreated cattle. Additionally, the carcass yield of cattle with severe lung disease at slaughter was 0.3 kg/ day less, being on average 14.3 kg lighter at slaughter, generating a loss of AUD $ 91.50 (US$ 65.2) compared to that of cattle without pulmonary disease (Blakebrough-Hall et al., 2020). Animals with subclinical (16 kg) and clinical (24.1 kg) BRD had lighter carcasses, and the revenue collected at slaughter was reduced by AUD $ 67.10 ($ 47.8) and AUD $ 213.90 ($152.3), respectively, when compared with healthy animals that were never treated and without pulmonary disease (Blakebrough- Hall et al., 2020).

5.4 ETIOLOGICAL AGENTS ASSOCIATED WITH THE DEVELOPMENT OF BRD IN BRAZIL

The most frequent viral agents of BRD include bovine alphaherpesvirus type 1, BoHV- 1 (Warren, Babiuk, & Campos, 1996; Muylkens, Thiry, Kirten, Schynts, & Thiry, 2007; Risalde, Molina, Sonchez-Cordon, Pedrera, & Romero- Palomo, 2013), bovine viral diarrhea virus, BVDV (Fulton, Purdy, Confer, Saliki, & Loan. 2000; Gagea et al., 2006), bovine parainfluenza virus type 3, BPIV-3 (Fulton, Briggs, Payton, Confer, & Saliki, 2004; Ceribasi, Ozkaraca, Ceribasi, & Ozer, 2014), bovine respiratory syncytial virus, BRSV (Fulton et al., 2000; Gagea et al., 2006) and bovine coronavirus, BCoV (Gagea et al., 2006; Fulton, Step, Wahrmund, Burge, & Payton, 2011). Most viral agents of cattle induce immunosuppression resulting in secondary or concomitant infections (Fulton, 2009; Caswell & Williams, 2016). HoBi- like pestivirus, an emerging agent initially identified in Europe in fetal bovine serum imported from Brazil, was related to respiratory manifestations, inducing viremia, leukopenia, fever, and nasal secretions (Bauermann, Ridpath, Weiblen, & Flores, 2013). Additionally, HoBi-like pestivirus was associated with the development of pneumonia in a calf from Italy (Decaro, Mari, Pinto, Lucente, & Sciarretta, 2012) and in several calves from an outbreak of BRD in São Paulo (Hoppe et al., 2019).

Other agents of viral origin not frequently associated with BRD are influenza D virus (IDV) and bovine adenovirus type 3 (BAV3). Serological studies demonstrated that IDV has a wide geographic distribution with high seroprevalence in many herds and has been circulated in the USA since 2003 (Ferguson, Eckard, Epperson, Long, & Smith, 2015; Luo, Ferguson, Smith, Woolums, & Epperson, 2017). This virus was also identified in the United Kingdom (Dane, Duffy, Guelbenzu, Hause, & Fee, 2019) and Mexico (Mitra, Cernicchiaro, Torres, Li, & Hause, 2016). BAV3 was first associated with the BRD complex in experimental studies in calves from Canada (Ide, Thomson, & Ditchfield, 1969) and Australia (Cole, 1971). Seroepidemiological studies demonstrated high seropositivity to BAV3 in calves in several countries including the USA (Mattson, Norman & Dunbar, 1988), Turkey (Ng, Kondov, Deng, Van Eenennaam, & Neibergs, 2015; Tuncer & Yeşilbağ, 2015). However, reports of infection associated with IFV and BAV3 have not been identified in Brazil.

The absence of reports of these agents in Brazil does not necessarily indicate that they do not infect cattle herds in the country, but it could simply indicate that they have not been diagnosed so far for not being included in the list of differentials.

Bacterial infections have been frequently associated with secondary bronchopneumonia in BRD. The main bacterial agents are *Mannheimia haemolytica, Pasteurella multocida, and Histophilus somni* (Fulton, 2009; Panciera & Confer, 2010; Grissett et al., 2015; Klima, Holman, Ralston, Stanford, & Zaheer, 2019), and the mollicute, *Mycoplasma bovis* (Caswell & Archambault, 2007; Fulton, 2009; López & Martinson, 2017). Most of the important infectious disease agents associated with the development of BRD worldwide have been diagnosed in herds from Brazil. The main pathological agents most frequently associated with the development of BRD, and the geographic regions found in Brazil are shown in Table 1. Although cattle farming occurs in several geographic regions of Brazil (Headley, Oliveira, & Cunha, 2020), the data collected suggest that most studies related to the investigation of BRD in Brazil were conducted mainly in the south and southeast regions of the country (Table 1). Additionally, BoAHV1, BVDV, and BRSV are endemic viral agents in most geographic regions of Brazil (Flores et al., 2000; Flores et al., 2005; Dias, Alfieri, Ferreira-Neto, Gonçalves, & Muller, 2013), resulting in BRD and other clinical manifestations in dairy and beef cattle.

Several studies done in Brazil have demonstrated concomitant action of respiratory agents of BRD (Baptista et al., 2017; Beuttemmuller et al., 2017; Magalhães et al., 2017; Tortorelli et al., 2017; Gaeta, Ribeiro, Alemán, Yoshihara, & Nassar, 2018; Oliveira & Pelaquim, et al. 2020; Oliveira & Dall Agnol, et al. 2020) using molecular identification with or without histopathological and immunohistochemical diagnoses. This trend of concomitant infections in BRD is evident in other countries such as in the USA (Fulton et al., 2009; Schneider, Tait, Busby, & Reecy, 2009; Taylor et al., 2010), Canada (Gagea et al., 2006; Booker, Abutarbush, Morley, Jim, & Pittman, 2008; Jim, 2009), and Australia (Cusack, McMeniman, & Lean, 2003; Moore et al., 2015; Hay et al., 2016). These data suggest that mixed infections in BRD are perhaps more frequent than have been reported.

The most accepted model of pathogenesis involves initial viral infection causing primary lung injury and/or immune dysfunction (López & Martinson, 2017) followed by a bacterial infection. However, *M. bovis* has been associated with singular infection (Oliveira, Pelaquim, et al., 2020), and could be classified as a primary infectious disease agent (Nicholas, 2011; Gershwin et al., 2015; Oliveira, Pelaquim, et al., 2020). This mollicute may predispose cattle to pulmonary infections induced by *P. multocida* (Nicholas, 2011), due to immunosuppression and immunomodulatory effects (Nicholas, 2011; Margineda, Zielinski, Jurado, Alejandra, & Mozgovoj, 2017). Furthermore, the association of *M. bovis* as a singular agent in the development of BRD has been confirmed in both dairy (Margineda et al., 2017; Oliveira & Pelaquim, 2020) and beef cattle (Ramírez Romero, Chavarría Martínez, Nevárez Garza, Rodríguez Tovar, & Dávila Martínez, 2010; Margineda et al., 2017). Consequently, the role of *M. bovis* as a possible primary agent in the development of BRD cannot be excluded.

Diverging from the trend of global literature (Martin, Bateman, Shewen, Rosendal, & Bohac, 1989; Fulton, Cook, Step, Confer, & Saliki, 2002; Murray, More, Sammin, Casey, & McElroy, 2017), there are few reports of BPIV-3 associated with the development of BRD in Brazil. This infectious disease agent was not identified in several studies (Brasil et al., 2013; Baptista et al., 2017; Headley, Okano, Balbo, Marcasso, & Oliveira, 2017; Oliveira & Dall Agnol, et al., 2020), but was associated with few cases of mixed infections in cattle from Brazil (Oliveira & Pelaquim, et al., 2020). A retrospective study done by our group identified singular BPIV-3 infections in only 6.6% (1/15 of all diagnoses) of interstitial pneumonia (Oliveira & Pelaquim, et al.,2020). These data suggest that BPIV-3 circulates in cattle herds from Brazil, with possibly low frequencies.

Additionally, there is evidence that ovine gammaherpesvirus-2, OvHV-2 (Headley, Oliveira, & Cunha, 2020) and HoBi-like pestiviruses (Hoppeetal., 2019) are candidates of emerging pathogens associated with the development of BRD. Recently, OvHV-2 was associated with the occurrence of respiratory disease in a calf infected simultaneously with BVDV, BoAHV1, and Aspergillus fumigatus (Headley, Müller, Oliveira, Duarte, & Pereira, 2020) and in two adult cattle with interstitial pneumonia (Headley, Oliveira, Li, Lisbôa, &

Queiroz, 2020). The proposed involvement of OvHV-2 in the development of BRD has been reviewed and can be consulted (Headley, Oliveira, & Cunha, 2020).

5.5 CLINICAL MANIFESTATIONS OF BRD

The first line of defense of the immune system is represented by the respiratory epithelium, which provides mechanical, chemical, and microbiological barriers to prevent infection by pathogens associated with BRD (Panciera & Confer, 2010). In asymptomatic cattle, the upper respiratory tract is colonized by a variety of bacteria, such as *M. haemolytica, H. somni,* and *P. multocida;* although these bacteria are part of the normal microbiota of the respiratory system, they are opportunistic and can result in severe respiratory disease (Griffin et al., 2010; Gershwin et al., 2015; Zhang, Hill, Godson, Ngeleka, & Fernando, 2020). Clinically, the BRD complex includes enzootic pneumonia of calves, a multifactorial etiologic entity that includes disease such as pneumonic mannheimiosis (*M. haemolytica*), respiratory histophilosis (*H. somni*), pneumonia by *M. bovis*, infectious bovine rhinotracheitis (BoAHV1), and viral pneumonia by BPIV-3 and BRSV (Andrews & Kennedy, 1997; Fulton, 2009; Fulton et al., 2009; Panciera & Confer, 2010; Klima, Zaheer, Cook, Booker, & Hendrick, 2014; Francoz et al., 2015; Gershwin et al., 2015; Murray et al., 2017; Zhang et al., 2020).

Clinical signs are usually observed after stressful events. Infection occurs by the inhalation of droplets containing microorganisms, direct contact with infected animals, or ingestion of water or food contaminated with nasal discharge from infected animals (Gagea et al., 2006; Fulton, 2009). Clinical manifestations may include nasal and ocular secretions, anorexia, fever of up to 42ºC, tachypnea, tachycardia, muzzle crusts, cough, dyspnea, and pulmonary rales (Griffin et al., 2010).

Pneumonic mannheimiosis is frequently associated with an acute respiratory disease that occurs a few days or weeks after animals are transported, hence the term “shipping fever” (López & Martinson, 2017), *M. haemolytica* associated with *P. multocida* can result in fatal pneumonia (Griffin et al., 2010),

that is pathologically characterized as severe fibrinous bronchopneumonia (López & Martinson, 2017).

P. multocida is easily isolated from nasal and pharyngeal secretions of cattle; isolation rates reported in clinically normal cattle are high and range from 20 to 60% (Griffin et al., 2010), since it is a commensal microorganism (Fulton, 2009). The isolation of *P. multocida* in animals clinically presenting respiratory disease is about two times higher than in healthy animals (Griffin et al., 2010). The development of pneumonia induced by this bacterium is associated with risk factors such as transport, stress, batch mixing, overcrowding, and primary viral or bacterial infections (Dabo, Taylor, & Confer, 2007).

Respiratory histophilosis is part of a complex of diseases associated with *H. somni* infection, termed histophilosis (Corbeil, 2007; O'Toole & Sondgeroth, 2016). This complex consists of several clinical manifestations including thrombotic meningoencephalitis, septicemia, pneumonia, pleuritis, myocarditis, arthritis, conjunctivitis, otitis, and abortions (Corbeil, 2007; O'Toole & Sondgeroth, 2016). The respiratory form is characterized by fibrinopurulent bronchopneumonia (Corbeil, 2007; O'Toole & Sondgeroth, 2016; Headley et al., 2017). All clinical manifestations of histophilosis are well known in the USA (Corbeil, 2007; O'Toole, Allen, Hunter, & Corbeil, 2009; O'Toole & Sondgeroth, 2016), Australia (Lancaster, McGillivery, Patterson, & Irwin, 1984; Hick, Read, Lugton, Busfield, & Dawood, 2012), with few descriptions in Brazil (Headley et al., 2014; Headley, Bracarense, Oliveira, Queiroz, & Okano, 2015; Headley, & Balbo, 2017; Headley, Pereira, Balbo, Santia, & Bracarense, 2018). The few cases or outbreaks of pulmonary histophilosis found in Brazil could be associated with the lack of diagnosis or confusion due to the similarity of the pathological findings observed in the lungs of cattle infected by *M. haemolytica*. Considering the multisystemic nature of histophilosis, which often results in mortality in affected animals, feedlot cattle in Brazil may be susceptible to infection since the pathological manifestations are not easily recognized (Headley et al., 2014).

M. bovis is the most common species of Mycoplasma sp. isolated from the lungs of cattle with pneumonia in Europe and North America (Caswell & Archambault, 2007; Nicholas, 2011), and pulmonary infection is aggravated by

457 the risk factors of BRD (Snowder, Van Vleck, Cundiff, & Bennett, 2006; Taylor et
458 al., 2010; Hay et al., 2016; Murray, Cassidy, Clegg, Tratalos, & McClure, 2016;
459 López & Martinson, 2017; Murray et al., 2017). The respiratory disease associated
460 with infection by *M. bovis* results in clinical manifestations that are similar to other
461 types of pneumonia; these alterations include fever, loss of appetite, nasal
462 discharge, cough, and increased respiratory rate, while in some cases, other
463 clinical manifestations such as otitis media, mastitis, and arthritis can be observed
464 (Maunsell & Donovan, 2009). Diseases associated with BoAHV1 infection are
465 endemic in most geographical regions of Brazil, with seroprevalence ranging from
466 11 to 74% (Dias, Alfieri, Médici, Freitas, & Ferreira, 2008; Pasqualotto, Sehnem, &
467 Winck, 2015). However, there are few studies that effectively evaluate the
468 respiratory manifestations associated with infections induced by BoAHV1 in cattle
469 from Brazil.

470 Depending on the immunological status of the host, BRSV
471 infections may be asymptomatic, with a tendency for greater severity observed in
472 younger cattle. The most frequently observed clinical signs are those of upper
473 airway infection, which may progress to bronchiolitis and interstitial pneumonia
474 (Philippou, Streckert, Otto, Reinhold, & Elschner, 1996; Sacco, McGill, Pillatzki,
475 Palmer, & Ackermann, 2014; Caswell & Williams, 2016). In a study in Denmark,
476 the clinical signs of BRSV infection included nasal secretion, pyrexia, cough,
477 elevated respiratory rate, and marked depression; the affected animals were
478 calves between four and seven months of age (Larsen, Tegtmeier, & Pedersen,
479 2001). In Brazil, cough, rales, and dyspnea were observed in calves infected with
480 BRSV (Driemeier et al., 1997).

481 BPIV-3 infections are commonly associated with discrete or
482 asymptomatic clinical manifestations, mainly in adult animals. However, BPIV-3
483 predisposes cattle to secondary respiratory infections and consequently, clinical
484 manifestations consistent with BRD will occur (Griffin et al., 2010). In Brazil, few
485 studies have identified the occurrence of BVPI-3 in associated with the
486 development of BRD (Gonçalves et al., 2003; Oliveira, & Pelaquim, et al., 2020).
487 In one of these studies, where the virus was isolated from a calf, clinical

manifestations of pulmonary disease including apathy, fever, tachypnea, and severe serous nasal discharge were reported (Gonçalves et al., 2003).

5.6 Methods and Challenges in the Diagnosis of the Agents of BRD

5.6.1 Clinical manifestations

There are several methods to obtain a diagnosis of BRD. These include the clinical history and laboratory analysis of animal tissues derived from live and dead animals. The diagnosis of BRD is a challenge for the clinician since numerous infectious disease agents are involved. The affected cattle suffer the effects of individual disease agents or more often by a combination of several pathogens (Fulton & Confer, 2012), so clinical evaluations must include the examination of several animals of the same farm (Caswell, Hewson, Slavić, DeLay, & Bateman, 2012). The DART system that evaluates the clinical signs (depression, appetite, respiratory, temperature) of pulmonary disease, is recommended for the clinical evaluation of animals with BRD (Wilson, Step, Maxwell, Wagner, & Richards, 2015), and has been used in several studies by our group (Baptista et al., 2017; Magalhães et al., 2017). However, the isolated use of clinical manifestations is not the best method to diagnose BRD (Fulton & Confer, 2012). Nevertheless, the clinical history should be concise, effective, and should contain specific observations that allow for the proper interpretation of laboratory results (Caswell et al., 2012). The clinical history should include biological data of the affected animals, duration of manifestations, morbidity and mortality data, common factors among the affected animals, changes in management, environment and climate, immunization history, and use of any quarantine measures at the herd (Cooper & Brodersen, 2010). Notwithstanding the above, there is always the necessity to discuss each situation with several persons involved to obtain complementary information and/or eliminate conflicting data.

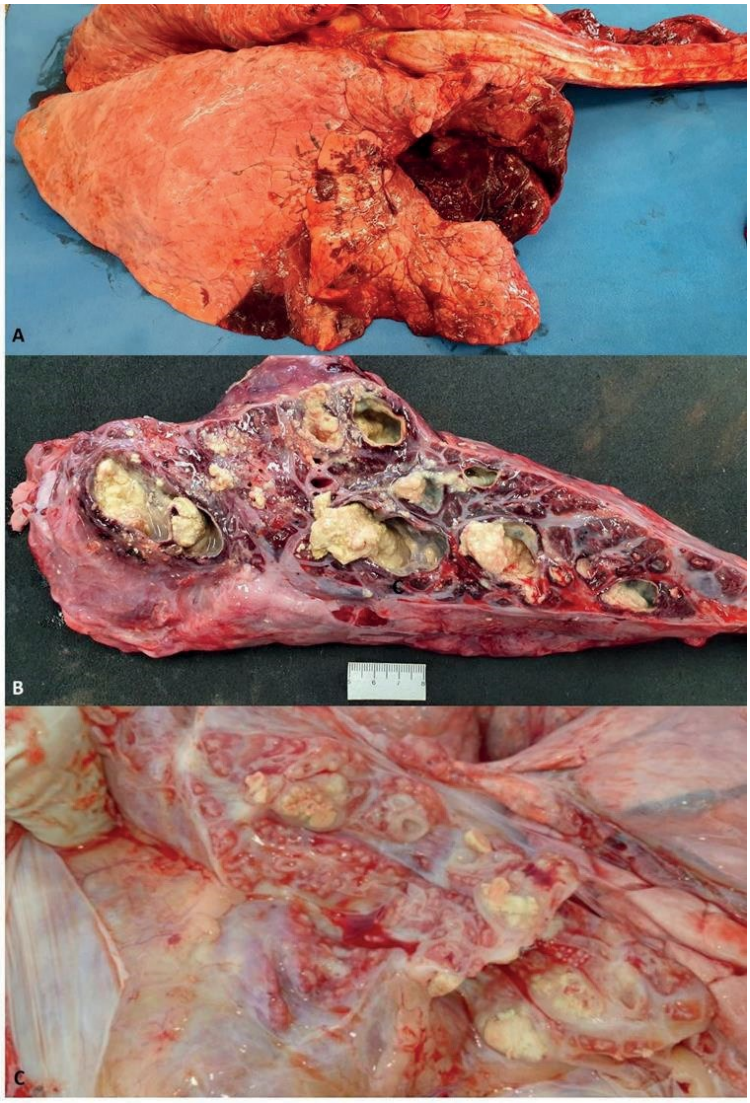
5.6.2 Pathological findings

The pathological pulmonary findings observed in cattle with BRD are diverse and vary according to the etiology and severity of the disease. In general, infections induced by agents of viral origin result in interstitial pneumonia, mainly in the initial phase of infection (Panciera & Confer, 2010). As the viral infection progresses, the animal often suffers from immunosuppression, which results in secondary bacterial infection, which is often fatal. Consequently, careful histopathological analysis often identifies the interfaces of the two pulmonary diseases. Lesions caused by bacterial infections are more exudative and accumulative, while viral lesions are more inflammatory with corresponding thickening of the alveolar wall (López & Martinson, 2017).

Suppurative bronchopneumonia is the most common form of BRD seen in young dairy calves and feedlot cattle, and is primarily associated with infection by *P. multocida*, although other bacteria can also produce this lesion (Griffin et al., 2010; Murray et al., 2017). Fibrinous bronchopneumonia is often associated with infections induced by *H. somni* (Headley et al., 2014; Headley et al., 2018), and *M. haemolytica* (Griffin et al., 2010; Gershwin et al., 2015; Zhang et al., 2020). Both types of bronchopneumonia are macroscopically characterized by cranioventral consolidation of the affected lungs (Figure 1A). The neutrophilic exudate is predominant in the suppurative manifestation (Figure 1B), with predominance of fibrinous exudate in fibrinous bronchopneumonia. However, the two patterns are frequently often seen concomitantly, resulting in fibrinopurulent bronchopneumonia. In acute lesions, the affected pulmonary lobes may vary in color from pink, pink-grey, dark red, red-grey, or grey, with interlobular septal edema (Caswell & Williams, 2016; López & Martinson, 2017). On the sectioned surface of the pulmonary lobes, brown to gray foci, indicative of bronchiolar and peribronchiolar inflammation (Caswell & Williams, 2016; López & Martinson, 2017), can be observed. The remaining areas of the lobules are pink to dark red and represent inflammation, congestion, and atelectasis (Caswell & Williams, 2016). As lesions become chronic, palpation reveals a more irregular distribution of consolidation and there is more evidence of purulent bronchitis, bronchiectasis, abscess formation, fibrosis, and adhesions (Panciera & Confer, 2010; Caswell &

Williams, 2016). Microscopically diffuse thickening of alveolar septa can be observed because of marked proliferation of type II pneumocytes, leukocytic infiltration within the interstitial and bronchioloalveolar junction, cellular degeneration (suppuration), and occasional intralesional bacterial colonies (Caswell & Williams, 2016; López & Martinson, 2017). These histopathological changes often demonstrate the coexistence of multiple infectious agents, viral followed by a secondary bacterial infection.

Figure 1 – Gross lesions associated with respiratory disease in cattle. There is cranioventral consolidation of the lung of a cow with purulent bronchopneumonia (A) and the accumulation of purulent exudate at the sectioned surface of the lung with bronchiectasis (B). Observe the typical caseonecrotic nodules seen in bronchopneumonia associated with infection by *Mycoplasma bovis* (C)



The lesions observed in the acute phase of bronchointerstitial pneumonia are caused predominantly by viruses (López & Martinson, 2017). At post-mortem evaluations of cattle with acute interstitial pneumonia, there is usually subcutaneous emphysema on the dorsal region, trachea filled with foamy fluid, and interlobular septa distended by pulmonary edema (Panciera & Confer, 2010). In the classic manifestation of interstitial pneumonia, exudates are not observed. Consequently, interstitial pneumonia is the most difficult pulmonary lesion to identify during gross evaluation because the affected tissue resembles the normal lung. Microscopically, interstitial pneumonia (Figure 2A) is characterized by hyperplasia of type II pneumocytes, necrotizing bronchiolitis, necrosis of type II pneumocytes, and alveolar edema (Caswell & Williams, 2016; López & Martinson, 2017), frequently with an influx of lymphoplasmacytic inflammatory infiltrate. Studies by our group confirmed that all viral agents associated with BRD in Brazil results in interstitial pneumonia (Oliveira, Pelaquim, et al., 2020). In infections associated with BRSV and BPIV-3, intracytoplasmic inclusion bodies and multinucleated syncytia can be identified; syncytia are formed by the fusion of infected bronchiolar epithelial cells (López & Martinson, 2017).

In an outbreak of BRSV-associated BRD in Rio Grande do Sul, the main macroscopic findings during gross evaluations were widespread alveolar emphysema with foci of atelectasis, interstitial emphysema and marked thickening of the interlobular septa (Driemeier et al., 1997). Microscopically, several syncytial cells were observed within the pulmonary lesion, in addition to dilation of lymphatic vessels, alveolar emphysema, atelectasis, hypertrophy of the peribronchiolar muscle layer, foci of squamous metaplasia of the bronchial and bronchiolar epithelium, mononuclear inflammatory infiltration with foci of infiltration by eosinophils, with fibrinous accumulation in some alveoli, but intracytoplasmic inclusion bodies were not observed (Driemeier et al., 1997).

Retrospective studies using immunohistochemistry (IHC) identified BRSV antigens in 24.4% (12/46) of calves with clinical and pathological manifestations of bronchopneumonia from the State of Rio Grande do Sul (Flores et al., 2000), while 34.4% (11/32) of pulmonary tissues demonstrated BRSV antigens in a study conducted in Paraná (Oliveira, Pelaquim, et al., 2020).

However, syncytial cell formation, a key element in the diagnosis of BRSV-induced interstitial pneumonia (Caswell & Williams, 2016; López & Martinson, 2017), was observed in only 36.6% (4/11) of infected animals (Oliveira, Pelaquim, et al., 2020). Interestingly, two animals with apparently normal pulmonary parenchyma but necrotic bronchitis contained intralesional BRSV antigens (Oliveira, Pelaquim, et al., 2020). Consequently, the confirmation of the histological diagnosis of infectious agents associated with the development of BRD should be based not only on histopathological changes but should be associated with analyses that detect intralesional antigens or proteins of the infectious agents such as IHC and in situ hybridization (ISH).

Pathological findings of lesions caused by *M. bovis* may be like those of viral infections but tend to progress to a chronic stage characterized by peribronchiolar lymphoid hyperplasia (Rodriguez, Bryson, Ball & Forster, 1996; Maunsell & Donovan, 2009). The pathological changes induced by *M. bovis* range from necrosuppurative, caseonecrotic (Figure 1C) to suppurative bronchopneumonia (Nicholas, 2011; Oliveira, Pelaquim, et al., 2020), with caseonecrotic bronchopneumonia being the typical finding associated with infection by *M. bovis.*

A retrospective study (Oliveira et al., manuscript in preparation) identified intralesional antigens from a malignant catarrhal fever virus (MCFV), probably OvHV- 2, in 53.3% (64/120) of all pathological lesions associated with BRD. Furthermore, this study has demonstrated that antigens of OvHV-2 were associated with pneumonia individually or concomitantly with other agents of BRD. Additionally, the possible participation of OvHV-2 in the pathogenesis of bovine pulmonary disease was proposed (Headley, Oliveira & Cunha, 2020), and a calf with fungal tracheitis due to Aspergillus fumigatus was concomitantly infected with BVDV, BoAHV1, and OvHV-2 (Headley, Müller, Oliveira, Duarte, & Pereira, 2020). Collectively, these data suggest that OvHV-2 can act individually or in association with other disease pathogens in the development of BRD and should be included in the differential diagnosis of cattle with pulmonary disease.

Although most infectious disease agents associated with the development of BRD are of bacterial and viral origin, fungi and protozoa can

630 concomitantly infect the lung and produce pulmonary disease. It is of fundamental
631 importance that the person responsible, when requesting a laboratory
632 examination, considers the clinical manifestations and requests a set of laboratory
633 tests to achieve the proper diagnosis of the suspected infectious disease
634 pathogens agents. A list of the main laboratory analyses used in the diagnosis
635 and/or identification of the infectious agents associated with BRD, as well as the
636 advantages and disadvantages, is provided in Table 2.
637
638 **Table 2** – Comparison of diagnostic methods and/or laboratory identification of
639 organisms related to the development of DRB[1]

Diagnostic methods	What is detected?	Differences	
		Advantages	**Disadvantages**
Serology	Antibody	Detects vaccine response and recent or old contact with the agent. Most respiratory pathogens induce a strong antibody response. Can be performed on several animals simultaneously. Results are processed in less time when compared to some tests.	Does not differentiate between vaccine antibodies and antibodies induced during infection. Elevated cost when the number of samples processed is large. Does not always indicate the state of the animal.
Culture: nasal, nasopharynx, tracheal and bronchoalveolar lavage	Bacterial, viral, and fungal organisms	Detects the presence of colonization and/or active infection.	Positive culture does not necessarily imply pulmonary disease/lesion. Prolonged time to obtain results. Specialized training is needed to perform bronchoalveolar lavage.

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Continuation…

Diagnostic methods	What is detected?	Differences	
		Advantages	Disadvantages
Culture of pulmonary tissues/lesion	Bacterial, viral, and fungal organisms	Isolation indicates elevated concentrations of the agent within the tissue. Antimicrobial resistance can be determined.	Concomitant infections and antimicrobial therapy can interfere with the outcome of the results. Time to obtain the results is extended.
Post-mortem evaluation and histopathological examination of pulmonary lesion	Pathologic findings within the pulmonary system	The causative agent of pulmonary disease can be suggested based on the pattern of pulmonary disease. Evaluation is comprehensive and can identify disease. or lesions not previously reported.	Frequently done with few animals. Animals that have died may not be representative of the affected herd. Sample collection and storage conditions are directly related to the quality and efficiency of the diagnosis. Specialized technical professional is needed.
Immunohistochemical evaluation of pulmonary lesion	Intralesional antigen in the affected pulmonary tissue	*In situ* location of the infectious disease pathogen within the lesion. Strong evidence that the infectious agent induced the disease. Recommended for retrospective studies using formalin fixed paraffin embedded tissues.	Sensitivity and specificity depend on the availability of polyclonal hyperimmune serum or commercial monoclonal antibody for the specific infectious disease agent. Prolonged and/or inadequate fixation could affect the result. Antigen retrieval must be enhanced for tissues fixed over prolonged periods. Specialized technical professional is needed.

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Diagnostic methods	What is detected?	Differences	
		Advantages	**Disadvantages**
In situ hybridization of pulmonary tissue/lesion	Genomic region of the infectious disease agent within the lesion	*In situ* identification of the infectious agent within the lesion. Strong evidence that the infectious agent caused the disease. No specific mono antiserum or monoclonal antibodies are required.	Dependent on the specific genomic region of the pathogen for the development of a specific primer. Elevated cost to acquire commercial reagents. Specialized technical professional is needed.
PCR/RT-PCR–nasal, nasopharynx, or trachea swabs or bronchoalveolar lavage collection	Genetic material of the infectious disease agent	Confirms that the amplified infectious disease agent is present in the sample.	Does not differentiate between concomitant, subclinical, or accidental infection from natural infection or vaccination. Does not always detect the agent in the sample. Does not determine resistance to antimicrobials. High cost to purchase reagents.
PCR/RT-PCR - pulmonary lesion and from formalin fixed paraffin embedded tissue fragments	Genomic region of the agent	Confirms that the infectious disease agent is associated with the disease/lesion	May not represent the causative infectious agent or differentiate between natural infection and modified live vaccine. Additional analysis(es) needed to determine the relationship between the agent amplified and tissue damage. Formalin fixation and/or paraffin embedding affects the amplification of the genetic material of the pathogen. Elevated cost to acquire reagents.

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Diagnostic methods	What is detected?	Differences	
		Advantages	Disadvantages
Multiplex PCR- nasal, nasopharynx, or trachea swabs or bronchoalveolar lavage collection	Genomic regions of several infectious disease agents	Confirms that one or more infectious disease agent is associated with the disease/ lesion.	May not represent the causative infectious agent or differentiate between natural infection and modified live vaccine. Additional analysis(es) needed to determine the relationship between the agent amplified and tissue damage. Elevated cost to acquire reagents.

[1]Adapted from Caswell et al., 2012; Fulton & Confer, 2012.

5.6.3 Serology and isolation of the agent

Serology is widely used to detect antibodies of infectious agents associated with BRD in Brazil (Gonçalves et al., 1993; Dias et al., 2013; Gaeta, Ribeiro, Alemán, Yoshihara, & Marques, 2018), and worldwide (Martin et al., 1989; Tuncer & Yeşilbağ, 2015; Luo et al., 2017). Seroepidemiological analyses are fundamental to determine the presence or spread of a particular agent(s) in herds; however, they do not always confirm whether animals are infected (Fulton & Confer, 2012). In general, seroepidemiological evaluations are effective in determining the agents associated with outbreaks of BRD (Caswell et al., 2012). The isolation of bacterial agents from secretions obtained from the nasal cavity, nasopharyngeal region, and bronchoalveolar lavage is usually done in live cattle (Fulton & Confer, 2012), while pulmonary tissues can be collected during post-mortem analysis (Caswell et al., 2012; Fulton & Confer, 2012). It is essential that the collected sample be refrigerated, but not frozen (Fulton & Confer, 2012), and that the sample be transported to the diagnostic laboratory as soon as possible. In contrast, biological samples collected for viral identification using different molecular biology techniques can be frozen (-20°C or preferably, -80°C) before being sent to the diagnostic laboratory. Viral isolation targets the growth of viral agents in specific cell cultures from collected samples, resulting in characteristic

cytopathic effects observed under the microscope (Leland & French, 1988; Fulton & Confer, 2012). However, the identity of the virus observed should be confirmed by another diagnostic technique (Leland & French, 1988; Fulton & Confer, 2012); several passages in cell culture are often required (Leland & French, 1988). Currently, viral isolation is being replaced by molecular biology techniques in most diagnostic laboratories.

5.6.4 Molecular characterization

There are several molecular methods used as tests for the identification of infectious disease agents from biological samples (collected in vivo or post-mortem) from cattle with BRD, that are being used frequently in studies worldwide. These tests confirm the presence of the amplified agents in the analyzed sample and, consequently, infection in the affected animal (Fulton & Confer, 2012). However, the presence of the agent identified by molecular biology tests does not necessarily indicate a morphological alteration resulting in cellular changes and finally a lesion and/or disease. Therefore, it is recommended to use histological and molecular analyses in combination when using pulmonary samples to confirm the participation of the agent found in the development of the pulmonary disease (Fulton & Confer, 2012; Maes, Langohr, Wise, Smedley, Thaiwong 2014). Additionally, one of the limitations of molecular tests for the identification of bacterial agents, is the inability to provide information related to bacterial resistance and susceptibility (Fulton & Confer, 2012); consequently, animals on the property cannot be treated effectively and adequately.

The greatest problem in the diagnosis of agents associated with BRD is the molecular identification of pulmonary samples derived from formalin-fixed paraffin-embedded tissues. Molecular results from these tissues could be negatively affected for several reasons, including: 1) prolonged time of fixation in formalin solution; 2) amount of fixative solution relative to tissue; 3) no buffering or inadequate buffering of formalin solution; 4) degradation of genetic material (DNA or RNA) of the infectious agent; 5) storage time of the embedded tissue; and 6) the quality of paraffin used (Maes et al., 2014). However, the molecular

amplification of any infectious agent from these lung tissues with the characteristic histological change confirms the participation of the amplified agent in the development of the pulmonary disease (Fulton & Confer, 2012). Consequently, all molecular identification from lung tissues must be accompanied by diagnostic methods such as histopathology, IHC, and/or ISH, to effectively differentiate between infection and disease (Maes et al., 2014).

Adequate post-mortem evaluation of pulmonary tissues should be performed for cattle at various stages of the disease from the same property or affected herd and must include asymptomatic animals (Cooper & Brodersen, 2010). Ideally, the lung should be evaluated in situ on the animal carcass; consequently, the pattern of pulmonary disease can be established and associated with a spectrum of infectious agents (Caswell et al., 2012). Lung fragments sent for pathological diagnosis should be acquired from several pulmonary lobes, containing normal and affected tissue.

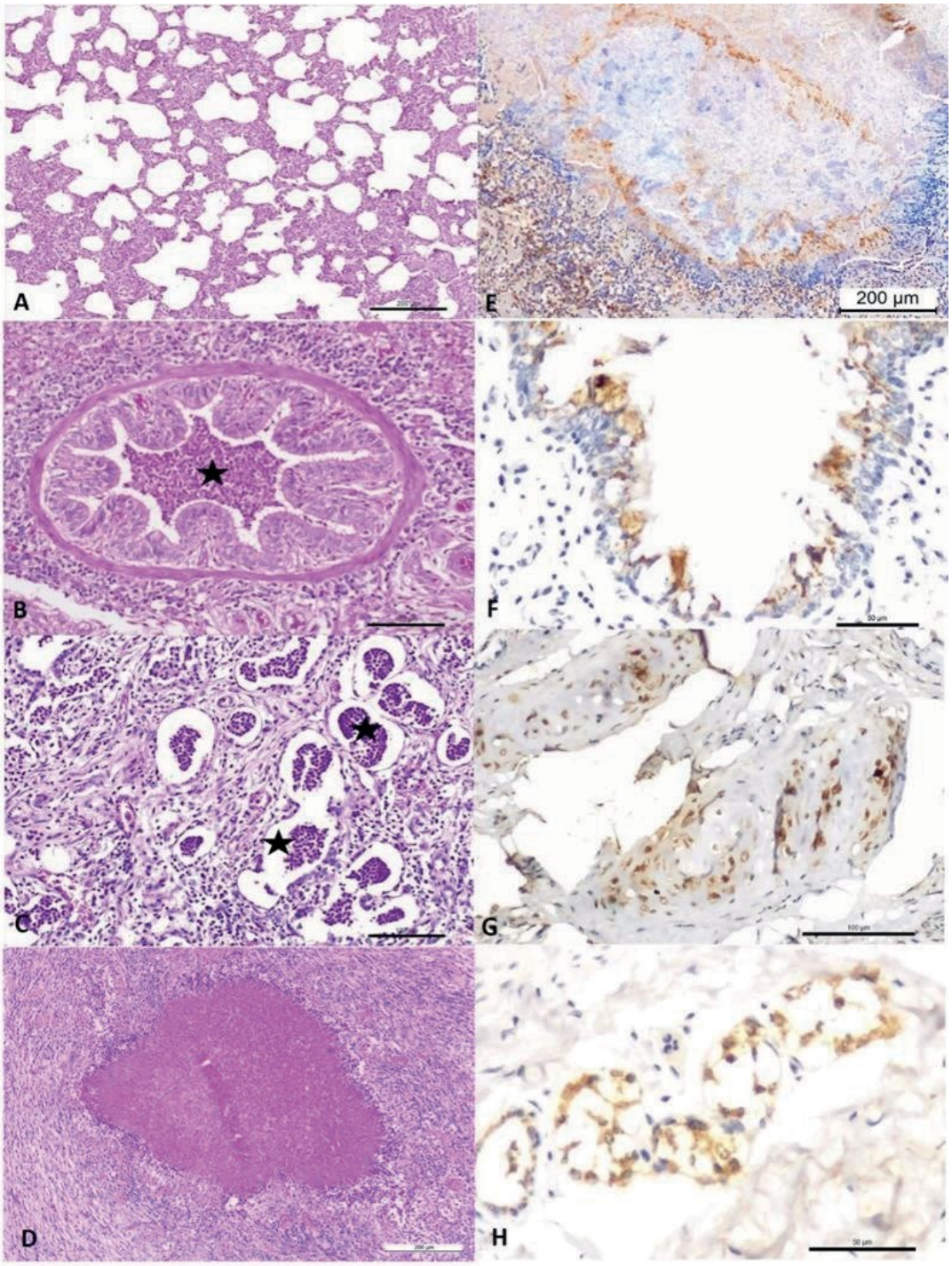
Histopathological evaluation is essential to recognize the pattern of tissue injury induced by infectious disease agents, as the associated patterns of most of the major pathogens of BRD are known. Additionally, there is a strong association between the histological pattern of pulmonary disease and the related infectious agents (Zhang et al., 2020). Interstitial pneumonia (Figure 2A) is the pattern of pulmonary injury characteristic of infections induced primarily by viral disease agents (Caswell & Williams, 2016; López & Martinson, 2017). However, histopathological evaluation alone is not sufficient to indicate the associated agent. Suppurative bronchopneumonia (Figure 2B-C) is the characteristic manifestation of some bacterial agents (Caswell et al., 2012; López & Martinson, 2017) associated with BRD. A metagenomic study identified multiple associations between pulmonary disease patterns and bacterial agents: *H. somni* and *M. haemolytica* with suppurative bronchopneumonia (Zhang et al., 2020). However, while caseonecrotic bronchopneumonia (Figure 2D) is the classic pattern of chronic *M. bovis*-induced pneumonia (Maunsell & Donovan, 2009; Margineda et al., 2017; Oliveira & Pelaquim, et al., 2020), other histopathological manifestations of bronchopneumonia and bronchointerstitial pneumonia have been observed (Caswell & Archambault, 2007; Nicholas, 2011; Oliveira & Pelaquim, et al., 2020).

Consequently, histopathological patterns alone should not be used to differentiate between infectious agents of BRD, since most viral disease pathogens induce interstitial pneumonia (Caswell & Williams, 2016; López & Martinson, 2017), further studies detailing the histologic lesions induced by each agent in the pulmonary tissue of cattle are needed. There are indications that BoAHV1-induced pulmonary disease can be differentiated from that associated with other viral agents due to bronchial angiogenesis, associated with the formation of granulation tissue, observed in cattle with BRD (Oliveira, Pelaquim, et al., 2020). However, additional studies are needed to confirm these associations between histological changes with specific viral agents of BRD.

The diagnosis of the agents of BRD using analyses that detect intralesional antigens or proteins, such as IHC and ISH, is perhaps one of the most efficient methods to define the association between infectious agents, the induced morphological alteration, and subsequent clinical manifestation. Additionally, the results of direct culture, IHC, and ISH are more easily interpreted regarding the cause and effect associated with any infectious agent of BRD (Fulton & Confer, 2012), is widely used to detect intralesional antigens of a vast majority of infectious agents (Figure 2E-H) of BRD in national (Flores et al., 2000; Oliveira, Pelaquim, et al., 2020), and international (Gagea et al., 2006; Ramírez Romero et al., 2010; Margineda et al., 2017) studies. Among the disadvantages of IHC (Fulton & Confer, 2012), availability and cost of monoclonal or polyclonal antibodies, especially for emerging agents (Fulton & Confer, 2012) must be highlighted. Another limiting factor of IHC is that prolonged fixation in formalin solution could affect the antigenic recovery and the expected result. Consequently, it is recommended for prolonged maintenance, that tissues already fixed in formalin solution be maintained in 70% alcohol solution, as alcohol interrupts the formation of cross-links and facilitates the detection of antigens for further evaluation by IHC (Ramos-Vara & Miller, 2014).

Figure 2 – Histopathological and immunohistochemical findings observed in bovine respiratory disease. There is interstitial pneumonia associated with viral infection (A) and the accumulation of neutrophilic exudate (star) within the lung of a cow with purulent bronchopneumonia (B-C). Observe the typical centrally located region of caseonecrotic bronchopneumonia that is characteristic for *Mycoplasma*

bovis (D) and positive immunoreactivity to antigens of *M. bovis* at the rim of the necrotic zone (E). There is positive immunolabeling for antigens of BRSV at the bronchial epithelium (F), for BoAHV1 within chondrocytes of the hyaline cartilage of the bronchus (G), and for BVDV within epithelial cells of the mixed peribronchial glands (H). A-D, Hematoxylin, and eosin stain; E-H, immunoperoxidase counterstained with hematoxylin. Bars, A, D, E, 200µm; B, C, G, 100µm; F, H, 50µm.



5.6.5 Prevention and Control of BRD

The implementation of BRD prophylaxis and control systems could substantially reduce economic losses due to morbidity and mortality in cattle. Control of BRD can best be accomplished by focusing on integrated programs of vaccination against pathogens causing BRD and improvements in nutritional status, especially for calves exposed to stress conditions.

Among the prevention strategies, vaccination against the main causative agents of BRD plays an important role in feedlot cattle, since animals immunized animals and booster vaccination prior to entering the feedlot were found to be 2.5 times less likely to develop BRD than those that received vaccination when entering the feedlot (Magalhães et al., 2017).

Another important factor is the reduction of stress caused by management practices (Urban-Chmiel & Grooms, 2012). Management that advocates the maintenance of animal welfare is considered an important tool for disease prevention, especially in confined cattle (Tucker, Coetzee, Stookey, Thomson, & Grandin, 2015). Measures to reduce exposure to pathogens of BRD should be taken by reducing the risk of infected animals being introduced into the herd by monitoring introduced animals via quarantine and the isolating of sick cattle (Urban-Chmiel & Grooms, 2012).

Mass treatment is based on the administration of antibiotics with prolonged action for the control of bovine respiratory disease. This practice is quite common and has proven effective in reducing morbidity and mortality rates arising from BRD (Taylor et al., 2010).

In Brazil, a study using oxytetracycline and tildipirosin in metaphylactic protocols for prophylaxis of BRD demonstrated that these drugs were efficient in reducing both morbidity and lung lesions in feedlot cattle (Magalhães et al., 2017). Florfenicol has also been shown to be efficient in the metaphylaxis of BRD in feedlot cattle, reducing clinical manifestations and improving the development of the animals economically viable (Catry, Duchateau, Van de Ven, Laevens, & Opsomer, 2008).

5.7 CONCLUSION

Bovine respiratory disease is endemic in the main cattle producing regions of Brazil. The principal causative agents identified in association with BRD in cattle from Brazil are BRSV, BVDV, BoAHV1, and *M. bovis*. Nevertheless, there are emerging evidence that OvHV-2 and HoBi-like pestivirus may be important agents in the development of BRD. Although, initial studies have identified high mortality and morbidity rates resulting in elevated costs related to economic losses in affected cattle from Brazil, additional surveys from other geographical regions are necessary to understand the exact situation locally.

5.8 REFERENCES

Andrews, G. A., & Kennedy, G. A. (1997). Respiratory diagnostic pathology. The Veterinary Clinics of North America. Food Animal Practice, 13(3), 515-547. doi: 10. 1016/s0749-0720(15)30312-1

Apley, M. (2006). Bovine respiratory disease: pathogenesis, clinical signs, and treatment in lightweight calves. The Veterinary Clinics of North America. Food Animal Practice, 22(2), 399-411. doi: 10.1016/j. cvfa.2006.03.009

Baptista, A. L., Rezende, A. L., Fonseca, P. A., Nogueira, G. M., Headley, S. A., Menezes, G. L., Alfieri, A. A., & Saut, J. P. E. (2017). Bovine respiratory disease complex associated mortality and morbidity rates in feedlot cattle from southeastern Brazil. Journal of Infection in Developing Countries, 11(10), 791-799. doi: 10.3855/jidc.9296

Bauermann, F. V., Ridpath, J. F., Weiblen, R., & Flores, E. F. (2013). HoBi-like viruses: an emerging group of pestiviruses. Journal of Veterinary Diagnostic Investigation, 25(1), 6-15. doi: 10.1177/10406387124 73103

Beuttemmuller, E. A., Alfieri, A. F., Headley, S. A., & Alfieri, A. A. (2017). Brazilian strain of bovine respiratory coronavirus is derived from dual enteric and respiratory tropism. Genetics and Molecular Research, 16(2), gmr16029580. doi: 10.4238/gmr16029580

Blakebrough-Hall, C., McMeniman, J. P., & González, L. A. (2020). An evaluation of the economic effects of bovine respiratory disease on animal performance, carcass traits, and economic outcomes in feedlot cattle defined using four BRD diagnosis methods. Journal of Animal Science, 98(2), 1-11. doi: 10.10 93/jas/skaa005

Booker, C. W., Abutarbush, S. M., Morley, P. S., Jim, G. K., Pittman, T. J., Schunicht, O. C., Janzen, E. D. (2008). Microbiological and histopathological

findings in cases of fatal bovine respiratory disease of feedlot cattle in Western Canada. Canadian Veterinary Journal, 49(5), 473-481. Retrieved from https://www.ncbi.nlm.nih. gov/pmc/articles/PMC2359492/pdf/cvj49 pg473.pdf

Brasil, N. D. A., Hinnah, F. L., Fiss, L., Sallis, E. S. V., Grecco, F. B., Ladeira, S. R. L., Marcolongo- Pereira, C., & Schild, A. L. (2013). Doenças respiratórias em bezerros na região sul do Rio Grande do Sul: estudo retrospectivo de 33 surtos. Pesquisa Veterinária Brasileira, 33(6), 745-751. doi: 10.1590/ S0100-736X2013000600010

Caswell, J. L., & Archambault, M. (2007). *Mycoplasma bovis* pneumonia in cattle. Animal Health Research Reviews, 8(2), 161- 186. doi: 10.1017/s1466252307001351

Caswell, J. L., Hewson, J., Slavić, Đ., DeLay, J., & Bateman, K. (2012). Laboratory and postmortem diagnosis of bovine respiratory disease. Veterinary Clinics of North America Food Animal Practice ,28 (3), 419-441. doi: 10.1016/j.cvfa.2012.07.004

Caswell, J. L., & Williams, K. J. (2016). Respiratory system. In: M. G. Maxie (Ed.), Jubb, Kennedy, and Palmer's Pathology of domestic animals (pp. 465-591). Pennsylvania, PA: Saunders/Elsevier.

Catry, B., Duchateau, L., Van de Ven, J., Laevens, H., Opsomer, G., Haesebrouck, F., & De Kruif, A. (2008). Efficacy of metaphylactic florfenicol therapy during natural outbreaks of bovine respiratory disease. Journal of Veterinary Pharmacology and Therapeutics, 31(5), 479-487. doi: 10.11 11/j.1365-2885.2008.00981.x

Ceribasi, A. O., Ozkaraca, M., Ceribasi, S., & Ozer, H. (2014). Histopathologic, immunoperoxidase and immunofluorescent examinations on natural cattle pneumonia originated from parainfluenza type 3, respiratory syncytial virus, adenovirus type 3 and herpesvirus type 1. Revue de Médecine Vétérinaire, 165(1), 201-212.

Cole, A. M. (1971). Experimental adenovirus pneumonia in calves. Australian Veterinary Journal, 47(7), 306-311. doi: 10.1111/j. 1751-0813.1971.tb15499.x

Cooper, V. L., & Brodersen, B. W. (2010). Respiratory disease diagnostics of cattle. Veterinary Clinics of North America. Food Animal Practice, 26(2), 409-416. doi: 10. 1016/j.cvfa.2010.04.009

Corbeil, L. B. (2007). *Histophilus somni* host- parasite relationships. Animal Health Research Reviews, 8(2), 151-160. doi: 10. 1017/S1466252307001417

Coutinho, A. d. S., Oliveira, J. P., Fo., Silva, D. P. G., Oliveira, A. P., Marcondes, J. S., Chiacchio, S. B.,.,... Gonçalves, R. C. (2009). Mannheimiose pulmonar experimental em bezerros: swab nasal e nasofaringeano como auxílio diagnóstico. Pesquisa Veterinária Brasileira, 29(1), 83-88. doi: 10.1590/S0100-736X2009000100013

878 Cusack, P., McMeniman, N., & Lean, I. (2003). The medicine and epidemiology of
879 bovine respiratory disease in feedlots. Australian Veterinary Journal, 81(8), 480-
880 487. doi: 10.1111/j.1751-0813.2003.tb13 367.x

881 Dabo, S. M., Taylor, J. D., & Confer, A. W. (2007).
882 and bovine respiratory disease. Animal Health Research Reviews, 8(2), 129-150.
883 doi: 10. 1017/s1466252307001399

884 Dane, H., Duffy, C., Guelbenzu, M., Hause, B., Fee, S., Forster, F., McMenamy,
885 M. J., & Lemon, K. (2019). Detection of influenza D virus in bovine respiratory
886 disease samples, UK. Transboundary and Emerging Diseases, 66(5), 2184-2187.
887 doi: 10.1111/tbed.13273

888 Decaro, N., Mari, V., Pinto, P., Lucente, M. S., Sciarretta, R., Cirone, F.,
889 Buonavoglia, C. (2012). Hobi-like pestivirus: both biotypes isolated from a
890 diseased animal. Journal of General Virology, 93(9), 1976-1983. doi:
891 10.1099/vir.0.044552-0

892 Dias, F. C., Médici, K. C., Alexandrino, B., Medeiros, A. S. R., Alfieri, A. A., &
893 Samara, S. I. (2010). Occurrence of persistently infected animals with bovine viral
894 diarrhoea virus in cattle herds from the States of Minas Gerais and São Paulo,
895 Brazil. Pesquisa Veterinária Brasileira, 30(11), 933-939. doi: 10.1590/S0100-73
896 6X2010001100006

897 Dias, J. A., Alfieri, A. A., Médici, K. C., Freitas, J. C., Ferreira, J. S. Neto, & Müller,
898 E. E. (2008). Fatores de risco associados à infecção pelo herpesvírus bovino 1 em
899 rebanhos bovinos da região Oeste do Estado do Paraná. Pesquisa Veterinária
900 Brasileira, 28(3), 161-168. Retrieved from: https://
901 www.scielo.br/pdf/pvb/v28n3/06.pdf

902 Dias, J. A., Alfieri, A. A., Ferreira-Neto, J. S., Gonçalves, V. S., & Muller, E. E.
903 (2013). Seroprevalence and risk factors of bovine herpesvirus 1 infection in cattle
904 herds in the state of Paraná, Brazil. Transboundary and Emerging Diseases, 60(1),
905 39-47. doi: 10.1111/j.1865-1682.2012.01316.x

906 Driemeier, D., Gomes, M. J. P., Moojen, V., Arns, C. W., Vogg, G., Kessler, L., &
907 Costa, U. M.D. (1997). Manifestação clínico-patológica de infecção natural pelo
908 vírus respiratório sincicial bovino (BRSV) em bovinos de criação extensiva no Rio
909 Grande do Sul, Brasil. Pesquisa Veterinária Brasileira, 17(2), 77-81. doi:
910 10.1590/S0100-736X19 97000200006

911 Dubrovsky, S. A., Van Eenennaam, A. L., Karle, B. M., Rossitto, P. V., Lehenbauer,
912 T. W., & Aly, S. S. (2019). Bovine respiratory disease (BRD) cause-specific and
913 overall mortality in preweaned calves on California dairies: The BRD 10K study.
914 Journal of Dairy Science, 102(8), 7320-7328. doi: 10.3168/ jds. 2018-15463

915 Ferguson, L., Eckard, L., Epperson, W. B., Long, L. P., Smith, D., Huston, C., Wan,
916 X. F. (2015). Influenza D virus infection in Mississippi beef cattle. Virology, 486(12),
917 28-34. doi: 10.1016/j.virol.2015.08.030

Flores, E. F., Weiblen, R., Medeiros, M., Botton, S. A., Irigoyen, L. F., Driemeier, D., Schuch, L. F., & Moraes, M. (2000). A retrospective search for bovine respiratory syncytial virus (BRSV) antigens in histological specimens by immunofluorescence and immunohistochemistry. Pesquisa Veterinária Brasileira, 20(4), 139-143. doi: 10.1590/S0100-736X2000000400002

Flores, E. F., Weiblen, R., Vogel, F. S. F., Roehe, P. M., Alfieri, A. A., & Pituco, E. M. (2005). A infecção pelo vírus da diarréia viral bovina (BVDV) no Brasil: histórico, situação atual e perspectivas. Pesquisa Veterinária Brasileira, 25(3), 125-134. doi: 10.1590/ S0100-736X2005000300002

Francoz, D., Buczinski, S., Belanger, A. M., Forte, G., Labrecque, O., Tremblay, D., Wellemans, V., & Dubuc, J. (2015). Respiratory pathogens in Quebec dairy calves and their relationship with clinical status, lung consolidation, and average daily gain. Journal of Veterinary Internal Medicine, 29(1), 381-387. doi: 10.1111/ jvim.12531

Fulton, R. W., Purdy, C. W., Confer, A. W., Saliki, J. T., Loan, R. W., Briggs, R. E., & Burge, L. J. (2000). Bovine viral diarrhea viral infections in feeder calves with respiratory disease: interactions with Pasteurella spp., parainfluenza-3 virus, and bovine respiratory syncytial virus. Canadian Journal of Veterinary Research, 64(3), 151-159. Retrieved from http://www.ncbi. nlm.nih.gov/pmc/articles/PMC1189606/

Fulton, R. W., Cook, B. J., Step, D. L., Confer, A. W., Saliki, J. T., Payton, M. E., … Blood, K. S. (2002). Evaluation of health status of calves and the impact on feedlot performance: assessment of a retained ownership program for postweaning calves. Canadian Journal of Veterinary Research, 66(3), 173-180. Retrieved from https:// pubmed.ncbi.nlm.nih.gov/12146889

Fulton, R. W., Briggs, R. E., Payton, M. E., Confer, A. W., Saliki, J. T., Ridpath, J. F., Burge, L. J., & Duff, G. C. (2004). Maternally derived humoral immunity to bovine viral diarrhea virus (BVDV) 1a, BVDV1b, BVDV2, bovine herpesvirus-1, parainfluenza-3 virus bovine respiratory syncytial virus, *Mannheimia haemolytica* and Pasteurella multocida in beef calves, antibody decline by half-life studies and effect on response to vaccination. Vaccine, 22(5-6), 643-649. doi: 10.1016/j.vaccine.2003.08.033

Fulton, R. W. (2009). Bovine respiratory disease research (1983-2009). Animal Health Research Reviews, 10(2), 131-139. doi: 10.1017/s146625230999017x

Fulton, R. W., Blood, K. S., Panciera, R. J., Payton, M. E., Ridpath, J. F., Confer, A. W.,… Reck, A. (2009). Lung pathology and infectious agents in fatal feedlot pneumonias and relationship with mortality, disease onset, and treatments. Journal of Veterinary Diagnostic Investigation, 21(4), 464-477. doi: 10.1177/104063870902100407

Fulton, R. W., Step, D. L., Wahrmund, J., Burge, L. J., Payton, M. E., Cook, B. J.,… Confer, A. W. (2011). Bovine coronavirus (BCV) infections in transported commingled beef cattle and sole-source ranch calves. Canadian Journal of

959 Veterinary Research, 75(3), 191-199. Retrieved from http://www.
960 ncbi.nlm.nih.gov/pmc/articles/PMC31 22965/

961 Fulton, R. W., & Confer, A. W. (2012). Laboratory test descriptions for bovine
962 respiratory disease diagnosis and their strengths and weaknesses: Gold standards
963 for diagnosis, do they exist? Canadian Veterinary Journal, 53(7), 754-761.
964 Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/ PMC3377458/

965 Gaeta, N. C., Ribeiro, B. L. M., Alemán, M. A. R., Yoshihara, E., Marques, E. C.,
966 Hellmeister, A. N., Pituco, E. M., & Gregory, L. (2018). Serological investigation of
967 antibodies against respiratory viruses in calves from Brazilian family farming and
968 their relation to clinical signs of bovine respiratory disease. Pesquisa Veterinária
969 Brasileira, 38(4), 642-648. doi: 10.1590/1678-5150- pvb-5234

970 Gaeta, N. C., Ribeiro, B. L. M., Alemán, M. A. R., Yoshihara, E., Nassar, A. F. C.,
971 Marques, L. M., Timenetsky, J., & Gregory, L. (2018). Bacterial pathogens of the
972 lower respiratory tract of calves from Brazilian rural settlement herds and their
973 association with clinical signs of bovine respiratory disease. Pesquisa Veterinária
974 Brasileira, 38(3), 374-381. doi: 10.1590/1678-5150-PVB-5323

975 Gagea, M. I., Bateman, K. G., van Dreumel, T., McEwen, B. J., Carman, S.,
976 Archambault, M., Shanahan, R. A., & Caswell, J. L. (2006). Diseases and
977 pathogens associated with mortality in Ontario beef feedlots. Journal of Veterinary
978 Diagnostic Investigation, 18(1), 18-28. Retrieved from https://
979 journals.sagepub.com/doi/pdf/10.1177/ 104063870601800104

980 Gershwin, L. J., Van Eenennaam, A. L., Anderson, M. L., McEligot, H. A., Shao,
981 M. X., Toaff-Rosenstein, R.,… Womack, J. (2015). Single pathogen challenge with
982 agents of the bovine respiratory disease complex. PLoS One, 10(11), e0142479.
983 doi: 10.1371/journal.pone.0142479

984 Gonçalves, D. A., Spilki, F. R., Chiminazzo, C., Oliveira, M. A., Franco, A. C., &
985 Roehe, P. M. (2003). Isolamento do vírus parainfluenza bovino tipo 3 no Rio
986 Grande do Sul, Brasil. Ciência Rural, 33(5), 953-956. doi:
987 10.1590/S010384782003000500025

988 Gonçalves, I., Simanke, A., Jost, H., Hôtzel, I., Soglio, A., & Moojen, V. (1993).
989 Detection of bovine respiratory syncytial virus in calves of Rio Grande do Sul,
990 Brazil. Ciência Rural, 23(3), 389-390. doi: 10.1590/S0103-84781993000300029

991 Griffin, D., Chengappa, M. M., Kuszak, J., & McVey, D. S. (2010). Bacterial
992 pathogens of the bovine respiratory disease complex. Veterinary Clinics of North
993 America. Food Animal Practice, 26(2), 381-394. doi: 10. 1016/j.cvfa.2010.04.004

994 Grissett, G. P., White, B. J., & Larson, R. L. (2015). Structured literature review of
995 responses of cattle to viral and bacterial pathogens causing bovine respiratory
996 disease complex. Journal of Veterinary Internal Medicine, 29(3), 770-780. doi:
997 10.1111/ jvim.12597

Hay, K. E., Morton, J. M., Mahony, T. J., Clements, A. C. A., & Barnes, T. S. (2016). Associations between animal characteristic and environmental risk factors and bovine respiratory disease in Australian feedlot cattle. Preventive Veterinary Medicine, 25(3), 66-74. doi:10.1016/j.prevetmed.2016.01.013

Headley, S. A., Oliveira, V. H., Figueira, G. F., Bronkhorst, D. E., Alfieri, A. F., Okano, W., & Alfieri, A. A. (2013). *Histophilus somni*-induced infections in cattle from Southern Brazil. Tropical Animal Health and Production, 45(7), 1579-1588. doi: 10.1007/s11250-013-0402-7

Headley, S. A., Alfieri, A. F., Oliveira, V. H., Beuttemmuller, E. A., & Alfieri, A. A. (2014). *Histophilus somni* is a potential threat to beef cattle feedlots in Brazil. Veterinary Record, 175(10), 249. doi: 10.11 36/ vr.102562.

Headley, S. A., Bracarense, A. P. F. R. L., Oliveira, V. H. S., Queiroz, G. R., Okano, W., Alfieri, A. F.,... Alfieri, A. A. (2015). *Histophilus somni*- induced thromboticmeningoencephalitis in cattle from northern Paraná, Brazil. Pesquisa Veterinária Brasileira, 35(4), 329- 336. doi: 10.1590/S0100-736X20150004 00003

Headley, S. A., Balbo, L. C., Alfieri, A. F., Saut, J. P. E., Baptista, A. L., & Alfieri, A. A. (2017). Bovine respiratory disease associated with *Histophilus somni* and bovine respiratory syncytial virus in a beef cattle feedlot from Southeastern Brazil. Semina: Ciências Agrárias, 38(1), 283-294. doi: 10.5433/16790359.2017v38n1p283

Headley, S. A., Okano, W., Balbo, L. C., Marcasso, R. A., Oliveira, T. E., Alfieri, A. F.,... Alfieri, A. A. (2017). Molecular survey of infectious agents associated with bovine respiratory disease in a beef cattle feedlot in southern Brazil. Journal of Veterinary Diagnostic Investigation, 30(2), 249-251. doi: 10.1177/1040638717739945

Headley, S. A., Pereira, A. H. T., Balbo, L. C., Santia, G. W. D., Bracarense, A. P. F. R. L., Cunha, L. F. C. F.,.. Alfieri, A. A. (2018). *Histophilus somni*-associated syndromes in sheep from Southern Brazil. Brazilian Journal of Microbiology, 49(3), 591-600. doi: 10.1016/j.bjm.2017.12.008

Headley, S. A., Müller, M. C., Oliveira, T. E. S., Duarte, C. A. B. G., Pereira, P. F. V., Vieira, M. V.,... Pretto-Giordano, L. G. (2020). Diphtheric aspergillosis tracheitis with gastrointestinal dissemination secondary to viral infections in a dairy calf. Microbial Pathogenesis, 149(12), 104497. doi: 10.10 16/j.micpath.2020.104497

Headley, S. A., Oliveira, T. E. S., & Cunha, C. W. (2020). A review of the epidemiological, clinical, and pathological aspects of malignant catarrhal fever in Brazil. Brazilian Journal of Microbiology, 51(3), 1405-1432. doi: 10.1007/s42770-020-00273-6

Headley, S. A., Oliveira, T. E. S., Li, H., Lisbôa, J. A. N., Queiroz, G. R., Fritzen, J. T. T., Flores, E. F., Alfieri, A. A., & Cunha, C. W. (2020). Immunohistochemical detection of intralesional antigens of ovine gammaherpesvirus-2 in cattle with

1038 sheep- associated malignant catarrhal fever. Journal of Comparative Pathology,
1039 174(1), 86-98. doi: 10.1016/j.jcpa.2019.11.002

1040 Hessman, B. E., Fulton, R. W., Sjeklocha, D. B., Murphy, T. A., Ridpath, J. F., &
1041 Payton, M. E. (2009). Evaluation of economic effects and the health and
1042 performance of the general cattle population after exposure to cattle persistently
1043 infected with bovine viral diarrhea virus in a starter feedlot. American Journal of
1044 Veterinary Research, 70(1), 73-85. doi: 10.2460/ajvr.70.1.73

1045 Hick, P. M., Read, A. J., Lugton, I., Busfield, F., Dawood, K. E., Gabor, L.,
1046 Hornitzky, M., & Kirkland, P. D. (2012). Coronavirus infection in intensively
1047 managed cattle with respiratory disease. Australian Veterinary Journal, 90(10),
1048 381-386. doi: 10.1111/j.1751-0813.2012.00978.x

1049 Hoppe, I., Souza-Pollo, A., Medeiros, A. S. R., Samara, S. I., & Carvalho, A. A. B.
1050 (2019). HoBi-like pestivirus infection in an outbreak of bovine respiratory disease.
1051 Research in Veterinary Science, 126, 184-191. doi: 10.1016/j.rvsc.2019.09.003

1052 Ide, P. R., Thomson, R. G., & Ditchfield, W.J. (1969). Experimental adenovirus
1053 infection in calves. Canadian Journal of Comparative Medicine, 33(1), 1-9.
1054 Retrieved from https://pubmed.ncbi.nlm. nih.gov/4304091

1055 Jim, K. (2009). Impact of bovine respiratory disease (BRD) from the perspective of
1056 the Canadian beef producer. Animal Health Research Reviews, 10(2), 109-110.
1057 doi: 10.1017/s1466252309990119

1058 Klima, C. L., Zaheer, R., Cook, S. R., Booker, C. W., Hendrick, S., Alexander, T.
1059 W., & McAllister, T. A. (2014). Pathogens of bovine respiratory disease in North
1060 American feedlots conferring multidrug resistance via integrative conjugative
1061 elements. Journal of Clinical Microbiology, 52(2), 438. doi: 10. 1128/JCM.0248513

1062 Klima, C. L., Holman, D. B., Ralston, B. J., Stanford, K., Zaheer, R., Alexander, T.
1063 W., & McAllister, T. A. (2019). Lower respiratory tract microbiome and resistome
1064 of bovine respiratory disease mortalities. Microbial Ecology, 78(2), 446-456. doi:
1065 10.1007/s00248-019-01361-3

1066 Lancaster, M. J., McGillivery, D. J., Patterson, R. M., & Irwin, S. (1984). Pneumonia
1067 associated with *Haemophilus somnus* in a calf. Australian Veterinary Journal,
1068 61(8), 269. https://doi.org/10.1111/j.1751-0813.1984.tb15545.x

1069 Larsen, L., Tegtmeier, C., & Pedersen, E. (2001). Bovine respiratory syncytial virus
1070 (BRSV) pneumonia in beef calf herds despite vaccination. Acta Veterinaria
1071 Scandinavica, 42, 113-121, doi:10.1186/1751-014742-113.

1072 Leland, D. S., & French, M. L. V. (1988). Virus Isolation and Identification. In E. H.
1073 Lennette, P. Halonen, F. A. Murphy, A. Balows, & W. J. Hausler Jr. (Eds.),
1074 Laboratory diagnosis of infectious diseases principles and practice (pp. 39- 59).
1075 New York, NY: Springer.

1076 Loneragan, G. H., Dargatz, D. A., Morley, P. S., & Smith, M. A. (2001). Trends in
1077 mortality ratios among cattle in US feedlots. Journal of the American Veterinary
1078 Medical Association, 219(8), 1122-1127. doi: 10. 2460/javma.2001.219.1122.

1079 López, A., & Martinson, S. A. (2017). Respiratory system, mediastinum, and
1080 pleurae. In: J. F. Zachary (Ed.), Pathologic basis of veterinary disease (pp. 471-
1081 560). St. Louis, Missouri: Elsevier.

1082 Luo, J., Ferguson, L., Smith, D. R., Woolums, A. R., Epperson, W. B., & Wan, X.
1083 F. (2017). Serological evidence for high prevalence of Influenza D Viruses in
1084 Cattle, Nebraska, United States, 2003-2004. Virology, 501(1), 88-91. doi:
1085 10.1016/j.virol.2016.11.004

1086 Maes, R. K., Langohr, I. M., Wise, A. G., Smedley, R. C., Thaiwong, T., & Kiupel,
1087 M. (2014). Beyond H&E: integration of nucleic acid- based analyses into diagnostic
1088 pathology. Veterinary Pathology, 51(1), 238-256. doi:
1089 10.1177/0300985813505878

1090 Magalhães, L. Q., Baptista, A. L., Fonseca, P. d. A., Menezes, G. L., Nogueira, G.
1091 M., Headley, S. A.,,,, Saut, J. P. E. (2017). Use of metaphylactic protocols based
1092 on the risk to develop bovine respiratory diseases in feedlot cattle. Ciência Rural,
1093 47(8), e20161110. doi: 10.1590/0103-8478cr20 161110

1094 Malafaia, P., Granato, T. A. L., Costa, R. M., Souza, V. C. d., Costa, D. F. A., &
1095 Tokarnia, C. H. (2016). Major health problems and their economic impact on beef
1096 cattle under two different feedlot systems in Brazil. Pesquisa Veterinária Brasileira,
1097 36(9), 837-843. doi: 10.1590/s0100-736x 2016000900008

1098 Margineda, C. A., Zielinski, G. O., Jurado, S., Alejandra, F., Mozgovoj, M., Alcaraz,
1099 A. C., & López, A. (2017). *Mycoplasma bovis* pneumonia in feedlot cattle and dairy
1100 calves in Argentina. Brazilian Journal of Veterinary Pathology, 10(2), 79-86. doi:
1101 10.24070/bjvp.1983-0246.v10i2p79-86

1102 Marques, L. M., Buzinhani, M., Oliveira, R. C., Yamaguti, M., Ferreira, J. B., Neto,
1103 R. L., & Timenetsky, J. (2007). Prevalence of mycoplasmas in the respiratory tracts
1104 of calves in Brazil. Veterinary Record, 161(20), 699-700. doi:
1105 10.1136/vr.161.20.699

1106 Martin, S. W., Bateman, K. G., Shewen, P. E., Rosendal, S., & Bohac, J. E. (1989).
1107 The frequency, distribution and effects of antibodies, to seven putative respiratory
1108 pathogens, on respiratory disease and weight gain in feedlot calves in Ontario.
1109 Canadian Journal of Veterinary Research, 53(3), 355-362. Retrieved from https://
1110 www.ncbi.nlm.nih.gov/pmc/articles/PMC 1255725/pdf/cjvetres00051-0103.pdf

1111 Mattson, D. E., Norman, B. B., & Dunbar, J. R. (1988). Bovine adenovirus type-3
1112 infection in feedlot calves. American Journal of Veterinary Research, 49(1), 67-69.
1113 Retrieved from https://pubmed.ncbi. nlm.nih.gov/2833138/

1114 Maunsell, F. P., & Donovan, G. A. (2009). *Mycoplasma bovis* infections in young
1115 calves. The Veterinary Clinics of North America. Food Animal Practice, 25(1), 139-
1116 177. doi: 10.1016/j.cvfa.2008.10.011

1117 Mitra, N., Cernicchiaro, N., Torres, S., Li, F., & Hause, B. M. (2016). Metagenomic
1118 characterization of the virome associated with bovine respiratory disease in feedlot
1119 cattleidentifiednovelvirusesandsuggests an etiologic role for influenza D virus. The
1120 Journal of General Virology, 97(8), 1771-1784. doi: 10.109 9/jgv.0.000492.

1121 Moore, S. J., O'Dea, M. A., Perkins, N., & O'Hara, A. J. (2015). Estimation of nasal
1122 shedding and seroprevalence of organisms known to be associated with bovine
1123 respiratory disease in Australian live export cattle. Journal of Veterinary Diagnostic
1124 Investigation, 27(1), 6-17. doi: 10.1177/104063871455 9741

1125 Murray, G. M., Cassidy, J. P., Clegg, T. A., Tratalos, J. A., McClure, J., O'Neill, R.
1126 G.,… S More, S. J. (2016). A retrospective epidemiological analysis of risk factors
1127 for a primary necropsy diagnosis of bovine respiratory disease. Preventive
1128 Veterinary Medicine, 132(9), 49-56. doi: 10.1016/j.prevetmed.20 16.08.009

1129 Murray, G. M., More, S. J., Sammin, D., Casey, M. J., McElroy, M. C., O'Neill, R.
1130 G.,… Cassidy, J. P. (2017). Pathogens, patterns of pneumonia, and epidemiologic
1131 risk factors associated with respiratory disease in recently weaned cattle in Ireland.
1132 Journal of Veterinary Diagnostic Investigation, 29(1), 20-34. doi:
1133 10.1177/1040638716674757

1134 Muylkens, B., Thiry, J., Kirten, P., Schynts, F., & Thiry, E. (2007). Bovine
1135 herpesvirus 1 infection and infectious bovine rhinotracheitis. Veterinary Research,
1136 38(2), 181-209. doi: 10.1051/vetres:2006059

1137 Ng, T. F., Kondov, N. O., Deng, X., Van Eenennaam, A., Neibergs, H. L., &
1138 Delwart, E. (2015). A metagenomics and case-control study to identify viruses
1139 associated with bovine respiratory disease. Journal of Virology, 89(10), 5340-
1140 5349. doi: 10.1128/ jvi.00064-15

1141 Nicholas, R. A. (2011). Bovine mycoplasmosis: silent and deadly. Veterinary
1142 Record, 168(17), 459-462. doi: 10.1136/vr.d2468O'Toole, D., Allen, T., Hunter, R.,
1143 & Corbeil, L. B. (2009). Diagnostic exercise: myocarditis due to *Histophilus somni*
1144 in feedlot and backgrounded cattle. Veterinary Pathology, 46(5), 1015-1017. doi:
1145 10.13 54/vp.08-VP-0332-O-DEX

1146 O'Toole, D., & Sondgeroth, K. S. (2016). Histophilosis as a natural disease. In: R.
1147 Ahmed, Akira, S., Aktories, K., Casadevall, A., Compans, R. W., Galan, J. E.,
1148 Garcia- Sastre, A., Malissen, B., Rappuoli, R. (Eds.), Current Topics in
1149 Microbiology and Immunology (pp. 15-48). Pennsylvania, PA: Springer.

1150 Oliveira, T. E. S., Pelaquim, I. F., Flores, E. F., Massi, R. P., Valdiviezo, M. J. J.,
1151 Pretto-Giordano, L. G.,... Headley, S. A. (2020). *Mycoplasma bovis* and viral
1152 agents associated with the development of bovine respiratory disease in adult

1153 dairy cows. Transboundary and Emerging Diseases, 67(Suppl 2), 82- 93. doi:
1154 10.1111/tbed.13223

1155 Oliveira, V. H. S., Dall Agnol, A. M., Fritzen, J. T. T., Lorenzetti, E., Alfieri, A. A., &
1156 Alfieri, A. F. (2020). Microbial diversity involved in the etiology of a bovine
1157 respiratory disease outbreak in a dairy calf rearing unit. Comparative Immunology,
1158 Microbiology and Infectious Diseases, 71(8), 101494. doi: 10.
1159 1016/j.cimid.2020.101494

1160 Panciera, R. J., & Confer, A. W. (2010). Pathogenesis and pathology of bovine
1161 pneumonia. The Veterinary Clinics of North America. Food Animal Practice, 26(2),
1162 191-214. doi: 10.1016/j.cvfa.2010.04.001

1163 Pasqualotto, W., Sehnem, S., & Winck, C. A. (2015). Incidência de rinotraqueíte
1164 infecciosa bovina (IBR), diarreia viral bovina (BVD) e leptospirose em bovinos
1165 leiteiros da região Oeste de Santa Catarina - Brasil. Revista em Agronegócio e
1166 Meio Ambiente, 8(2), 249-270. Retrieved from https://
1167 periodicos.unicesumar.edu.br/index.php/ rama/article/view/ 3034/2595

1168 Philippou, S., Streckert, H. J., Otto, P., Reinhold, P., Elschner, M., Werchau, H., &
1169 Morgenroth, K. (1996). Morphological studies of the respiratory syncytial virus
1170 induced bronchiolitis in experimentally infected calves. Pathology, Research and
1171 Practice, 192(10), 1045-1056. doi: 10.1016/s0344-0338(96)80048-3

1172 Pretto, G. L., Müller, E. E., Freitas, J. C., Mettifogo, E., Buzinhanr, M., & Yamaguti,
1173 M. (2001). Isolamento de *Mycoplasma bovis* de bezerros com pneumonia.
1174 Veterinária Notícias, 7(1), 69-21.

1175 Ramírez Romero, R., Chavarría Martínez, B., Nevárez Garza, A. M., Rodríguez
1176 Tovar, L. E., Dávila Martínez, C., Hernández Vidal, G., Hernández Escareño, J. J.,
1177 & López Mayagoitia, A. (2010). Demostración inmunohistoquímica de
1178 *Mycoplasma bovis* em lesiones neumónicas crónicas enganado en corral de
1179 engorda. Veterinaria México, 41(4), 289-296. Retrieved from: http://
1180 www.scielo.org.mx/scielo.php?script=sci_ arttext&pid=S0301-5092201000040
1181 0005&nrm=iso

1182 Richter, V., Lebl, K., Baumgartner, W., Obritzhauser, W., Käsbohrer, A., & Pinior,
1183 B. (2017). A systematic worldwide review of the direct monetary losses in cattle
1184 due to bovine viral diarrhoea virus infection. Veterinary Journal, 220(2), 80-87. doi:
1185 10.1016/j.tvjl.2017.01.005

1186 Risalde, M. A., Molina, V., Sonchez-Cordon, P. J., Pedrera, M., Romero-Palomo,
1187 F., Bautista, M. J., Moreno, A., & Gomez- Villamandos, J. C. (2013). Comparison
1188 of pathological changes and viral antigen distribution in tissues of calves with and
1189 without preexisting bovine viral diarrhea virus infection following challenge with
1190 bovine herpesvirus-1. American Journal of Veterinary Research, 74(4), 598-610.
1191 doi: 10.2460/ajvr.74.4.598

1192 Rodriguez, F., Bryson, D. G., Ball, H. J., & Forster, F. (1996). Pathological and
1193 immunohistochemical studies of natural and experimental *Mycoplasma bovis*
1194 pneumonia in calves. Journal of Comparative Pathology, 115(2), 151-162. doi:
1195 10.1016/s0021-9975(96)80037-5

1196 Sacco, R. E., McGill, J. L., Pillatzki, A. E., Palmer, M. V., & Ackermann, M. R.
1197 (2014). Respiratory syncytial virus infection in cattle. Veterinary Pathology, 51(2),
1198 427-436. doi: 10.1177/03009858135013 41

1199 Schibrowski, M. L., Gibson, J. S., Hay, K. E., Mahony, T. J., & Barnes, T. S. (2018).
1200 *Mycoplasma bovis* and bovine respiratory disease: A risk factor study in Australian
1201 feeder cattle. Preventive Veterinary Medicine, 157(9), 152-161. doi: 10.1016/j.
1202 prevetmed.2018.06.005

1203 Schneider, M. J., Tait, R. G., Jr., Busby, W. D., & Reecy, J. M. (2009). An
1204 evaluation of bovine respiratory disease complex in feedlot cattle: Impact on
1205 performance and carcass traits using treatment records and lung lesion scores.
1206 Journal of Animal Science, 87(5), 1821-1827. doi: 10.2527/ jas.2008-1283

1207 Snowder, G. D., Van Vleck, L. D., Cundiff, L. V., & Bennett, G. L. (2006). Bovine
1208 respiratory disease in feedlot cattle: environmental, genetic, and economic factors.
1209 Journal of Animal Science, 84(8), 1999-2008. doi: 10.2527/jas.2006-046

1210 Taylor, J. D., Fulton, R. W., Lehenbauer, T. W., Step, D. L., & Confer, A. W. (2010).
1211 The epidemiology of bovine respiratory disease: What is the evidence for
1212 predisposing factors? Canadian Veterinary Journal, 51(10), 1095-1102.

1213 Timsit, E., Hallewell, J., Booker, C., Tison, N., Amat, S., & Alexander, T. W. (2017).
1214 Prevalence and antimicrobial susceptibility of *Mannheimia haemolytica,*
1215 *Pasteurella multocida*, and *Histophilus somni* isolated from the lower respiratory
1216 tract of healthy feedlot cattle and those diagnosed with bovine respiratory disease.
1217 Veterinary Microbiology, 208(9), 118-125. doi: 10.10 16/j.vetmic.2017.07.013

1218 Tortorelli, G., Carrillo Gaeta, N., Mendonça Ribeiro, B. L., Miranda Marques, L.,
1219 Timenetsky, J., & Gregory, L. (2017). Evaluation of Mollicutes microorganisms in
1220 respiratory disease of cattle and their relationship to clinical signs. Journal of
1221 Veterinary Internal Medicine, 31(4), 1215- 1220. doi: 10.1111/ jvim.14721.

1222 Tucker, C. B., Coetzee, J. F., Stookey, J. M., Thomson, D. U., Grandin, T., &
1223 Schwartzkopf-Genswein, K. S. (2015). Beef cattle welfare in the USA: identification
1224 of priorities for future research. Animal Health Research Reviews, 16(2), 107-124.
1225 doi: 10.1017/s1466252315000171

1226 Tuncer, P., & Yeşilbağ, K. (2015). Serological detection of infection dynamics for
1227 respiratory viruses among dairy calves. Veterinary Microbiology, 180(3-4), 180-
1228 185. doi: 10.1016/j.vetmic.2015.08.025

1229 Urban-Chmiel, R., & Grooms, D. L. (2012). Prevention and control of bovine
1230 respiratory disease. Journal of Livestock Science, 3(1), 27-36. Retrieved from

https://livestockscience.in/wp-content/ uploads/2012/ Bovine_Respiratory_ Disease. Pdf

USDA. Death loss in U.S. cattle and calves due to predator and nonpredator causes, 2015. Centre Avenue: Animal and Plant Health Inspection Service (APHIS), Veterinary Services (VS), National Animal Health Monitoring System (NAHMS), United States Department of Agriculture, 2017.77 p. Retrieved from https://www.aphis. usda.gov/animal_health/nahms/general/ downloads/cattle_calves_deathloss_ 2015.pdf

Vaucher,R. A.,Dezen,D.,Simonetti,A.B.,Spilki, F. R., & Roehe, P. M. (2011). Phylogenetic characterization of bovine parainfluenza 3 from contaminated cell cultures and field isolates from Brazil. Brazilian Journal of Microbiology, 42(4), 1440-1444. doi: 10.1590/S1517-83822011000400029

Warren, L. M., Babiuk, L. A., & Campos, M. (1996). Effects of BHV-1 on PMN adhesion to bovine lung endothelial cells. Veterinary Immunology and Immunopathology, 55(1-3), 73-82. doi: 10.1016/s0165-2427(96)05630-9

Wilson, B. K., Step, D. L., Maxwell, C. L., Wagner, J. J., Richards, C. J., & Krehbiel, C. R. (2015). Evaluation of multiple ancillary therapies used in combination with an antimicrobial in newly received high-risk calves treated for bovine respiratory disease. Journal of Animal Science, 93(7), 3661-3674. doi: 10.2527/jas.2015-9023

Yarnall, M. J., & Thrusfield, M. V. (2017). Engaging veterinarians and farmers in eradicating bovine viral diarrhoea: a systematic review of economic impact. The Veterinary Record, 181(13), 347. doi: 10.1136/ vr.104370.

Zhang, M., Hill, J. E., Godson, D. L., Ngeleka, M., Fernando, C., & Huang, Y. (2020). The pulmonary virome, bacteriological and histopathological findings in bovine respiratory disease from western Canada. Transbound Emerg Dis, 67(2), 924-934. doi: 10.1111/tbed.13419

6 ARTIGO 2 – *MYCOPLASMA BOVIS* AND VIRAL AGENTS ASSOCIATED WITH THE DEVELOPMENT OF BOVINE RESPIRATORY DISEASE IN ADULT DAIRY COWS

Thalita Evani Silva Oliveira, Isadora Fernanda Pelaquim, Eduardo Furtado Flores, Rodrigo Pelisson Massi, Milton James Jiménez Valdiviezo, Lucienne Garcia Pretto-Giordano, Amauri Alcindo Alfieri, João Paulo Elsen Saut, Selwyn Arlington Headley. **Transboundary Emerging Disease**. 2020 Jul;67 Suppl 2:82-93. doi: 10.1111/tbed.13223.

6.1 ABSTRACT

The aetiology and pathologic findings of bovine respiratory disease (BRD) in adult dairy cows (*n*=35) from a commercial dairy herd in Southern Brazil were investigated. Pulmonary samples were examined for histopathologic patterns and specific features within these patterns, while immunohistochemical (IHC) assays were designed to detect the intralesional antigens of viral infectious disease agents and *Mycoplasma bovis*. Pneumonia was diagnosed in 91.4% (32/35) of these cases; neither pneumonia nor any of the infectious disease pathogens evaluated occurred in three cows. The presence of multiple respiratory pathogens in 75% (24/32) of these cases indicated the complex origin of pneumonia in cattle. Interstitial pneumonia, necrosuppurative bronchopneumonia and suppurative bronchopneumonia were the principal patterns of pulmonary disease identified by histopathology. The most frequent pathogens identified by IHC were bovine viral diarrhoea virus (BVDV; *n*=18), *M. bovis* (*n*=16) and bovine alphaherpesvirus type 1 (BoAHV1; *n*=14), followed by bovine respiratory syncytial virus (BRSV; *n*=11) and bovine parainfluenza virus type 3 (BPIV-3; *n*=5). Obliterative bronchiolitis and peribronchial lymphocytic cuffings were the characteristic histopathologic features associated with *M. bovis*. Necrohemorrhagic bronchitis with bronchial angiogenesis was associated with BoAHV1. Necrotizing bronchitis and bronchiolitis were associated with BVDV, BoAHV1 and BRSV. Ballooning degeneration of the bronchial and bronchiolar epithelia was associated with BRSV and BoAHV1. This is the first report from Brazil that correlated the histopathologic findings of BRD with the associated infectious disease agents by immunohistochemistry. *M. bovis* was frequently detected in the tissues of cows with

fatal pulmonary disease during this study and may be a possible primary disease pathogen associated with the development of BRD in dairy cows. Additionally, the histopathologic features identified within patterns of pulmonary disease during this investigation may be an efficient diagnostic tool to associate histopathologic findings with specific agents of BRD in dairy cows.

Key words: BoAHV1, bovine pulmonary mycoplasmosis, bovine respiratory syncytial virus, bovine viral diarrhoea virus, diagnostic immunohistochemistry, interstitial pneumonia, respiratory pathogens.

6.2 INTRODUCTION

The bovine respiratory disease (BRD) is a multifactorial and multi-aetiological disease associated with several infectious disease agents (Fulton et al., 2009; Panciera & Confer, 2010). Data relative to the occurrence of BRD in Brazil are scarce and incipient. Most of these investigations used polymerase chain reaction (PCR) and identified infectious agents of BRD such as *Histophilus somni* (Headley, Alfieri, Oliveira, Beuttemmuller, & Alfieri, 2014; Headley et al., 2018), bovine alphaherpesvirus type 1, BoAHV1 (Suarez Heinlein et al., 1993), bovine respiratory syncytial virus, BRSV (Arns et al., 2003; Headley et al., 2017), bovine viral diarrhoea virus, BVDV (Cortez et al., 2006; Flores, Ridpath, Weiblen, Vogel, & Gil, 2002; Otonel et al., 2014; Silveira et al., 2017), bovine coronavirus, BCoV (Headley et al., 2018), *Pasteurella multocida* (Baptista et al., 2017; Headley et al., 2018), *Mannheimia haemolytica* (Baptista et al., 2017; Headley et al., 2018) and *Mycoplasma bovis* (Tortorelli et al., 2017). Furthermore, studies done in Brazil using serology identified seropositivity to infectious disease agents including BoAHV1 (Barbosa, Brito, & Alfaia, 2005; Fernandes, Pimenta, Pituco, Brasil, & Azevedo, 2016), bovine parainfluenza virus type 3, BPIV-3 (Gonçalves et al., 2003), BRSV (Driemeier et al., 1997), and BVDV (Flores et al., 2005; Wageck Canal, Strasser, Hertig, Masuda, & Peterhans, 1998) and *M. bovis* (Pretto et al., 2001). Additionally, there is the isolation of *M. bovis* (Pretto et al., 2001), while few studies from Brazil have investigated only BRSV by immunohistochemistry, IHC (Brasil et al., 2013; Peixoto et al., 2000).

The detection of antigen coding sequences in tissues by PCR in the absence of histopathologic findings does not necessarily indicate that the identified

agent is associated with a specific lesion or disease (Maes et al., 2013). Disease due to infectious agents associated with BRD is confirmed by the simultaneous presence of pathogens within the affected tissues (Fulton & Confer, 2012). The IHC assay is a sensitive diagnostic technique that can be used to identify the intralesional presence of specific protein of infectious disease agents associated with histopathologic lesions in formalin-fixed paraffin embedded (FFPE) tissues sections (Fulton & Confer, 2012; Maes et al., 2013), and the results obtained are strong evidence of an associated disease process within the affected tissues (Fulton & Confer, 2012).

The disease pathogens associated with BRD have been evaluated extensively mainly in North America (Fulton, 2009; Fulton et al., 2009; Panciera & Confer, 2010; Wolfger, Timsit, White, & Orsel, 2015) and Australia (Cusack, McMeniman, & Lean, 2003; Hay, Morton, Mahony, Clements, & Barnes, 2016). However, only a few studies have used histopathologic diagnosis with related IHC assays to confirm the participation of infectious disease agents as- sociated with BRD (Gershwin et al., 2015; Haines, Martin, Clark, Jim, & Janzen, 2001; Haines et al., 2004; Rodríguez, Bryson, Ball, & Forster, 1996). This study describes the histopathologic patterns with associated histologic features and the IHC findings associated with *M. bovis* and four viral agents of BRD in a commercial dairy herd from Southern Brazil.

6.3 MATERIAL AND METHODS

6.3.1 Animals, clinical history, and study location

This study investigated the occurrence of infectious disease agents of BRD and the pathologic findings in Holstein cows (*n*=35) from a commercial dairy establishment in Eastern Central Paraná, Southern Brazil. This establishment consisted of 1,500 Holstein dairy cows with milk production of 45,000 L/day and an average of 29.2 L/cow/ day. Due to the purchase of heifer and cows from different neighbouring herds, as well as from farms from neighbouring cities, this farm is considered as an open dairy cattle herd.

Between January and September 2017, there were reports of recurrent respiratory distress of the affected dairy cows that demonstrated clinical signs of inappetence, reluctance to walk and pulmonary distress (dyspnea, extended head and neck, and audible noise when breathing) associated with BRD, and

eventually died spontaneously. Respiratory diseases were predominant in recently calved cows that demonstrated low morbidity (10%; 150/1,500) and mortality (2.3%; 35/1,500). Autopsies were performed by on-site veterinarians during this seven-month period as mortality occurred; pulmonary samples were collected and submitted for laboratory diagnosis after autopsy. The clinical course of the respiratory disease and possible antibiotic therapies are not known.

6.3.2 Histopathologic examination

Refrigerated pulmonary sections were submitted for pathologic diagnostics; tissues sections were fixed by immersion in 10% buffered formalin solution for 24 hr and then routinely processed for histopathologic evaluation with the haematoxylin and eosin (H&E) stain. Histopathologic patterns were classified (TESO, SAH) and recorded according to the presence/absence of bronchopneumonia (suppurative, necrosuppurative, fibrinous or fibrinosuppurative) and interstitial pneumonia. Additionally, the occurrence of specific histopathologic features associated with these patterns of pulmonary disease such as obliterative bronchiolitis, syncytial formation, necrotizing bronchitis/bronchiolitis/alveolitis, necrohemorrhagic bronchiolitis, abscesses and intralesional bacterial accumulations was identified and recorded. Furthermore, whenever necessary new histologic slides were evaluated with the Brown–Brenn modified staining technique to detect Gram-positive or Gram-negative bacteria, the Giemsa stain was used to detect intralesional accumulations of *Mycoplasma* spp.

6.3.3 Immunohistochemical identification of infectious agents associated with BRD

Immunohistochemistry assays were performed on lung sections to investigative the presence of five pathogens associated with BRD: BoAHV1, BRSV, BVDV, BPIV-3 and *M. bovis*. Selected FFPE tissue sections from the lung of each cow were prepared on silanized slides with Poly-l-lysine 0,1% (Sigma-Aldrich, St. Louis, MO, USA), deparaffinized, hydrated in alcohol baths and subjected to antigen retrieval. The dilutions of the monoclonal antibodies used during this investigation are shown in Table 1. Antigen retrieval (Table 1) was achieved by using citrate buffer (pH 6.0) or Tris-EDTA buffer with 0.05% Tween (pH 9.0). Both solutions were utilized with the

pressure cooker system (Electrolux Pressure Cooker PCC10, São Paulo, Brazil) for 5 min. Endogenous peroxidase was blocked with distilled water and hydrogen peroxide (6%) for 30 min in a dark chamber.

The primary incubation was achieved with the monoclonal antibodies shown in Table 1 during 24 hr at 4°C. Incubation with the secondary antibody SuperPicture™ Polymer Detection kit (Invitrogen Corporation, Camarillo, CA, USA) was done in a humid chamber for 30 min at 25°C, after which the chromo- gen 3,3′-diaminobenzidine (DAB, Invitrogen® Life Technologies, Frederick, MD, USA) was added for 4 min. The slides were counterstained with Harris' haematoxylin, dehydrated in successive alcohol baths to xylol and then assembled with commercial resins and coverslips. Positive controls consisted of pulmonary sections from other cases known to be infected with BRSV (Headley et al., 2018), BoAHV1 (Oliveira, Lorenzetti, Alfieri, & Lisbôa, 2015), BVDV (Lunardi, Headley, Lisboa, Amude, & Alfieri, 2008) and *M. bovis* (Pretto et al., 2001); positive controls for BPIV-3 were obtained from tissue culture maintained within our laboratory. Negative control consisted of using the same tissue, with substitution of the primary antibody by its diluent. Positive and negative controls were included in each IHC assay.

Table 1 – List of antibodies, dilutions, method of antigen retrieval and source manufactures of the immunohistochemical assays

Antibody (clone)	Antigen retrieval	Dilutio	Source
BoAHV1 (Mab gC-gIII)	Citrate buffer (pH 6.0)	1:700	VRMD (Pullman, WA, USA)
BPIV-3	EDTA buffer (pH 9.0)	1:40	Gently ceded by Dr. Eduardo F. Flores, UFSM
BRSV (15c7)	Citrate buffer (pH 6.0)	1:300	Gently ceded by Dr. Eduardo F. Flores, UFSM
BVDV (15c5)	Citrate buffer (pH 6.0)	1:1,500	Gently ceded by Dr. Eduardo F. Flores, UFSM
Mycoplasma bovis	Citrate buffer (pH 6.0)	1:10	Gently ceded by Dr. Lucienne Pretto-Giordano, UEL

Abbreviations: BoAHV1: bovine alphaherpesvirus type 1; BPIV-3: bovine parainfluenza virus type 3; BRSV: bovine respiratory syncytial virus; BVDV: bovine viral diarrhoea virus.

6.4 RESULTS

6.4.1 Histopathologic patterns and features associated with BRD

An overview of the principal histopathologic patterns of pulmonary disease observed in adult dairy cows during this study and their associated histologic features are given in Table 2. Pneumonia was diagnosed in 91.4% (32/35) of the affected cows, and at least one infectious disease agent was identified in each animal by IHC (46.8%; 15/32) was the most predominant pattern of pulmonary disease observed, followed by necrosuppurative bronchopneumonia (28.1%; 9/32; Figure 1a) with peribronchial lymphocytic cuffings (21.9%; 7/32), and suppurative bronchopneumonia (18.7%; 6/32). Additionally, two cows without histopathologic evidence of pneumonia had necrotizing bronchitis. Accumulations of intralesional, Giemsa-stained, coccoid bacteria were associated with necrosuppurative and suppurative bronchopneumonia; Gram-positive or Gram-negative bacteria were not detected by the modified Brown–Brenn stain.

Table 2 – Association of patterns of pulmonary disease with specific histologic features observed in dairy cows with bovine respiratory disease

Pulmonary patterns	Agent	Associated features
Interstitial pneumonia (*n*=15)	BVDV	Necrotizing bronchitis
	BoAHV1	Necrotizing bronchiolitis
	BRSV	Necrohemorrhagic bronchitis with angiogenesis
	BPIV-3	Syncytial formation
		Ballooning degeneration of bronchial/bronchiolar epithelium
		Hyperplasia of type II pneumocytes
Necrosuppurative bronchopneumonia (*n*=9)	*M. bovis*	Accumulations of intralesional Giemsa-stained coccoid bacteria
		Peribronchial lymphocytic cuffings
Suppurative bronchopneumonia (*n*=6)	*M. bovis*	Accumulations of intralesional Giemsa- stained coccoid bacteria
		Peribronchial lymphocytic cuffings
Without pneumonia (*n*=2)	BRSV	Necrotizing bronchitis

Abbreviations: BoAHV1: bovine alphaherpesvirus type 1; BPIV-3: bovine parainfluenza virus type 3; BRSV: bovine respiratory syncytial virus; BVDV: bovine viral diarrhoea virus; and *M. bovis: Mycoplasma bovis*.

The histopathologic features associated with the patterns of pneumonia are summarized in Table 3; these lesions included obliterative bronchiolitis (28.6%; 10/35; Figure 1b), necrotizing bronchi- tis (18.7%; 6/32) and bronchiolitis (12.5%; 4/32; Figure 1c), syncytial formation (12.5%; 4/32), ballooning degeneration of the bronchial (12.5%; 4/32; Figure 1d), and bronchiolar (3.1%; 1/32; Figure 1e) epithelia, hyperplasia of type II pneumocytes (6.2%; 2/32) and necrohemorrhagic bronchitis with angiogenesis at the lamina propria of the bronchus (6.2%; 2/32; Figure 1f).

Figure 1 – Histopathologic features observed in dairy cattle with bovine respiratory disease. (a) Observe obliterative bronchiolitis and (b) necrosuppurative bronchopneumonia with large areas of necrosis filled with hypereosinophilic (pink–red) granular debris associated with *M. bovis.* (c) Observe BVDV associated with necrotizing bronchiolitis (arrows) while (d) ballooning degeneration of bronchial (arrow) © and bronchiolar epithelium (arrow) (f) and focal area of necrohemorrhagic bronchitis with angiogenesis (asterisk) at the lamina propria (arrow) with detachment of the bronchial epithelium within the lumen were seen with BoAHV1. *M. bovis*-associated lesions at A and B; BVDV associated lesion at C; and BoAHV1-associated lesions at D-F. Haematoxylin and eosin stain. A, E 50 µm; B-D, F 200 µm.

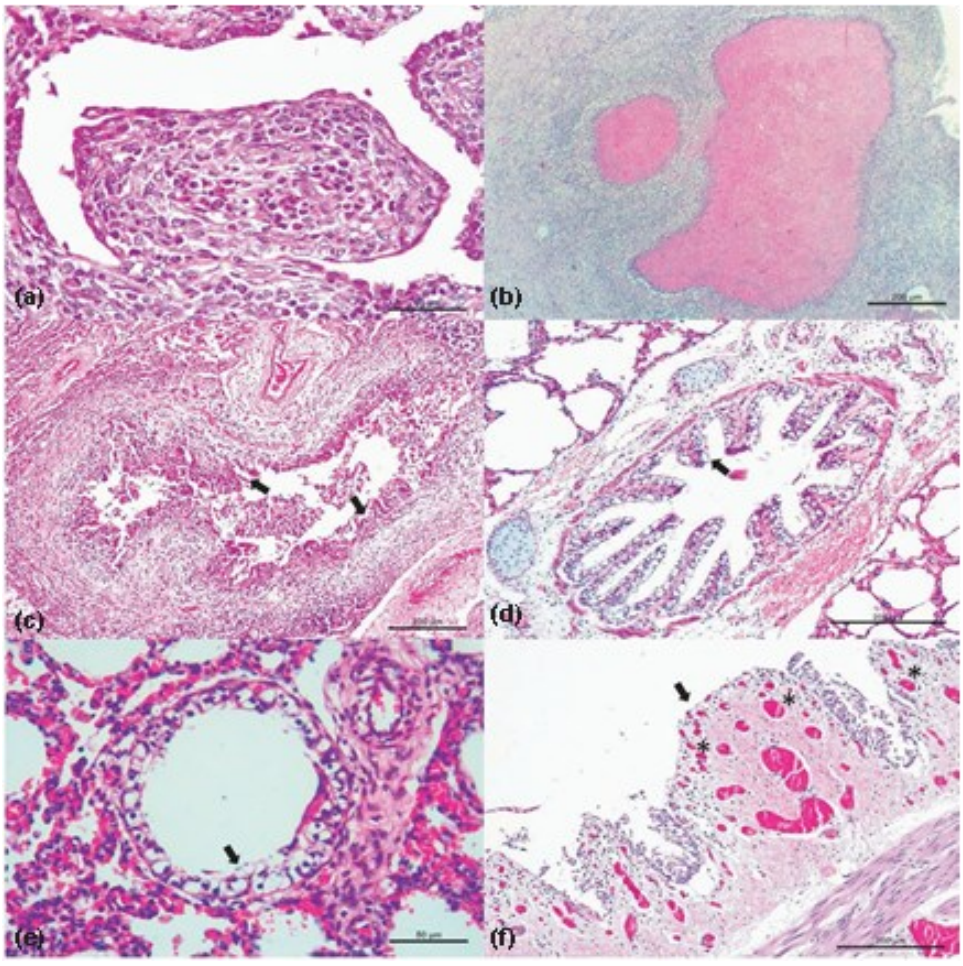


Table 3 – Principal histopathologic diagnosis/features and immunohistochemical findings of bovine respiratory disease in 32 dairy cows

Histopathologic diagnosis/features	Detection of pathogens in the lungs (number/total)
Obliterative bronchiolitis	*M. bovis* (10/10)
Accumulations of intralesional Giemsa-stained coccoid bacteria	*M. bovis* (9/9)
Necrosuppurative bronchopneumonia	*M. bovis* (9/9)
Peribronchial lymphocytic cuffings	*M. bovis* (8/8)
Suppurative bronchopneumonia	*M. bovis* (6/6)
Interstitial pneumonia	BVDV (10/15) BoAHV1 (9/15) BRSV (4/15) BPIV-3 (1/15)
Necrotizing bronchitis	BVDV (4/6) BRSV (4/6) BoAHV1 (2/6)
Necrotizing bronchiolitis	BVDV (4/4) BRSV (2/4)
Necrohemorrhagic bronchitis with bronchial angiogenesis	BoAHV1 (2/2)
Syncytial formation	BRSV (4/4)
Ballooning degeneration of bronchial epithelium	BoAHV1 (2/3) BRSV (1/3)
Hyperplasia of type II pneumocytes	BRSV (2/2)
Ballooning degeneration of bronchial epithelium of bronchiolar epithelium	BoAHV1 (1/1)

Abbreviations: BoAHV1: bovine alphaherpesvirus type 1; BPIV-3: bovine parainfluenza virus type 3; BRSV: bovine respiratory syncytial virus; BVDV: bovine viral diarrhoea virus; *M. bovis: Mycoplasma bovis.*

6.4.2 Immunohistochemical identification of viral agents associated with BRD

Tissues from 32 positive cows contained antigens of at least one of the infectious disease pathogens evaluated. The most frequent infectious disease agents identified were BVDV (56.2%; 18/32), *M. bovis* (50%; 16/32) and BoAHV1 (43.7%; 14/32), followed by BRSV (34.4%; 11/32) and BPIV-3 (15.6%; 5/32). Viral infections were identified in 50% (16/32) of the cases without any association with *M. bovis*, while the other cases (50%; 16/32) were associated with intralesional ac-

cumulations of *M. bovis*. In six cases (18.7%; 6/32), there was the IHC detection of *M. bovis* in simultaneous disease without the presence of BVDV.

During this investigation, singular and mixed infections were identified (Table 4). However, dual (31.3%; 10/32) and triple (18.7%; 6/32) associations were the most frequent forms at this outbreak, followed by quadruple (6.3%; 2/32) and quintuple (3.1%; 1/32, cow #34) simultaneous association of infectious disease agents associated with BRD. Singular infections were caused by BVDV (15.6%; 5/32), *M. bovis* (12.5%; 4/32), BRSV (9.4%; 3/32) and BoAHV1 (3.1%; 1/32); singular infections were not associated with BPIV-3.

Table 4 – Distribution of the infectious disease agents observed in adult dairy cows with bovine respiratory disease

Positive immunoreactivity for single and/or combined infections disease pathogens	Number of cows (*n*=35)	Age[a]
BVDV, BoAHV1, BRSV, BPIV-3, *M. bovis*	1	4
BVDV, BoAHV1, BRSV, *M. bovis*	1	5
BRSV, BoAHV1, BPIV-3, *M. bovis*	1	5
BVDV, BRSV, BPIV-3	2	NI
BVDV, BoAHV1, *M. bovis*	3	4, 4, 5
BRSV, BoAHV1, *M. bovis*	1	NI
BVDV, BoAHV1	4	2, NI
BVDV, BPIV-3	1	2
BVDV, *M. bovis*	1	2
BRSV, *M. bovis*	2	2, 5
BoAHV1, *M. bovis*	2	2, NI
BVDV	5	2, 2, NI, NI, 4
M. bovis	4	2, 3, NI, NI
BRSV	3	NI, 2, 2
BoAHV1	1	3
Negative to all	3	2, 2, 2

Note: NI: not informed.
Abbreviations: BoAHV1: bovine alphaherpesvirus type 1; BPIV-3: bovine parainfluenza virus type 3; BRSV: bovine respiratory syncytial virus; BVDV: bovine viral diarrhoea virus; *M. bovis: Mycoplasma bovis.*
[a] Estimated age based on number of calving seasons.

6.4.3 Correlation between the immunohistochemical identification of BRD pathogens and histopathologic patterns

All antibodies demonstrated cytoplasmic immunolabelling within epithelial cells of bronchi and/or bronchioles of the infected cows. When the histopathologic patterns were correlated with the immunohistochemical identification (Table 5), all cows with obliterative bronchiolitis (100%; 10/10; Figure 2a), necrosuppurative bronchopneumonia with intralesional, Giemsa-positive, bacterial

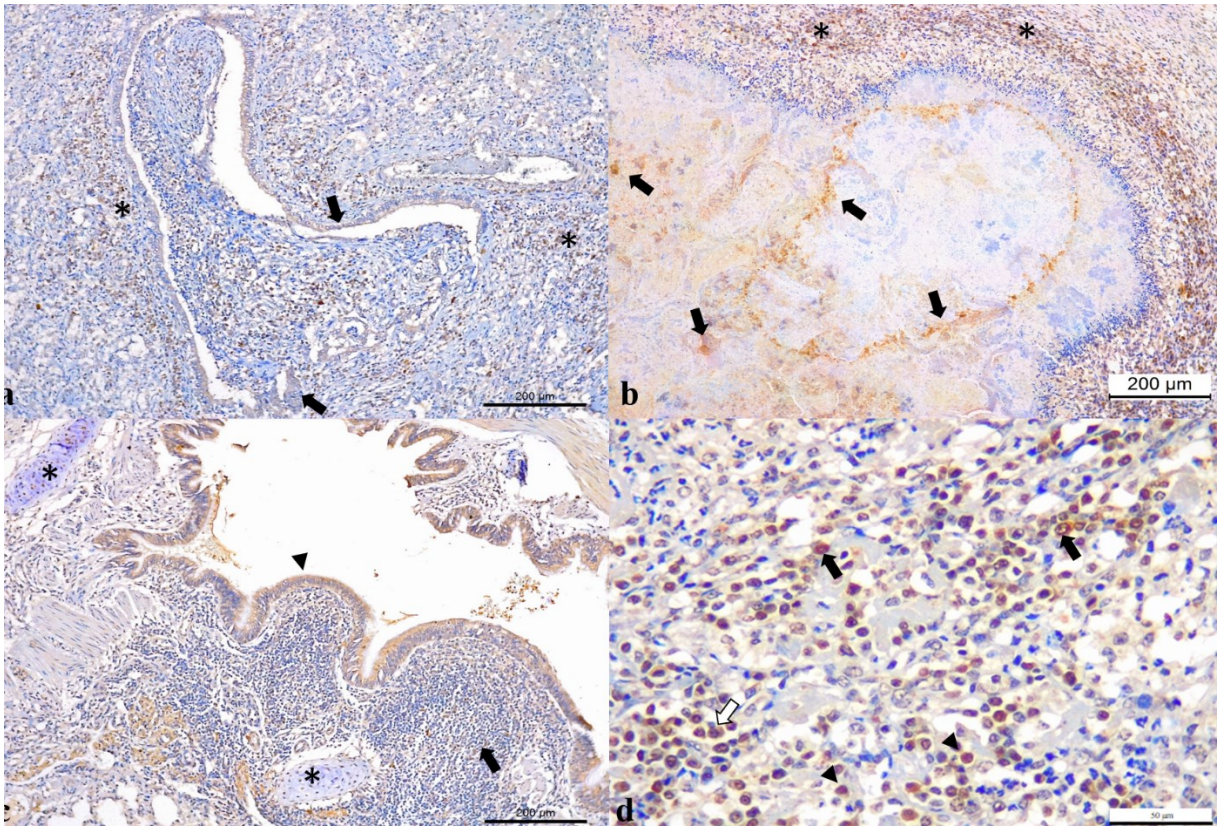
accumulations within the foci of necrosis (100%; 9/9; Figure 2b) and peribronchial lymphocytic cuffings (100%; 7/7; Figure 2c,d) demonstrated positive immunoreactivity for *M. bovis.*

Table 5 – Association of principal histopathologic patterns/features and positive immunoreactivity with infectious disease agents of bovine respiratory disease in dairy cows

Histopathologic patterns/features	Positive immunoreactivity				
	M. bovis	**BVDV**	**BoAHV1**	**BRSV**	**BPIV-3**
Necrossupurative and suppurative bronchopneumonia	X				
Obliterative bronchiolitis	X				
Peribronchial lymphocytic cuffings	X				
Bronchial angiogenesis			X		
Ballooning degeneration of bronchial, bronchiolar and alveolar epithelium			X	X	
Necrotizing bronchitis and bronchiolitis		X	X	X	
Necrohemorrhagic bronchitis		X	X		
Hyaline cartilage of the bronchus	X	X	X	X	
Mixed peribronchial glands		X	X	X	
Syncytial formation				X	
Interstitial pneumonia		X	X	X	X

Abbreviations: BoAHV1, bovine alphaherpesvirus type 1; BPIV-3, bovine parainfluenza virus type 3; BRSV, bovine respiratory syncytial virus; BVDV, bovine viral diarrhoea virus; *M. bovis, Mycoplasma bovis.*

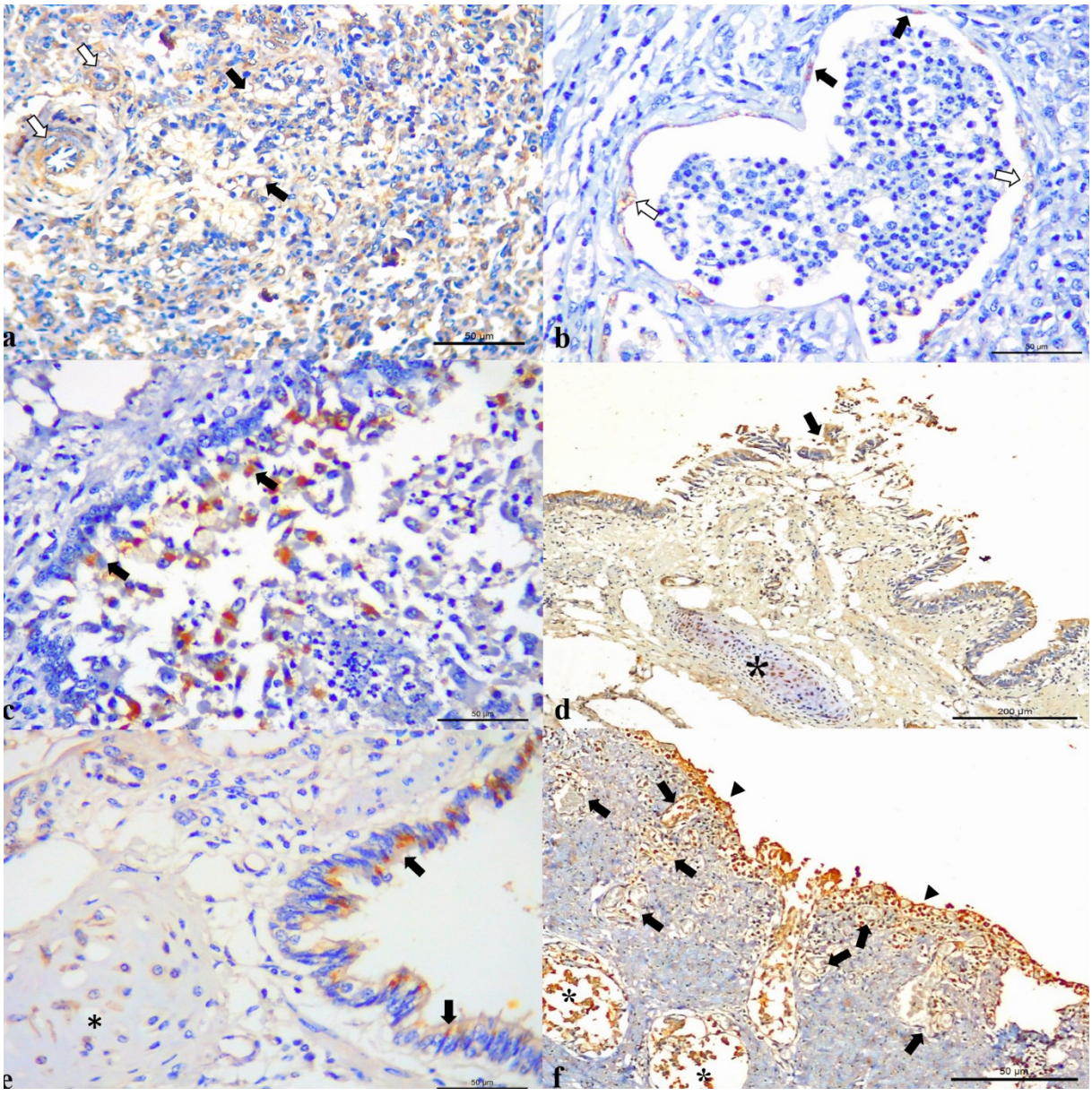
Figure 2 – Immunohistochemical findings observed in dairy cattle with bovine respiratory disease associated with *Mycoplasma bovis*. (a) There is positive intracytoplasmic immunoreactivity to antigens of *M. bovis* in obliterative bronchiolitis; observe immunoreactivity within epithelial cells of the bronchiole (arrows) and peripheral macrophage (asterisk). (b) There is necrosuppurative bronchopneumonia with intralesional immunolabelling of bacteria within foci of necrosis (arrows), (c) bronchiolar epithelium (arrowhead), at the bronchial hyaline cartilage (asterisk) and peribronchial lymphocytic cuffings (arrow). (d) Closer view of peribronquial lymphocytic cuffing demonstrating positive immunoreactivity for *M. bovis* with macrophages (black arrows), lymphocytes (arrowhead) and plasma cells (white arrow). Immunoperoxidase counterstained with haematoxylin. Bar, A–C 200 µm; D 50 µm



Interstitial pneumonia was associated with all viral disease pathogens evaluated. Positive immunostaining of the vascular endothelia was related with BoAHV1 (64.3%; 9/14; Figure 3a) and BVDV (11.1%; 2/18; Figure 3b), while hydropic degeneration of vascular endothelia was associated only with positive immunolabelling for BVDV (Figure 3b). Additionally, necrotizing bronchitis/bronchiolitis was associated with BVDV (44.4%; 8/18; Figure 3c), BRSV (54.5%; 6/11; Figure 3d) and BoAHV1 (15.4%; 2/13). Positive immunoreactivity to chondrocytes of hyaline cartilage of the bronchi was associated with BoAHV1 (28.6%; 4/14), BRSV (21.4%; 3/14; Figure 3d), BVDV (11.1%; 2/18; Figure 3e) and *M. bovis* (12.5%; 2/16; Figure 2c). It must be highlighted that one BVDV-positive cow (#34) was carrying a foetus in the final third semester of gestation when it died, and there was positive immunoreactivity for BVDV at the hyaline cartilage of the bronchus (Figure S1) of this foetus. Syncytial formation (100%; 4/4) and hyperplasia of type II pneumocytes (18.2%; 2/11) demonstrated positive immunoreactivity only for antigens of BRSV.

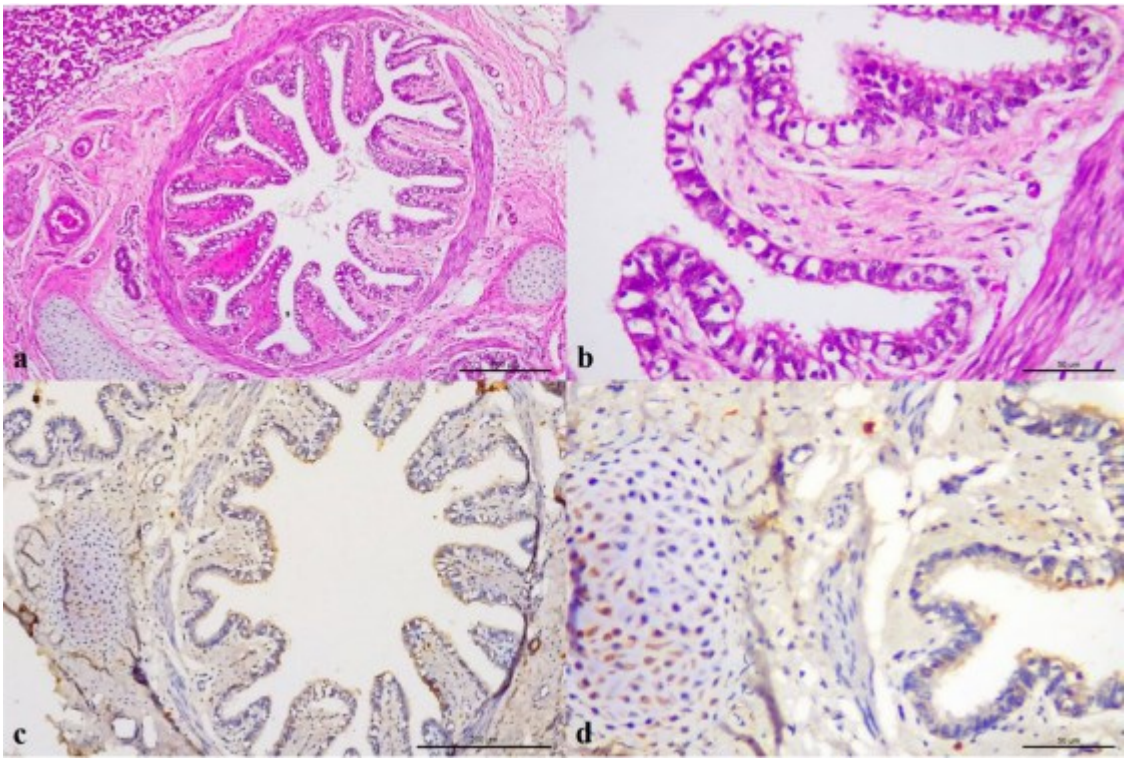
Figure 3 – Immunohistochemical findings observed in dairy cattle with bovine respiratory disease. (a) There is positive immunoreactivity to antigens of BoAHV1 ballooning degeneration of the bronchiole (black arrows) and endothelial staining in

alveolar capillaries (white arrows). (b) There is positive immunoreactivity for BVDV at the endothelium of alveolar venule (black arrows), within degenerated endothelial cells (white arrows), and (c) necrotizing bronchiolitis (arrow). (d) Observe positive immunoreactivity for antigens of BRSV at the bronchial epithelium, within detached bronchial epithelial cells (arrow), in bronchial hyaline cartilage (asterisk). (e) Observe positive immunolabelling for antigens of BVDV at the bronchial epithelium and within chondrocytes of the bronchial hyaline cartilage (asterisk). (f) There is positive immunoreactivity associated with BoAHV1 at the bronchial epithelium of a cow with diffused ulcerative bronchitis and epithelial necrotizing bronchitis (arrows heads), at the newly formed capillaries (arrows) and mixed peribronchial glands (asterisks). Immunoperoxidase counterstained with haematoxylin. Bar, A–C, E 50 µm; D, F 200 µm



Supplementary File 1 – Histopathologic and immunohistochemical findings associated with BVDV in a bovine foetus. (a) Observe ballooning degeneration of the

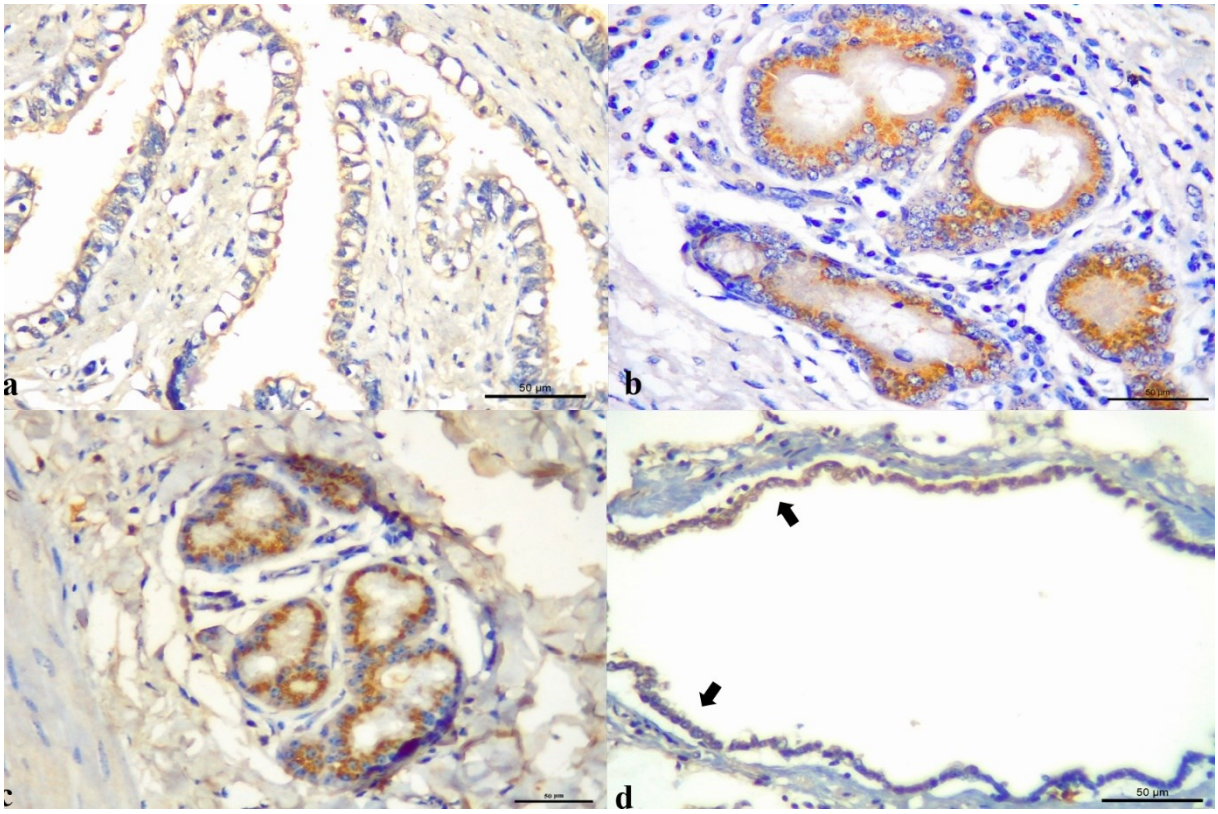
epithelium of the bronchus; (b) closer view of the degenerated epithelium. (c) There is positive immunoreactivity to antigens of BVDV at the chondrocytes of the hyaline cartilage and at the bronchial epithelium; (d) closer view of the positive immunoreactivity at the cartilage and epithelium. Haematoxylin and Eosin stain. Bar, A, 200 µm; B, 50 µm. Immunoperoxidase counterstained with Haematoxylin. Bar, C, 200 µm; D, 50 µm.



In cases of necrohemorrhagic bronchitis (14.3%; 2/14), an unusual lesion was identified, which was histologically characterized as angio- genesis of the capillaries within the lamina propria of the bronchus and trachea (Figure 3f) that demonstrated positive immunolabelling at the epithelia and endothelial cells for BoAHV1. Ballooning degeneration of the bronchial and bronchiolar epithelium was associated with positive immunoreactivity for BoAHV1 (14.3%; 2/14; Figure 3a) and BRSV (1/11; Figure 4a); mixed bronchial glands demonstrated positive immunoreactivity for BVDV (9.1%; 9/18; Figure 4b), BoVH-1 (21.4%; 3/14) and BRSV (1/11; Figure 4c). Mild interstitial pneumonia was as- sociated with positive immunolabelling for BPIV-3 at alveolar macrophages (100%; 5/5) and the detached bronchiolar epithelia (60%; 3/5; Figure 4d).

Figure 4 – Immunohistochemical findings observed in dairy cows with bovine respiratory disease. (a) There is positive immunoreactivity to antigens of BoAHV1

within ballooning degenerated bronchial epithelial cells. Observe positive immunoreactivity associated with (b) BVDV and (c) BRSV at the mixed peribronchial glands. (d) There is positive immunolabelling of mildly detached bronchiole epithelium associated with BPIV-3. Immunoperoxidase counterstained with haematoxylin. Bar, A–D 50 µm



6.5 DISCUSSION

The findings of this study have demonstrated the multifactorial nature of BRD during which 59.4% (19/32) of the dairy cows submitted by veterinary clinicians for diagnostic were concomitantly infected by two or more infectious disease agents. Although viral infectious disease agents were the predominant causes (87.5%; 28/32) of BRD, fatal pneumonia associated with *M. bovis* occurred in 12.5% (4/32) of these cases.

Primary pulmonary infections due to BVDV were demonstrated experimentally (Fulton et al., 2016; Gershwin et al., 2015; Rodríguez et al., 1996) and observed in field outbreaks of BRD (Fulton et al., 2000, 2004; Szeredi, Janosi, & Palfi, 2010). The role of BVDV in the development of the mixed infections herein described, especially with respect to those of bovine pneumonic mycoplasmosis, must be

highlighted. Several studies have suggested that synergism between BVDV and *M. bovis* (Bürgi, Josi, Bürki, Schweizer, & Pilo, 2018; Haines et al., 2001, 2004; Shahriar, Clark, Janzen, West, & Wobeser, 2002) and BVDV and BRSV (Liu, Lehmkuhl, & Kaeberle, 1999) may affect the severity of BRD due to the increased virulence of pathogens caused by synergy in coinfections (Ridpath, 2010). This aspect is relevant not only for the interpretation of experimental data, but because BVDV may interact with *M. bovis* in field conditions (Bürgi et al., 2018). Additionally, some strains of BVDV are immunemunosuppressive in cattle, due to the loss of alveolar macrophage functionality (Bürgi et al., 2018; Welsh, Adair, & Foster, 1995) and severe and extensive apoptosis of lymphocytes (Chase, 2013). Consequently, immunosuppression due to BVDV associated with BoAHV1, BRSV and *M. bovis* may have resulted in an increased severity of disease in this dairy herd, due to incapacity of the innate immune system to control these infections.

In our study, several chondrocytes of the hyaline cartilage of the bronchus demonstrated positive immunoreactivity for BRSV, BoAHV1 and BVDV, with diffused immunoreactivity to BVDV occurring within the chondrocytes of two cows. Similar positive immunoreactivity to BVDV was described in 60% (6/10) of persistently infected (PI) calves, where it was proposed that the presence of BVDV antigen in respiratory cartilage is an indication that this viral disease pathogen predisposes cows to secondary bacterial infection (Confer, Fulton, Step, Johnson, & Ridpath, 2005). In the cases herein described, we were unable to confirm these cows as being PI animals since two biological samples were not available for testing. However, there was positive immunoreactivity to BVDV (using Mab 15c5) within the chondrocytes of the hyaline cartilage of the bronchus of the foetus of one of these cows. Collectively, these findings may suggest that the foetus of the gravid cow and probably the cow itself were PI animals; moreover, positive immunoreactivity to BVDV within the pulmonary cartilage of these two cows can correlate with the occurrence of simultaneous infections in these animals (Confer et al., 2005). Additionally, it must be highlighted that the MAb used during this study to identify intralesional antigens of BVDV has elevated specificity and sensitivity for the diagnosis of this infectious disease pathogen (Haines, Clark, & Dubovi, 1992).

The results of this study suggest that *M. bovis*-associated pneumonias might be important causes of BRD associated mortality in dairy cattle from Brazil. Until the present moment, the authors are unaware of studies done in Brazil that confirmed

BRD due to infections by *M. bovis* using pulmonary tissues derived from adult dairy cattle with fatal pulmonary disease. Two studies have previously identified the presence of *M. bovis* associated with BRD in Brazil. The first identified *M. bovis*-associated pulmonary disease in 26.1% (12/46) of calves due to a combination of bacterial culture and direct immunofluorescence using lung sections (Pretto et al., 2001). While the other amplified nucleic acids of *M. bovis* from 5.6% (1/18) of nasal swabs from dairy cows (Tortorelli et al., 2017). The marked difference between these and the current study was the confirmation of disease (Fulton & Confer, 2012), in our investigation due to the intralesional detection of antigens of *M. bovis* by IHC and intralesional accumulations of bacteria by the Giemsa stain, while the frequency of disease was more elevated. Additionally, during this investigation IHC assays were done from pulmonary tissues with gross evidence of disease, which may have also contributed towards the elevated number of positive cases, considering the elevated sensitivity of this diagnostic technique to identify intralesional antigens of infectious disease pathogens (Fulton & Confer, 2012). Since positive Giemsa staining was observed in most pulmonary sections that contained intralesional antigens of *M. bovis* by IHC, we postulate that Giemsa staining may be an adequate but simple method to characterize infections associated with this pathogen.

Necrosuppurative bronchopneumonia and peribronchial lymphocytic cuffings are the hallmarks of chronic pulmonary mycoplasmosis in cattle (Nicholas & Ayling, 2003) and calves (Hermeyer et al., 2012; Khodakaram-Tafti & López, 2004), and were the main findings observed in the affected dairy cows during this study. These findings were previously described (Caswell & Archambault, 2007; Gagea et al., 2006; Haines et al., 2004) and demonstrate the ability of *M. bovis* to invade the pulmonary parenchyma, as was observed in experimental (Gershwin et al., 2015; Hermeyer et al., 2012; Rodríguez et al., 1996; Thomas, Howard, Stott, & Parsons, 1986) and spontaneous (Gagea et al., 2006) cases of pulmonary disease in cattle. Furthermore, *M. bovis* may induce cytotoxicity and induction of apoptosis of the alveolar macrophage (Bürgi et al., 2018), and neutrophils, inhibit the production of nitric oxide in cows (Jimbo et al., 2017) and infects a wide range of epithelial and immune cells (Bürki, Frey, & Pilo, 2015). These mechanisms then favour the dissemination of *M. bovis* and would have facilitated simultaneous infections in this herd with BVDV, BoAHV1 and BRSV.

The silent immunomodulatory and immunosuppressive effects of *M. bovis* predisposes the respiratory tract of calves to other bacterial infections (Margineda et al., 2017; Nicholas, 2011; Poumarat et al., 2001). These effects have been demonstrated experimentally (Poumarat et al., 2001; Rodríguez et al., 1996; Vanden Bush & Rosenbusch, 2003) and were described in spontaneous cases (Haines et al., 2004; Rodríguez et al., 1996; Yİlmaz et al., 2016), and there are reports of pneumonic diseases in which *M. bovis* was the only pathogen identified (Gershwin et al., 2015; Nicholas, 2011). These findings may suggest that *M. bovis* can act as a primary disease pathogen and produce BRD. However, the possibility of *M. bovis* being a primary disease agent is still controversial, since this agent may colonize and intralesional antigens of *M. bovis* by IHC, we postulate that Giemsa staining may be an adequate but simple method to characterize in- fections associated with this pathogen.

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of pneumonic diseases in which *M. bovis* was the only pathogen identified (Gershwin et al., 2015; Nicholas, 2011). These findings may suggest that *M. bovis* can act as a primary disease pathogen and produce BRD. However, the possibility of *M. bovis* being a primary disease agent is still controversial, since this agent may colonize and perpetuate pulmonary lesions that were initiated by other bacteria, such as *M. haemolytica* or others virus (Caswell, Bateman, Cai, & Castillo-Alcala, 2010). Nevertheless, the immunosuppressive nature of BVDV-associated infections cannot be overlooked as contributing towards the development of BRD in these cows, since in most cases of combined infections associated with *M. bovis*, intralesional anti- gens of BVDV were detected.

As far as the authors are aware, this manuscript may represent the first description of *M. bovis*-related BRD in dairy cows. This pathogen was frequently associated with several disease syndromes in dairy calves (Mahmood et al., 2017) and feedlot cattle (Caswell & Archambault, 2007; Haines et al., 2001), including descriptions of *M. bovis*-related pneumonia (Gagea et al., 2006; Mahmood et al., 2017), but we did not locate reports of *M. bovis*-induced BRD in adult dairy cows on searching major databases. Furthermore, *M. bovis* was not identified in recent studies done by our group to identify this pathogen associated with BRD in feedlot cattle from different geographical regions of Brazil (Baptista et al., 2017; Headley et al., 2014, 2018). Additionally, there are only two reports (Pretto et al., 2001; Tortorelli et al., 2017) of *M. bovis*-associated pulmonary disease in cattle from this country. Although the exact reasons for the low detection rate of *M. bovis*-associated BRD in Brazil are unknown, we believe that the reduced identification of this agent may be related to the diagnostic strategy used and the type of cattle evaluated, that is beef against dairy. All previous cases of *M. bovis*-associated BRD were diagnosed in dairy cows (Pretto et al., 2001; Tortorelli et al., 2017), while this bacterial pathogen was not identified in studies done with beef cattle (Headley et al., 2014, 2017, 2018). It must be highlighted that nasal swabs were tested by PCR in the beef cattle surveys done by our group which resulted in negative results; however, these negative results could have been associated with the sporadic elimination of *M. bovis* so that PCR testing would not be an efficient method to detect this pathogen (Nicholas, 2011). This sporadic shedding of *M. bovis* may also explain the low results (5.6%; 1/18) obtained in a study done by another group from Brazil that used the same molecular testing strategy in dairy cattle (Tortorelli et al., 2017), as compared with the elevated results (26.1%; 12/46) obtained

by bacterial culture and immunofluorescence assay using pulmonary tissue with characteristic lesions (Pretto et al., 2001). Since pulmonary mycoplasmosis is a chronic disease (Caswell & Archambault, 2007), dairy cows that are maintained for longer durations at a herd would be more prone to develop this infection as com- pared with beef cattle which are younger on entering feedlots and are maintained on feed for approximately 120–150 days. Moreover, the higher detection rate in dairy cattle as opposed to beef cattle may be because the dairy cows, herein described, could have prob- ably demonstrated extra-pulmonary manifestations of mycoplasmosis, such as mastitis and reproductive diseases, with concomitant pulmonary disease. Lastly, pulmonary mycoplasmosis of cattle may probably be underdiagnosed in Brazil, considering that isolation can be difficult in severe chronic cases (Nicholas & Ayling, 2003), the sporadic shedding of *M. bovis* (Nicholas, 2011) in affected cows, and the molecular testing frequently used to identify this pathogen in association with BRD. Consequently, this pathogen should be suspected in dairy cows with clinical manifestations of respiratory distress and be included in the differential diagnosis of infectious disease agents associated with BRD in adult dairy cattle from Brazil. Moreover, pulmonary tissue rather than nasal swabs may be more efficient to diagnose *M. bovis* in dairy cattle.

Necrohemorrhagic bronchitis with bronchial angiogenesis was exclusively associated with infections induced by BoAHV1 in this study. We have not seen this lesion previously described in the development of pulmonary disease. It was demonstrated that BoAHV1 infects bronchial epithelial cells, induced increase in neutrophil adhesion and activation, and the infection elicits a rapid secreting proinflammatory cytokines such as interleukin 1 (IL-1), IL-8 and tumour necrosis factor (TNF-α), causing the influx of neutrophils, degranulation and tissue necrosis (Rivera-Rivas, Kisiela, & Czuprynski 2009). Although the pathogenesis of angiogenesis observed at the lamina propria of the bronchus with ulcerative or necrosis induced by BoAHV1 is not fully elucidated, these cytokines may be associated with the development of this lesion due to inflammatory reaction, and necrosis; in addition, IL-8 has angiogenic and repair qualities (Koch et al., 1992). Consequently, further studies are needed to understand the pathophysiology and the importance of this unique lesion.

Few studies have demonstrated synergism of BRSV with BVDV (Liu et al., 1999), *H. somni* (Agnes et al., 2013; Headley et al., 2017) and *M. bovis* (Thomas

et al., 1986). Experimentally it was demonstrated that this synergism induced alveolar cell retraction and in- creased degradation of collagen by TNF-α (Agnes et al., 2013). Both mechanisms may facilitate pulmonary damage resulting in subsequent pneumonia and the dissemination of the infectious pathogen (Agnes et al., 2013). Additionally, BRSV infects type I and type II pneumocytes (Bryson, McConnell, McAliskey, & McNulty, 1991), with consequent apoptosis to these cells (Viuff et al., 2002), and produces necrosis of alveoli and small airways (Bryson et al., 1991), due to alveolar neutrophilic exudation (Agnes et al., 2013), resulting in suppurative alveolitis/bronchiolitis and necrotizing bronchiolitis/bronchitis (Andrews & Kennedy, 1997; Brasil et al., 2013; Gershwin et al., 2015; Peixoto et al., 2000). Moreover, during this investigation, hyperplasia of type II pneumocytes was observed in association only with positive immunoreactivity to BRSV; similar findings were previously associated with chronic epithelial injury in the proximal alveolar region (Barry, Miller, & Crapo, 1985) and identified in beef cattle with chronic pulmonary disease (Driemeier et al., 1997). The low number of cases with syncytial formation (4/11) in the present study may be since the manifestations were chronic (Driemeier et al., 1997). These histopathologic findings were observed in cattle infected by BRSV during this investigation and may have contributed to the increased severity of pulmonary lesions observed in the dairy cows herein described.

6.6 CONCLUSIONS

This is the first report from Brazil that demonstrated and correlated the histopathologic findings of bovine respiratory disease by immunohistochemistry. Necrosuppurative bronchopneumonia, obliterative bronchiolitis and peribronchial lymphocytic cuffings were the characteristic histopathologic features associated with mycoplasma pneumonia. Necrotizing bronchitis and bronchiolitis were associated with BVDV, BoAHV1 and BRSV. Necrohemorrhagic bronchitis with bronchial angiogenesis was associated with infection by BoAHV1.

M. bovis was commonly detected in the tissues of fatal pulmonary disease during this outbreak and may be a possible primary disease pathogen associated with the development of bovine respiratory disease. Moreover, the histopathologic features observed within the patterns of pulmonary disease may be an

1804 excellent diagnostic tool to identify some infectious disease agents of BRD in dairy
1805 cattle.
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1808 **6.7** REFERENCES
1809

1810 Agnes, J. T., Zekarias, B., Shao, M., Anderson, M. L., Gershwin, L. J., & Corbeil, L. B.
1811 (2013). Bovine respiratory syncytial virus and *Histophilus somni* interaction at the
1812 alveolar barrier. Infection and Immunity, 81, 2592–2597.

1813 Andrews, G. A., & Kennedy, G. A. (1997). Respiratory diagnostic pathology. Veterinary
1814 Clinics of North America: Food Animal Practice, 13, 515–547.

1815 Arns, C. W., Campalans, J., Costa, S. C. B., Domingues, H. G., D'Arce, R. C. F., &
1816 Almeida, R. S. (2003). Characterization of bovine respiratory syncytial virus isolated in
1817 Brazil. Brazilian Journal of Medical and Biological Research, 36, 213–218.

1818 Baptista, A. L., Rezende, A. L., Fonseca, P. A., Massi, R. P., Nogueira, G. M.,
1819 Magalhães, L. Q., … Saut, J. P. E. (2017). Bovine respiratory dis- ease complex
1820 associated mortality and morbidity rates in feedlot cattle from southeastern Brazil. The
1821 Journal of Infection in Developing Countries, 11, 791–799.

1822 Barbosa, A. C. V. C., Brito, W. M. E. D., & Alfaia, B. T. (2005). Seroprevalence and
1823 risk factors to the infectious by bovine herpes- virus type 1 (BHV-1) in Goiás State,
1824 Brazil (in Portuguese). Ciência Rural, 35, 1368–1373.

1825 Barry, B. E., Miller, F. J., & Crapo, J. D. (1985). Effects of inhalation of 0.12 and 0.25
1826 parts per million ozone on the proximal alveolar region of juvenile and adult rats.
1827 Laboratory Investigation, 53, 692–704.

1828 Brasil, N. D. A., Hinnah, F. L., Fiss, L., Sallis, E. S. V., Grecco, F. B., Ladeira, S. R.
1829 L., … Schild, A. L. (2013). Respiratory diseases in calves in southern Rio Grande do
1830 Sul: Study of 33 outbreaks (in Portuguese). Pesquisa Veterinária Brasileira, 33, 745–
1831 751.

1832 Bryson, D. G., McConnell, S., McAliskey, M., & McNulty, M. S. (1991). Ultrastructural
1833 features of alveolar lesions in induced respiratory syncytial virus pneumonia of calves.
1834 Veterinary Pathology, 28, 286–292.

1835 Bürgi, N., Josi, C., Bürki, S., Schweizer, M., & Pilo, P. (2018). *Mycoplasma bovis* co-
1836 infection with bovine viral diarrhea virus in bovine macrophages. Veterinary Research,
1837 49, 2.

1838 Bürki, S., Frey, J., & Pilo, P. (2015). Virulence, persistence and dissemination of
1839 *Mycoplasma bovis*. Veterinary Microbiology, 179, 15–22.

1840 Caswell, J. L., & Archambault, M. (2007). *Mycoplasma bovis* pneumonia in cattle.
1841 Animal Health Research Reviews, 8, 161–186.Caswell, J. L., Bateman, K. G., Cai, H.

1842 Y., & Castillo-Alcala, F. (2010). *Mycoplasma bovis* in respiratory disease of feedlot
1843 cattle. Veterinary Clinics of North America: Food Animal Practice, 26, 365–379.

1844 Chase, C. C. (2013). The impact of BVDV infection on adaptive immunity. Biologicals:
1845 Journal of the International Association of Biological Standardization, 41, 52–60.

1846 Confer, A. W., Fulton, R. W., Step, D. L., Johnson, B. J., & Ridpath, J. F. (2005). Viral
1847 antigen distribution in the respiratory tract of cattle persistently infected with bovine
1848 viral diarrhea virus subtype 2a. Veterinary Pathology, 42, 192–199.

1849 Cortez, A., Heinemann, M. B., Castro, A. M. M. G., Soares, R. M., Pinto, A. M. V.,
1850 Alfieri, A. A., … Richtzenhain, L. J. (2006). Genetic characterization of Brazilian bovine
1851 viral diarrhea virus Genetic characterization of Brazilian bovine viral diarrhea virus
1852 isolates by partial nucleotide sequencing of the 5′-UTR region. Pesquisa Veterinária
1853 Brasileira, 26, 211–216.

1854 Cusack, P. M., McMeniman, N., & Lean, I. J. (2003). The medicine and epidemiology
1855 of bovine respiratory disease in feedlots. Australian Veterinary Journal, 81, 480–487.

1856 Driemeier, D., Gomes, M. J. P., Moojen, V., Arns, C. W., Vogg, G., Kessler, L., & Costa,
1857 U. M. (1997). Clinic-pathological aspects in the natural infection of Bovine Respiratory
1858 Syncytial Virus (BRVS) in extensive management of cattle in Rio Grande do Sul, Brazil
1859 (in Portuguese). Pesquisa Veterinária Brasileira, 17, 77–81.

1860 Fernandes, L. G., Pimenta, C. L. R. M., Pituco, E. M., Brasil, A. W. L., & Azevedo, S.
1861 S. (2016). Risk factors associated with BoAHV1 and BVDV seropositivity in buffaloes
1862 (Bubalus bubalis) from the State of Paraiba, Northeastern Brazil. Semina: Ciências
1863 Agrárias, 37, 1929–1936.

1864 Flores, E. F., Ridpath, J. F., Weiblen, R., Vogel, F. S., & Gil, L. H. (2002). Phylogenetic
1865 analysis of Brazilian bovine viral diarrhea virus type 2 (BVDV-2) isolates: Evidence for
1866 a subgenotype within BVDV-2. Virus Research, 87, 51–60.

1867 Flores, E. F., Weiblen, R., Flores-Vogel, F. S., Roehe, P. M., Alfieri, A. A., & Pituco, E.
1868 M. (2005). Bovine viral diarrhea virus (BVDV) infection in Brazil: History, current
1869 situation and perspectives (in Portuguese). Pesquisa Veterinária Brasileira, 25, 125–
1870 134.

1871 Fulton, R. W. (2009). Bovine respiratory disease research (1983-2009). Animal Health
1872 Research Reviews, 10, 131–139.

1873 Fulton, R. W., Blood, K. S., Panciera, R. J., Payton, M. E., Ridpath, J. F., Confer, A.
1874 W., … Reck, A. (2009). Lung pathology and infectious agents in fatal feedlot
1875 pneumonias and relationship with mortal- ity, disease onset, and treatments. Journal
1876 of Veterinary Diagnostic Investigation, 21, 464–477.

1877 Fulton, R. W., Briggs, R. E., Payton, M. E., Confer, A. W., Saliki, J. T., Ridpath, J. F.,
1878 … Duff, G. C. (2004). Maternally derived humoral im- munity to bovine viral diarrhea
1879 virus (BVDV) 1a, BVDV1b, BVDV2, bovine herpesvirus-1, parainfluenza-3 virus bovine
1880 respiratory syncytial virus, *Mannheimia haemolytica* and *Pasteurella multocida* in beef

calves, antibody decline by half-life studies and effect on response to vaccination. Vaccine, 22, 643–649.

Fulton, R. W., & Confer, A. W. (2012). Laboratory test descriptions for bovine respiratory disease diagnosis and their strengths and weaknesses: Gold standards for diagnosis, do they exist? Canadian Journal of Veterinary Research, 53, 754–761.

Fulton, R. W., d'Offay, J. M., Landis, C., Miles, D. G., Smith, R. A., Saliki, J. T., …Payton, M. E. (2016). Detection and characterization of viruses as field and vaccine strains in feedlot cattle with bovine respiratory disease. Vaccine, 34, 3478–3492.

Fulton, R. W., Purdy, C. W., Confer, A. W., Saliki, J. T., Loan, R. W., Briggs, R. E., & Burge, L. J. (2000). Bovine viral *diarrhea* viral infections in feeder calves with respiratory disease: Interactions with Pasteurella spp., parainfluenza-3 virus, and bovine respiratory syncytial virus. Canadian Journal of Veterinary Research, 64, 151–159.

Gagea, M. I., Bateman, K. G., Shanahan, R. A., van Dreumel, T., McEwen, B. J., Carman, S., … Caswell, J. L. (2006). Naturally occurring *Mycoplasma bovis*-associated pneumonia and polyarthritis in feedlot beef calves. Journal of Veterinary Diagnostic Investigation, 18, 29–40.

Gershwin, L. J., Van Eenennaam, A. L., Anderson, M. L., McEligot, H. A., Shao, M. X., Toaff-Rosenstein, R., … Womack, J. (2015). Single pathogen challenge with agents of the bovine respiratory disease complex. PLoS ONE, 10, e0142479.

Gonçalves, D. A., Spilki, F. R., Chiminazzo, C., Oliveira, M. A., Franco, A. C., & Roehe, P. M. (2003). Isolation of bovine Parainfluenza virus type 3 in Rio Grande do Sul, Brazil (in Portuguese). Ciência Rural, 33, 953–956.

Haines, D. M., Clark, E. G., & Dubovi, E. J. (1992). Monoclonal antibody- based immunohistochemical detection of bovine viral diarrhea virus in formalin-fixed, paraffin-embedded tissues. Veterinary Pathology, 29, 27–32.

Haines, D. M., Martin, K. M., Clark, E. G., Jim, G. K., & Janzen, E. D. (2001). The immunohistochemical detection of *Mycoplasma bovis* and bovine viral diarrhea virus in tissues of feedlot cattle with chronic, unresponsive respiratory disease and/or arthritis. Canadian Journal of Veterinary Research, 42, 857–860.

Haines, D. M., Moline, K. M., Sargent, R. A., Campbell, J. R., Myers, D. J., & Doig, P. A. (2004). Immunohistochemical study of Hemophilus somnus, *Mycoplasma bovis*, *Mannheimia haemolytica*, and bovine viral diarrhea virus in death losses due to myocarditis in feedlot cattle. Canadian Journal of Veterinary Research, 45, 231–234.

Hay, K. E., Morton, J. M., Mahony, T. J., Clements, A. C., & Barnes, T. S. (2016). Associations between animal characteristic and environmental risk factors and bovine respiratory disease in Australian feedlot cattle. Preventive Veterinary Medicine, 125, 66–74.

1919 Headley, S. A., Alfieri, A. F., Oliveira, V. H., Beuttemmuller, E. A., & Alfieri, A. A. (2014).
1920 *Histophilus somni* is a potential threat to beef cattle feedlots in Brazil. Veterinary
1921 Record, 175, 249.

1922 Headley, S. A., Balbo, L. C., Alfieri, A. F., Baptista, A. L., Saut, J. P. E., & Alfieri, A. A.
1923 (2017). Bovine respiratory disease associated with *Histophilus somni* and bovine
1924 respiratory syncytial virus in a beef cattle feedlot from Southeastern Brazil. Semina:
1925 Ciências Agrárias, 38, 283–294.

1926 Headley, S. A., Okano, W., Balbo, L. C., Marcasso, R. A., Oliveira, T. E., Alfieri, A. F.,
1927 … Alfieri, A. A. (2018). Molecular survey of infectious agents associated with bovine
1928 respiratory disease in a beef cattle feedlot in southern Brazil. Journal of Veterinary
1929 Diagnostic Investigation, 30, 249–251.

1930 Hermeyer, K., Buchenau, I., Thomasmeyer, A., Baum, B., Spergser, J., Rosengarten,
1931 R., & Hewicker-Trautwein, M. (2012). Chronic pneu- monia in calves after experimental
1932 infection with *Mycoplasma bovis* strain 1067: Characterization of lung pathology,
1933 persistence of variable surface protein antigens and local immune response. Acta
1934 Veterinaria Scandinavica, 54, 9.

1935 Jimbo, S., Suleman, M., Maina, T., Prysliak, T., Mulongo, M., & Perez- Casal, J. (2017).
1936 Effect of *Mycoplasma bovis* on bovine neutrophils. Veterinary Immunology and
1937 Immunopathology, 188, 27–33.

1938 Khodakaram-Tafti, A., & López, A. (2004). Immunohistopathological findings in the
1939 lungs of calves naturally infected with *Mycoplasma bovis*. Journal of Veterinary
1940 Medicine A, Physiology, Pathology, Clinical Medicine, 51, 10–14.

1941 Koch, A. E., Polverini, P. J., Kunkel, S. L., Harlow, L. A., DiPietro, L. A., Elner, V. M.,
1942 … Strieter, R. M. (1992). Interleukin-8 as a macrophage- derived mediator of
1943 angiogenesis. Science, 258, 1798–1801.

1944 Liu, L., Lehmkuhl, H. D., & Kaeberle, M. L. (1999). Synergistic effects of bovine
1945 respiratory syncytial virus and non-cytopathic bovine viral diarrhea virus infection on
1946 selected bovine alveolar mac- rophage functions. Canadian Journal of Veterinary
1947 Research, 63, 41–48.

1948 Lunardi, M., Headley, S. A., Lisboa, J. A., Amude, A. M., & Alfieri, A. A. (2008).
1949 Outbreak of acute bovine viral diarrhea in Brazilian beef cattle: Clinicopathological
1950 findings and molecular characterization of a wild-type BVDV strain subtype 1b.
1951 Research in Veterinary Science, 85, 599–604.

1952 Maes, R. K., Langohr, I. M., Wise, A. G., Smedley, R. C., Thaiwong, T., & Kiupel, M.
1953 (2013). Beyond H&E: Integration of nucleic acid– based analyses into diagnostic
1954 pathology. Veterinary Pathology, 51, 238–256.

1955 Mahmood, F., Khan, A., Hussain, R., Khan, I. A., Abbas, R. Z., Ali, H. M., & Younus,
1956 M. (2017). Patho-bacteriological investigation of an out- break of *Mycoplasma bovis*
1957 infection in calves - emerging stealth assault. Microbial Pathogenesis, 107, 404–408.

Margineda, C. A., Zielinski, G. O., Jurado, S., Alejandra, F., Mozgovoj, M., Alcaraz, A. C., & López, A. (2017). *Mycoplasma bovis* pneumonia in feedlot cattle and dairy calves in Argentina. Brazilian Journal of Veterinary Pathology, 10, 79–86.

Nicholas, R. A. (2011). Bovine mycoplasmosis: Silent and deadly. Veterinary Record, 168, 459–462.

Nicholas, R. A., & Ayling, R. D. (2003). *Mycoplasma bovis*: Disease, diagno- sis, and control. Research in Veterinary Science, 74, 105–112.

Oliveira, R. A. M., Lorenzetti, E., Alfieri, A. A., & Lisbôa, J. A. N. (2015). Prevalence of latent infection with BoAHV1 and BoHV-5 in beef cat- tle of Parana, Brazil (in Portuguese). Arquivo Brasileiro de Medicina Veterinária e Zootecnia, 67, 1217–1225.

Otonel, R. A., Alfieri, A. F., Dezen, S., Lunardi, M., Headley, S. A., & Alfieri, A. A. (2014). The diversity of BVDV subgenotypes in a vaccinated dairy cattle herd in Brazil. Tropical Animal Health and Production, 46, 87–92.

Panciera, R. J., & Confer, A. W. (2010). Pathogenesis and pathology of bovine pneumonia. Veterinary Clinics of North America: Food Animal Practice, 26, 191–214.

Peixoto, P. V., Mota, R. A., Brito, M. F., Corbellini, L. G., Driemeier, D., & Souza, M. I. (2000). Spontaneous BRSV infection in cattle of the state of Alagoas, Brazil Spontaneous BRSV infection in cattle of the state of Alagoas, Brazil (in Portuguese). Pesquisa Veterinária Brasileira, 20, 171–175.

Poumarat, F., Le Grand, D., Philippe, S., Calavas, D., Schelcher, F., Cabanie, P., … Navetat, H. (2001). Efficacy of spectinomycin against *Mycoplasma bovis* induced pneumonia in conventionally reared calves. Veterinary Microbiology, 80, 23–35.

Pretto, L. G., Müller, E. E., Freitas, J. C., Mettifoga, E., Buzinhani, M., & Yamaguti, M. (2001). *Mycoplasma bovis* isolation from calves with pneumonia (in Portuguese). Veterinária Notícias, 7, 69–73.

Ridpath, J. (2010). The contribution of infections with Bovine viral diarrhea viruses to bovine respiratory disease. Veterinary Clinics of North America: Food Animal Practice, 26, 335–348.

Rivera-Rivas, J. J., Kisiela, D., & Czuprynski, C. J. (2009). Bovine herpes- virus type 1 infection of bovine bronchial epithelial cells increases neutrophil adhesion and activation. Veterinary Immunology and Immunopathology, 131, 167–176.

Rodríguez, F., Bryson, D. G., Ball, H. J., & Forster, F. (1996). Pathological and immunohistochemical studies of natural and experimental *Mycoplasma bovis* pneumonia in calves. Journal of Comparative Pathology, 115, 151–162.

Shahriar, F. M., Clark, E. G., Janzen, E., West, K., & Wobeser, G. (2002). Coinfection with bovine viral diarrhea virus and *Mycoplasma bovis* in feedlot cattle with chronic pneumonia. Canadian Journal of Veterinary Research, 43, 863–868.

Silveira, S., Weber, M. N., Mosena, A. C., da Silva, M. S., Streck, A. F., Pescador, C. A., … Canal, C. W. (2017). Genetic diversity of brazilian bovine pestiviruses detected between 1995 and 2014. Transboundary and Emerging Diseases, 64, 613–623.

Suarez Heinlein, A., Metzler, A. E., Weiblen, R., Berrios, P., Schudel, A. A., & Rodriguez, M. (1993). Molecular characterization of South American bovine herpesvirus-1 isolates with monoclonal antibodies and SDS-PAGE. Journal of Veterinary Medicine B, Infectious Diseases and Veterinary Public Health, 40, 125–130.

Szeredi, L., Janosi, S., & Palfi, V. (2010). Microbiological and pathological examination of fatal calf pneumonia cases induced by bacterial and viral respiratory pathogens. Acta Veterinaria Hungarica, 58, 341–356. Thomas, L. H., Howard, C. J., Stott, E. J., & Parsons, K. R. (1986). *Mycoplasma bovis* infection in gnotobiotic calves and combined infection with respiratory syncytial virus. Veterinary Pathology, 23, 571–578.

Tortorelli, G., Carrillo Gaeta, N., Mendonça Ribeiro, B. L., Miranda Marques, L., Timenetsky, J., & Gregory, L. (2017). Evaluation of Mollicutes microorganisms in respiratory disease of cattle and their relationship to clinical signs. Journal of Veterinary Internal Medicine, 31, 1215–1220.

Vanden Bush, T. J., & Rosenbusch, R. F. (2003). Characterization of the immune response to *Mycoplasma bovis* lung infection. Veterinary Immunology and Immunopathology, 94, 23–33.

Viuff, B., Tjornehoj, K., Larsen, L. E., Rontved, C. M., Uttenthal, A., Ronsholt, L., & Alexandersen, S. (2002). Replication and clearance of respiratory syncytial virus: Apoptosis is an important pathway of virus clearance after experimental infection with bovine respiratory syncytial virus. The American Journal of Pathology, 161, 2195–2207.

Wageck Canal, C., Strasser, M., Hertig, C., Masuda, A., & Peterhans, E. (1998). Detection of antibodies to bovine viral diarrhoea virus (BVDV) and characterization of genomes of BVDV from Brazil. Veterinary Microbiology, 63, 85–97.

Welsh, M. D., Adair, B. M., & Foster, J. C. (1995). Effect of BVD virus infection on alveolar macrophage functions. Veterinary Immunology and Immunopathology, 46, 195–210.

Wolfger, B., Timsit, E., White, B. J., & Orsel, K. (2015). A systematic re- view of bovine respiratory disease diagnosis focused on diagnostic confirmation, early detection, and prediction of unfavorable outcomes in feedlot cattle. Veterinary Clinics of North America: Food Animal Practice, 31, 351–365.

Yİlmaz, R., Cangul, I. T., Onat, K., Akkoc, A., Ozyİgİt, M. O., & Akdesİr, E. (2016). Histopathological, immunohistochemical and bacteriological characterization of *Mycoplasma bovis* pneumonia in cattle. Pakistan Veterinary Journal, 36, 316–321.

7 ARTIGO 3 – THE PARTICIPATION OF A MALIGNANT CATARRHAL FEVER VIRUS AND *MYCOPLASMA BOVIS* IN THE DEVELOPMENT OF SINGLE AND MIXED INFECTIONS IN BEEF AND DAIRY CATTLE WITH BOVINE RESPIRATORY DISEASE

Thalita Evani Silva Oliveira, Gabriela Sanches Scuisato, Isadora Fernanda Pelaquim, Cristina Wetzel Cunha, Lucas Santana Cunha, Eduardo Furtado Flores, Lucienne Garcia Pretto-Giordano, Júlio Augusto Naylor Lisbôa, Amauri Alcindo Alfieri, João Paulo Elsen Saut, Paulo Henrique Jorge da Cunha, Selwyn Arlington Headley. **Frontier Veterinary Science**. 2021; 8:691448. doi: 10.3389/fvets.2021.691448.

7.1 SUMMARY

The bovine respiratory disease (BRD) complex is a multietiological and multifactorial disease associated with a wide range of viral and bacterial pathogens. This study evaluated the contribution of specific infectious disease agents in the development of BRD in cattle from Brazil and determined if a virus within the MCF group (MCFV) and *Mycoplasma bovis*, acting individually or in conjunction, can be associated with the development of BRD. Formalin-fixed paraffin embedded pulmonary sections were used in immunohistochemical assays to determine the intralesional presence of six antigens associated with BRD: bovine alphaherpesvirus 1 (BoAHV1), bovine parainfluenza virus 3 (BPIV-3), bovine viral diarrhea virus (BVDV), bovine respiratory syncytial virus (BRSV), MCFV, and *M. bovis*. Pneumonia was diagnosed in 82.7% (120/145) of all cattle evaluated. Interstitial pneumonia (60%; 72/120) and suppurative bronchopneumonia (25.8%; 31/113) were the most frequent patterns of pneumonia identified. Intralesional antigens of MCFV (53.3%; 64/120) were the most frequently associated with BRD, followed by *M. bovis* (47.5%; 57/120), BVDV (42.5%; 51/120), BoAHV1 (28.3%; 34/120), BRSV (24.2%; 29/120), and BPIV-3 (8.3%; 10/120). Additionally, antigens of BVDV, MCFV, and *M. bovis* were the most frequently identified agents associated with singular and concomitant infections. The MCFV identified during this study is more likely ovine gammaherpesvirus 2 (OvHV-2), since OvHV-2 is the only MCFV identified within the geographical region of this study. Interstitial pneumonia with proliferative vascular lesions may be a useful histologic feature to differentiate MCFV-induced pneumonia from other viral pneumonias of

cattle. These results demonstrate that MCFV (OvHV-2) and *M. bovis*, in single or in mixed infections, can produce pneumonia in cattle and should therefore be considered as primary agents in the development of BRD.

Keywords: diagnostic immunohistochemistry, fibrinoid change, histopathologic patterns, caseonecrotic bronchopneumonia, proliferative vascular alterations, respiratory disease pathogens, ovine gammaherpesvirus 2

7.2 INTRODUCTION

Bovine respiratory disease (BRD) is a complex multifactorial and multietiological disease entity that is associated with viral and bacterial pathogens coupled with unfavorable management practices and environmental conditions. The principal viral disease pathogens associated with the development of BRD are bovine coronavirus (BCoV), bovine alphaherpesvirus 1 (BoAHV1), bovine parainfluenza virus 3 (BPIV-3), bovine viral diarrhea virus (BVDV), and bovine respiratory syncytial virus, BRSV (FULTON, 2009; O'NEILL *et al.*, 2014; GERSHWIN *et al.*, 2015; HEADLEY *et al.*, 2018). The major bacterial pathogens of BRD include *Mannheimia haemolytica, Pasteurella multocida, Histophilus somni* (FULTON *et al.*, 2009; PANCIERA & CONFER, 2010; GRISSETT *et al.*, 2015; KLIMA *et al.*, 2019), and *Mycoplasma bovis* (FULTON *et al.*, 2009; LÓPEZ & MARTINSON, 2017). All of these pathogens have been associated with outbreaks of BRD in beef and dairy cattle herds from Brazil (CASTRO *et al.*, 2021).

More recently, ovine gammaherpesvirus 2 (OvHV-2) was suggested as a possible infectious disease pathogen associated with the development of BRD (HEADLEY *et al.*, 2020b). Additionally, it must be highlighted that although the malignant catarrhal fever virus (MCFV) complex, is known to be composed of eight members (ICTV, 2021), only OvHV-2 was associated with malignant catarrhal fever (MCF) in ruminants from Brazil (HEADLEY *et al.*, 2020b; HEADLEY *et al.*, 2020c). Although, spontaneous cases of interstitial pneumonia associated with the amplification of OvHV-2 DNA was previously described in cattle (HEADLEY *et al.*, 2020a; HEADLEY *et al.*, 2020c), bison (O'TOOLE *et al.*, 2002; O'TOOLE & LI, 2014),

and buffaloes (COSTA *et al.*, 2009), the participation of MVFV/OvHV-2 in the development of BRD is a novel finding. Moreover, a MCFV, more likely OvHV-2, has been associated with respiratory disease in a calf simultaneously infected with three additional agents (O'TOOLE & LI, 2014).

Viral disease pathogens of BRD may cause primary infections, acting either in single or mixed infections with other pathogens (GERSHWIN *et al.*, 2015; OLIVEIRA *et al.*, 2020a; ZHANG *et al.*, 2020). A significant role for BRD-related viruses is their interaction with bacteria (GRIFFIN *et al.*, 2010; SZEREDI *et al.*, 2010; ZHANG *et al.*, 2020) and mycoplasma (NICHOLAS & AYLING, 2003; ARCANGIOLI *et al.*, 2008; CASWELL *et al.*, 2010) in the development of bronchopneumonia. Moreover, studies have shown that *M. bovis* was the only pathogen identified in pneumonic lungs, resulting in fatal pneumonia in calves (RODRIGUEZ *et al.*, 1996; GERSHWIN *et al.*, 2015; MARGINEDA *et al.*, 2017) and adult cattle (OLIVEIRA *et al.*, 2020a).

In Brazil, information relative to the occurrence of BRD is limited when compared with studies from North America (GAGEA *et al.*, 2006b; TAYLOR *et al.*, 2010; KLIMA *et al.*, 2014; FRANCOZ *et al.*, 2015) and Australia (CUSACK *et al.*, 2003; HAY *et al.*, 2016). Previous studies done in Brazil were designed to investigate a single (DRIEMEIER *et al.*, 1997; FLORES *et al.*, 2000; PEIXOTO *et al.*, 2000; PRETTO *et al.*, 2001; SILVA *et al.*, 2007; BRASIL *et al.*, 2013; BEUTTEMMULLER *et al.*, 2017) or several agents (BAPTISTA *et al.*, 2017; HEADLEY *et al.*, 2017; MAGALHÃES *et al.*, 2017; TORTORELLI *et al.*, 2017; GAETA *et al.*, 2018; HEADLEY *et al.*, 2018; OLIVEIRA *et al.*, 2020a; OLIVEIRA *et al.*, 2020b) associated with BRD. These studies focused on the molecular identification of agents, with and without the histopathologic diagnosis of the patterns of pulmonary disease. It must be highlighted, that associating the pattern of pulmonary disease with intralesional presence of the agents is fundamental to confirm infection. Recently, we have identified the occurrence of infectious disease pathogens of BRD by using a combination of the histopathologic patterns and the *in situ* identification of the infectious disease pathogen by immunohistochemistry (IHC) and have shown that *M. bovis* may be a potential primary disease agent of pneumonia in cattle (OLIVEIRA *et al.*, 2020a).

Consequently, the aims of this study were to determine the occurrence of respiratory infectious disease agents in cattle from several geographical regions of Brazil, evaluate the contribution of specific infectious disease agents in the

development of BRD, and determine if a MCFV, and *M. bovis*, acting individually or mixed infections, can be associated with the development of BRD.

7.3 MATERIAL AND METHODS

7.3.1 Study design

A cross-sectional study focusing on two units of observation was designed: the first investigated respiratory agents associated with the development of BRD, while the second determined the occurrence of pneumonia. The first was designed to associate the occurrence of pneumonia with specific infectious disease pathogens irrespective of the pattern of pulmonary disease. Subsequently, predetermined patterns of pneumonia were classified and related with specific agent (s) associated with BRD.

7.3.2 Study area, animal selection, and histopathologic analyses

A review of all pulmonary tissues of cattle submitted for histopathologic diagnosis between 2015-2018 was done to determine the occurrence of pneumonia. These samples originated from diagnostic laboratories located within the states of São Paulo (Southeastern), Paraná, and Santa Catarina (Southern) Brazil; some of the pulmonary tissues derived from cows from Paraná were part of a larger study that investigated the occurrence of neurological disease of cattle (QUEIROZ *et al.*, 2018). Archival records of all animals were retrieved, reviewed, and tabulated to obtained information relative to gender, type of animal (beef, dairy, mixed), age, and cause of death (natural x slaughter). These data were then associated with the occurrence of pulmonary disease. The age of all cattle was divided into two predetermined categories: calves (cattle up to 15 month-old) and adults (animals 16 month-old or older) (VAZ *et al.*, 2011). Only data relative to the age of cattle with pulmonary disease, irrespective of the patterns of pneumonia were included in the analysis.

Formalin-fixed paraffin-embedded (FFPE) tissue sections of these pulmonary sections were used to produce new histological slides when necessary. All sections were stained by the hematoxylin and eosin (H&E) method and reviewed for histopathologic patterns of pulmonary disease as outlined (OLIVEIRA *et al.*, 2020a);

the histopathologic review was done by two veterinary pathologists (TESO; SAH). In addition, new histological slides were made for all tissues containing intralesional pleomorphic organisms; these were colored with the Giemsa histochemical method for the identification of organisms consistent with *M. bovis,* and the Brown Brenn Gram histochemical stain to differentiate from other accumulations of Gram-positive or negative bacteria; both methods were based on previous protocols (LUNA, 1968). Giemsa staining was used to identify *M. bovis*, since we have previously suggested that this histochemical stain may efficiently identify these intralesional organisms (OLIVEIRA *et al.*, 2020a).

Additionally, the pulmonary tissues evaluated were divided into three categories based on the predominant histologic alterations observed: 1) pulmonary tissues with predominantly cellular and vascular alterations (congestion, reversible and irreversible cellular lesions); 2) interstitial pneumonia, and 3) bronchopneumonia. These categories were then used as inputs to correlate these histologic findings with the intralesional localization of antigens of the evaluated agents identified by IHC.

7.3.3 Immunohistochemical identification of infectious disease agents associated with BRD

Immunohistochemical assays were performed on pulmonary sections of each animal to determine the intralesional presence of antigens six agents potentially associated with the development of BRD: BoAHV1, BRSV, BVDV, BPIV-3, MCFV, and *M. bovis*. Selected FFPE tissue sections of the lungs were prepared on silanized slides with Poly-L-lysine 0.1% (Sigma-Aldrich, St. Louis, MO, USA) and submitted to IHC assays designed to identify the antigens of these agents. The IHC assays to detect antigens of BoAHV1, BVDV, BRSV, BPIV-3, and *M. bovis* as previously described (OLIVEIRA *et al.*, 2020a). MCFV-specific antigens were identified by using the monoclonal antibody 15A, MAb-15A (HEADLEY *et al.*, 2020c). Positive controls included FFPE tissue sections known to contain antigens of BoAHV1, BVDV, BRSV, BPIV-3, *M. bovis* (OLIVEIRA *et al.*, 2020a), and MCFV/OvHV-2 (HEADLEY *et al.*, 2020c). Two negative controls were used: the first consisted of substituting the primary antibodies with their respective diluents; the second consisted of utilizing the primary antibodies on FFPE tissues with known negative immunoreactivity to the BRD

antigens derived from the studies cited above. Positive and negative controls were included in each IHC assay.

7.3.4 Data analysis

The association between the biological data of the cattle and the occurrence of pneumonia and the frequencies of infection (absolute and relative) were determined using descriptive statistics. The determination of the comparative distribution of IHC antigens in normal and affected tissues was obtained due to the comparative frequency relative to each agent. Consequently, the frequency of each agent evaluated in a determined histologic element was obtained by calculating the occurrence of positive immunolabelling within the histologic element with the total IHC identification of each disease pathogen. Additionally, the association between the occurrence of pathogens and the number of cattle infected was determined. When appropriated, association between the presence of pulmonary disease and specific variables was analyzed using the Chi-square test, using a free software R 4.0.3 (2021), differences were considered significant when the resulting *p*-value was less than 0.05.

7.4 RESULTS

7.4.1 Occurrence of pneumonia and biological data

During the four-year period, the lungs from 145 cattle submitted for histopathologic diagnosis were reviewed. Pneumonia was diagnosed in 82.7% (120/145) of these lungs, 25 animals had no histopathologic lesions consistent with pneumonia. No significant differences ($p>0.05$) were identified between the type of cattle, gender, and age relative to the occurrence of pneumonia (Table 1). The median age of the calves was 2 months (range: 2 days to 13 months), while that of adult animals were four was (range: 1 year and 8 months to 10 years).

Table 1 – Epidemiological data of pulmonary tissue of cattle submitted for histopathologic diagnosis.

Variable	Pulmonary tissue without pneumonia (%)	Pulmonary tissue with pneumonia (%)	Chi-square test (p-value)
Gender (*n*)			
Male (19)	6	13	0.406
Female (54)	13	41	
Not reported (72)	13	59	
Type of cattle (*n*)			
Beef (49)	10	39	0.366
Dairy (34)	5	29	
Mixed (62)	17	45	
Age (*n*)			
Calves (51)	12	39	0.097
Adult (94)	20	74	
Form of death (*n*)			
Natural (108)	27	81	0.146
Slaughter (37)	5	32	

7.4.2 Patterns of pulmonary disease and histologic features of pneumonia

Most of the lungs evaluated (82.7%; 120/145) had at least one pattern of pneumonia and some contained more than one pattern of pulmonary disease (Table 2). Consequently, from the 120 animals with pneumonia evaluated, 139 patterns of pulmonary disease were observed, with animals presenting one (72.5%; 87/120) or several patterns (27.5%; 33/120) of pneumonia. Nevertheless, interstitial pneumonia (60%; 72/120) was the most predominant pattern observed (Table 2), followed by suppurative (25.8%; 31/120), caseonecrotic bronchopneumonia (8.3%; 10/120), and fibrinosuppurative bronchopneumonia (4.2%; 5/120). A few cows (1.7%; 2/120) had cuffing pneumonia.

Table 2 – The frequency of the patterns of pneumonia identified in the lungs of cattle

Patterns of pneumonia	#Number of cattle	%
Without pneumonia	32	22.1
Interstitial pneumonia	70	48.3
Suppurative bronchopneumonia	26	17.9
Caseonecrotic bronchopneumonia	10	6.9
Fibrinosuppurative bronchopneumonia	5	3.4
Cuffing pneumonia	2	1.4
Total	**145**	**100**

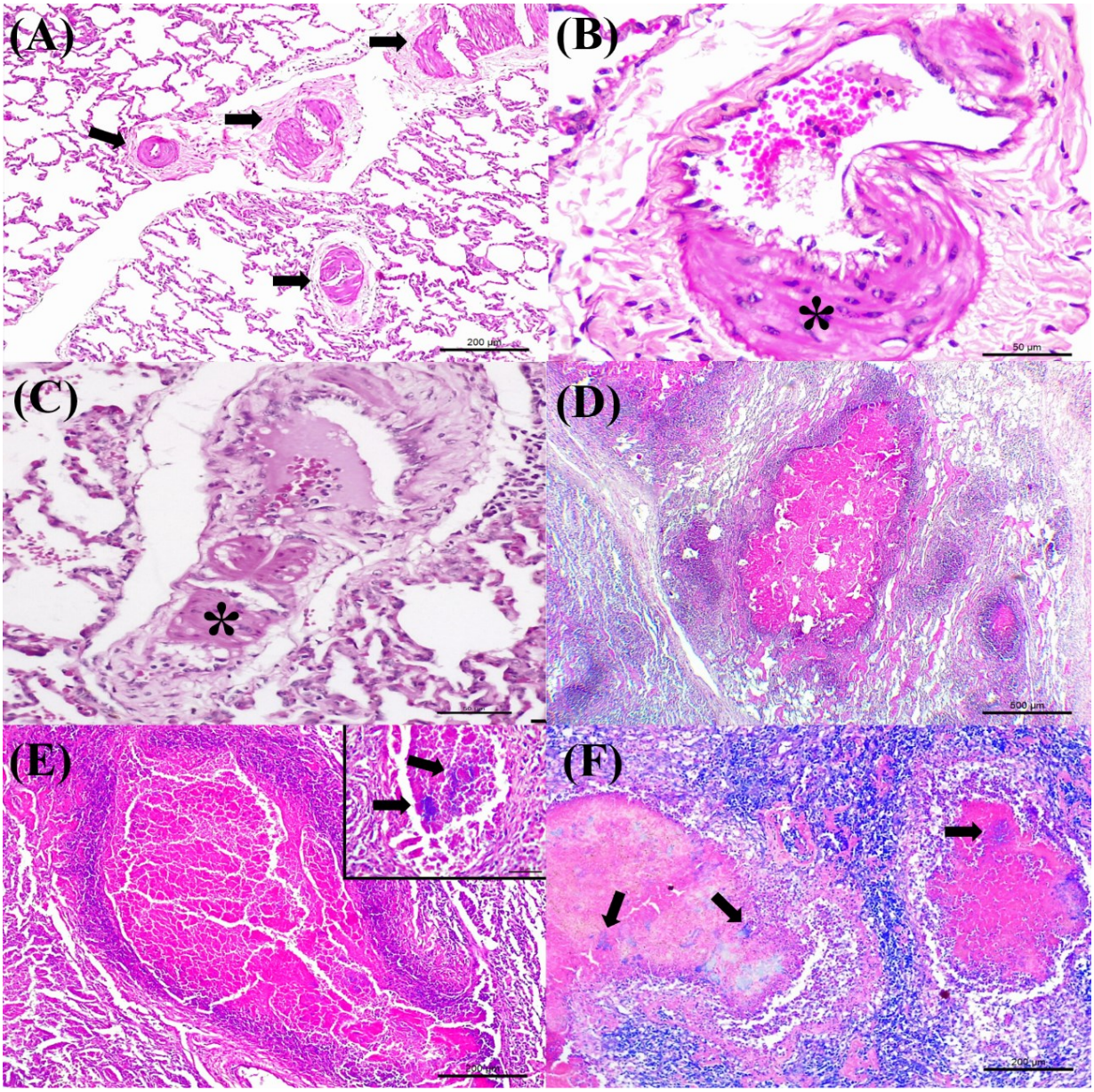
Antigens of all agents investigated were identified in all three categories of pulmonary lesions evaluated (Table 3). However, antigens of MCFV were more frequently associated with all categories (53.3%; 64/120), including pulmonary tissues with interstitial pneumonia (28.3%; 34/120; Table 3) and vascular disease resulting in arterial proliferation (Figure 1A-C). Other agents frequently associated with the development of interstitial pneumonia were BVDV (25%; 30/120) and BoAHV1 (17.5%; 21/120; Table 3). As expected, antigens of *M. bovis* were more frequently (25%; 30/120) associated with the development of bronchopneumonia (Table 3; Figure 1D), suppurative infiltrate predominantly in the terminal and respiratory bronchioles (Figure 1E), and frequently associated with obliterative bronchiolitis (Figure 1F). Accumulations of intralesional, Giemsa-positive (Figure 1G-H), bacterial accumulations were only observed in cases of bronchopneumonia (66%; 31/47; Table 2). Gram-positive or Gram-negative bacteria were not detected by the modified Brown–Brenn stain.

Table 3 – Occurrence of infectious disease pathogens within the categories of pulmonary disease in cattle with BRD (*n*=145)[1]

Disease pathogens	Cellular/vascular alterations (%)	Interstitial pneumonia (%)	Broncho-pneumonia (%)	Total (%)
Malignant catarrhal fever virus	11 (7.6)	34 (23.4)	19 (13.1)	64 (44.1)
Mycoplasma bovis	13 (9)	14 (9.7)	30 (27)	57 (39.3)
Bovine viral diarrhea virus	8 (5.5)	30 (20.7)	13 (9)	51 (35.2)
Bovine alphaherpesvirus-1	3 (2.1)	21 (14.5)	10 (6.9)	34 (23.4)
Bovine respiratory syncytial virus	6 (4.1)	17 (11.7)	6 (4.1)	29 (20)
Bovine parainfluenza virus-3	4 (2.8)	4 (2.8)	2 (1.4)	10 (6.9)

[1]Based on the occurrence of singular and simultaneous pathogens.

Figure 1 – Histopathological findings observed in the lungs of cattle with bovine respiratory disease associated with intralesional antigens of MCFV (A–C) and *M. bovis* (D–H). (A) There is interstitial pneumonia, (B) containing areas of arterial proliferation (arrows); (C) higher magnification demonstrating proliferation of the tunica media of pulmonary arteries (asterisk). (D) Typical demonstration of caseonecrotic bronchopneumonia associated with infection by *M. bovis*; observe the well-demarcated, centrally located, foci of necrosis containing a large necrotic hyper-eosinophilic debris and pleomorphic bacteria (arrows). (E) There is a neutrophilic exudate within a terminal bronchiole (asterisk) in a cow with suppurative bronchopneumonia. (F) Observe areas of obliterative bronchiolitis (asterisk). (G) There are intralesional bacterial aggregates positive for *M. bovis* (arrows). (H) Closer view demonstrating intralesional mollicutes (arrows). Hematoxylin and eosin (A–F) and Giemsa stain (G,H). Bar: (A,B,D–F) 200 µm; (C,G,H) 50 µm.



7.4.3 IHC identification of infectious disease antigens in cattle with bovine respiratory disease

The associations between intralesional antigens of infectious disease pathogens of BRD with the specific histologic element of the lung are summarized in Figure 2. When IHC results were associated with a single pathogen (Table 4), *M. bovis* (31.8%; 14/44) was the most frequently identified agent, followed by BVDV (22.7%; 10/44), MCFV (18.2%; 8/44), BRSV (13.6%; 6/44), BoAHV1 (11.4%; 5/44) and BPIV-3 (2.3%; 1/44).

Figure 2 – Comparative distribution of the immunohistochemical identification of antigens of single infectious disease pathogens within the lungs of cattle with BRD due to **(A)** *Mycoplasma bovis,* **(B)** bovine viral diarrhea virus, **(C)** malignant catarrhal fever viruses, **(D)** bovine respiratory syncytial virus, and **(E)** bovine alphaherpesvirus type 1. Bar; Green: cellular and vascular alterations; Blue: interstitial pneumonia and Red: bronchopneumonia.

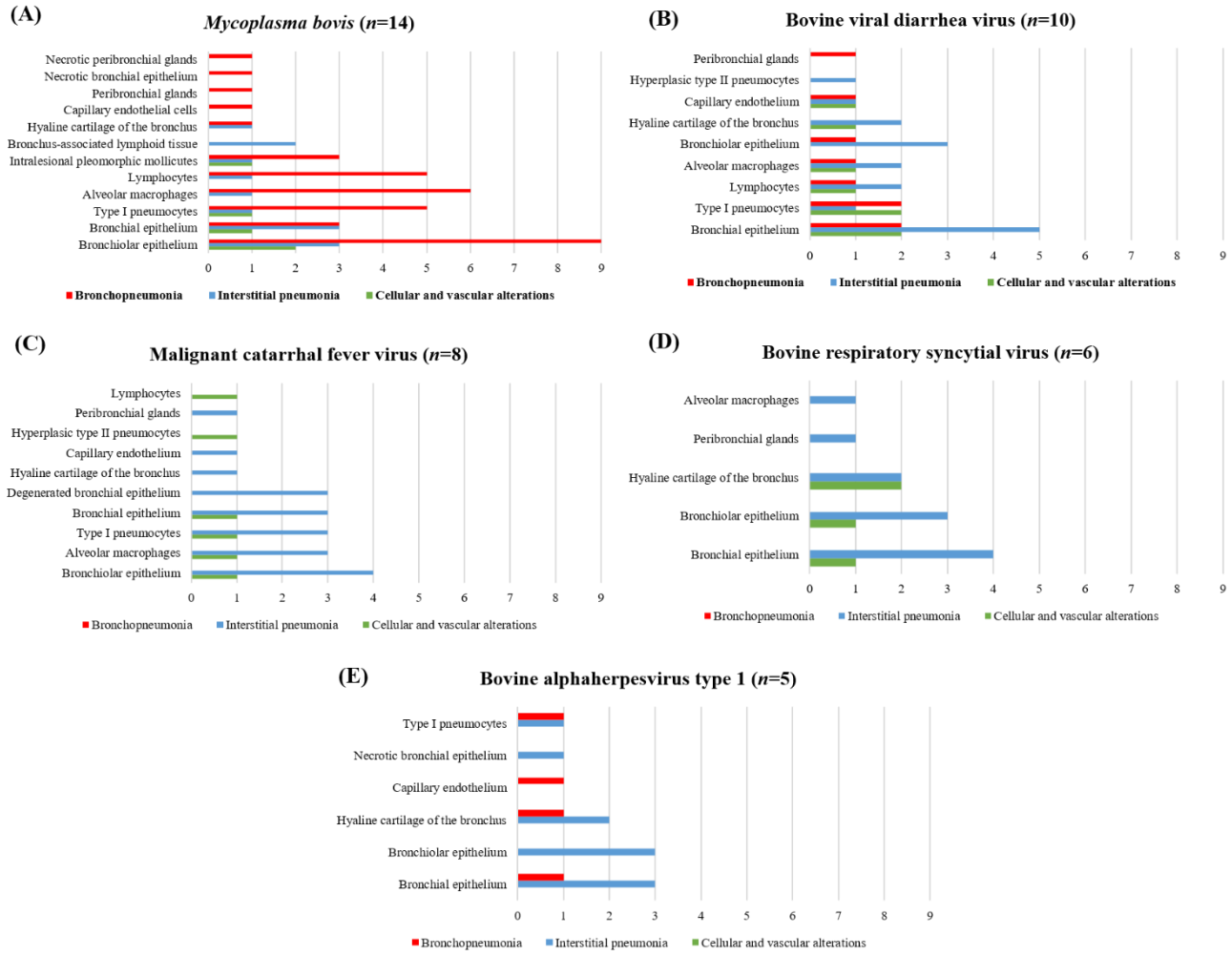


Table 4 – Distribution of the immunohistochemical identification of intralesional antigens in the development of singular and concomitant infections in the lungs of cattle with BRD

Summary of singular and dual infections						
Agents	**MCFV**	***M. bovis***	**BVDV**	**BoAHV1**	**BRSV**	**BPIV-3**
MCFV	8	10	12	3	3	0
M. bovis	-	14	2	2	2	2
BVDV	-	-	10	0	2	0
BoAHV1	-	-	-	5	1	1
BRSV	-	-	-	-	6	0
BPIV-3	-	-	-	-	-	1

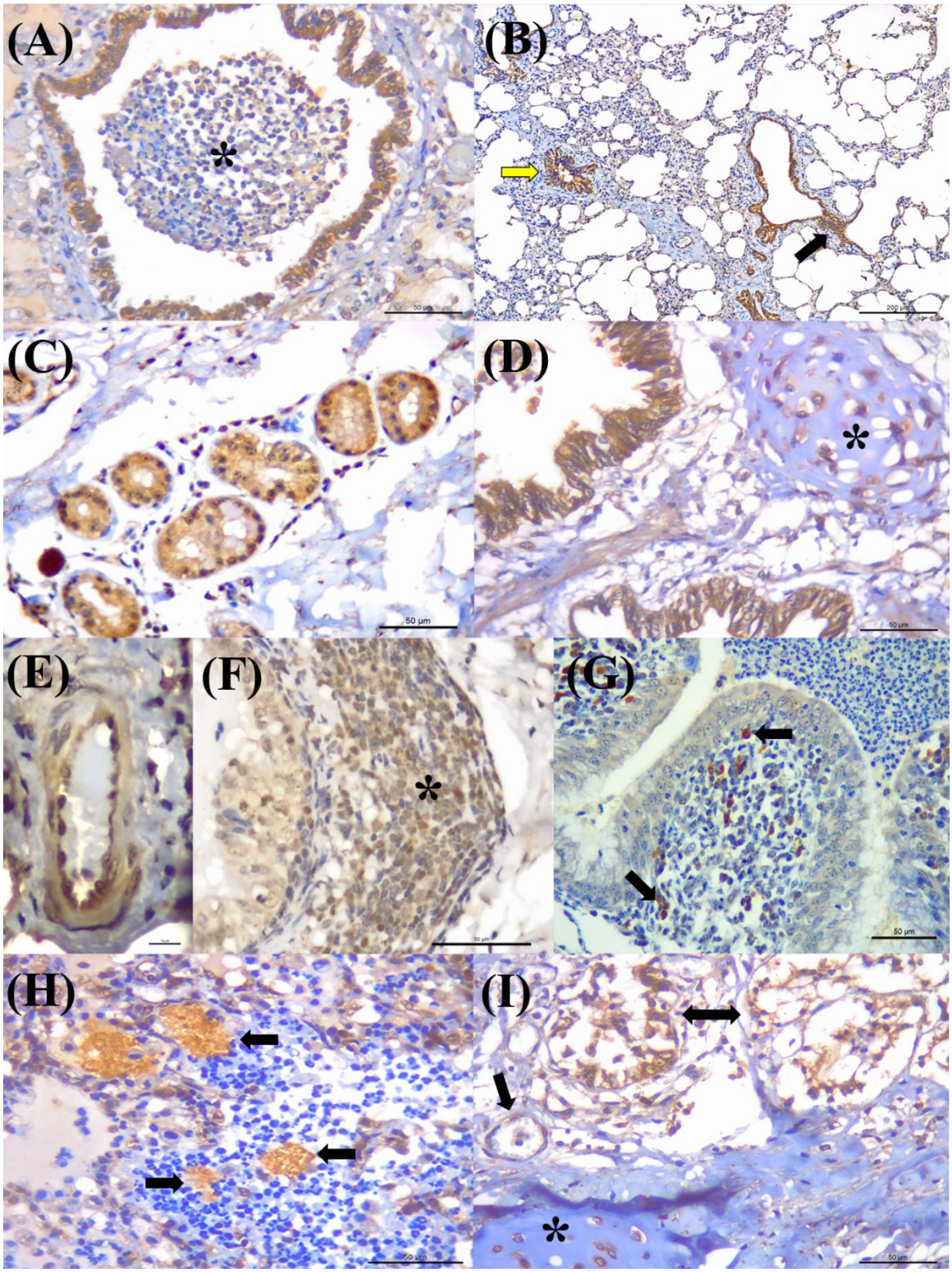
Association of intralesional antigens	**Number of cattle**
Triple infections	
BoAHV1, BPIV-3, BRSV	1
BoAHV1, BRSV, *M. bovis*	2
BoAHV1, BVDV, *M. bovis*	3
BoAHV1, BVDV, MCFV	6
BoAHV1, *M. bovis*, MCFV	1
BPIV-3, BRSV, *M. bovis*	1
BRSV, BVDV, MCFV	1
BRSV, *M. bovis*, MCFV	3
BVDV, *M. bovis*, MCFV	7
Total	**25**
Quadruple infections	
BoAHV1, BPIV-3, BVDV, *M. bovis*	1
BoAHV1, BRSV, BVDV, MCFV	3
BoAHV1, BRSV, MCFV, *M. bovis*	1
BoAHV1, BVDV, *M. bovis*, MCFV	2
BPIV-3, BRSV, *M. bovis*, MCFV	1
BRSV, BVDV, *M. bovis*, MCFV	1
Total	**9**
Quintuple infections	
BoAHV1, BPIV-3, BRSV, *M. bovis*, MCFV	1
BoAHV1, BPIV-3, BVDV, *M. bovis*, MCFV	1
Total	**2**
Without pathogens (*n*=25)	

BoAHV1: bovine alphaherpesvirus type 1; BPIV-3: bovine parainfluenza virus type 3; BRSV: bovine respiratory syncytial virus; BVDV: bovine viral diarrhea virus; *M. bovis*: *Mycoplasma bovis*; MCFV: malignant catarrhal fever virus complex; and OvHV-2: ovine gammaherpesvirus-2.

Positive immunoreactivity to *M. bovis* antigens was widely distributed within the lung during this study (Figure 2A). These include the epithelial cells of the bronchiole (Figure 3A-B) and bronchus, alveolar macrophages, type I pneumocytes, mixed peribronchial glands (Figure 3C), chondrocytes of the bronchial hyaline cartilage (Figure 3D), endothelium (Figure 3E). Furthermore, *M. bovis* was the only agent associated with positive immunolabeling on BALT lymphoid tissue (Figure 3F-G) and intralesional pleomorphic mollicutes (Figure 3H). Antigens of *M. bovis* were also

identified within necrotic peribronchial glands (Figure 3I) and necrotic bronchial epithelial cells.

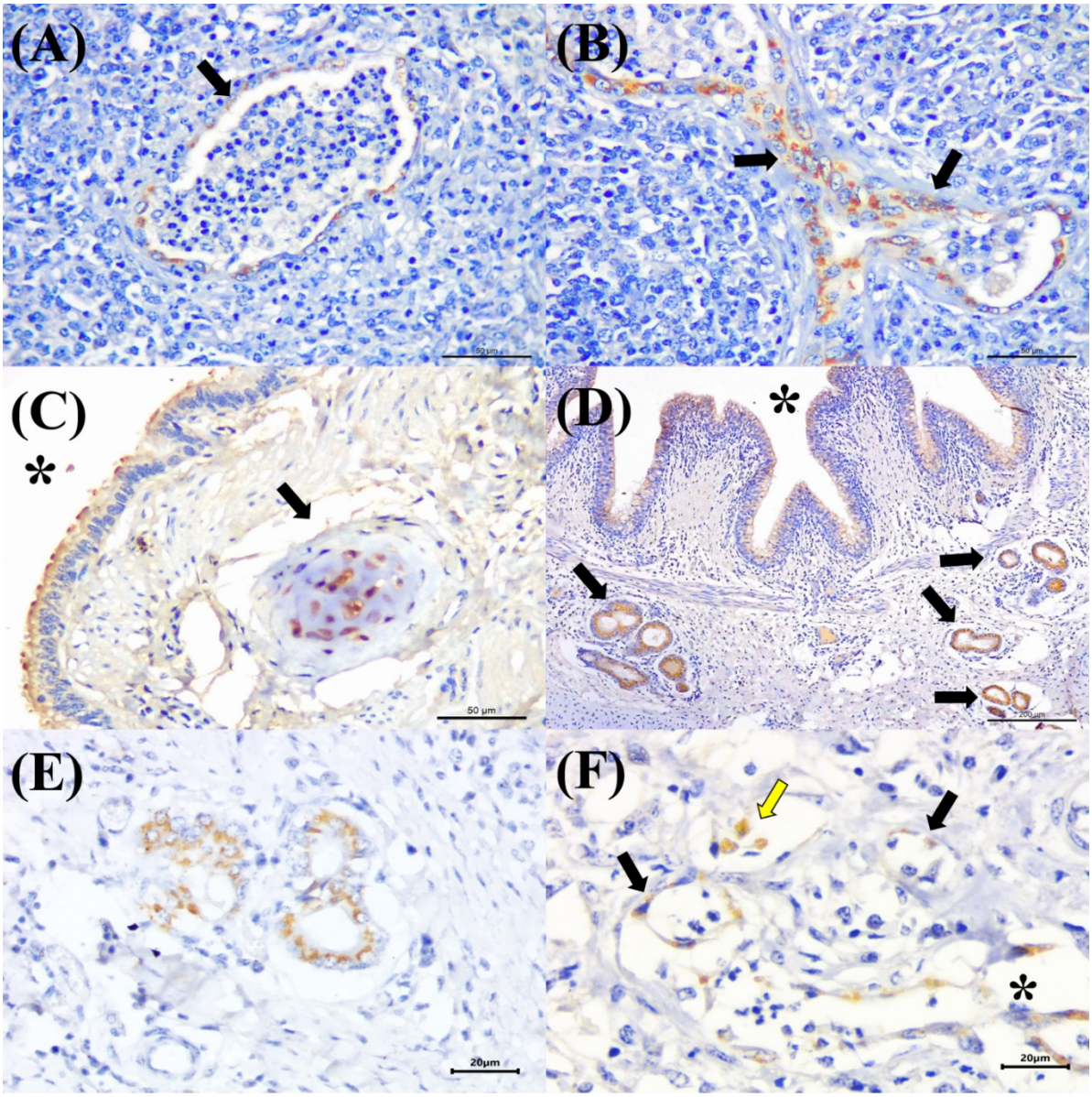
Figure 3 – Immunohistochemical identification of *Mycoplasma bovis* antigens in cattle with bovine respiratory disease. (A) There is positive intracytoplasmic immunoreactivity to *M. bovis* antigens in the normal bronchiolar epithelium (arrows) of a cow with bronchopneumonia. (B) Observe positive immunoreactivity in respiratory (yellow arrow) and terminal bronchiole (black arrow) of a cow with interstitial pneumonia. (C) There is positive reactivity at the peribronchial glands. (D) Observe positive immunoreactivity within chondrocytes of the hyaline cartilage (asterisk), (E) within endothelium cells, (F) lymphocytes (asterisk), (G) macrophages, (arrow), and bronchus-associated lymphoid tissue. (H) There is positive immunolabeling within accumulations of the pleomorphic organism (arrows) in a lung with suppurative bronchopneumonia. (I) Observe positive immunolabeling in necrotic peribronchial glands (two-headed arrow), normal endothelium cells (arrow), and chondrocytes of the hyaline cartilage (asterisk). Immunoperoxidase counterstained with hematoxylin. Bar: A, C-D, F-I 50 µm; B 200 µm; E 10 µm.



BVDV antigens were observed in the three categories studied (Figure 2B). Immunoreactivity was observed within the epithelial cells of the bronchus and bronchiole (Figure 4A-B), type I pneumocytes, alveolar lymphocytes and macrophages, chondrocytes of the bronchial hyaline cartilage (Figure 4C),

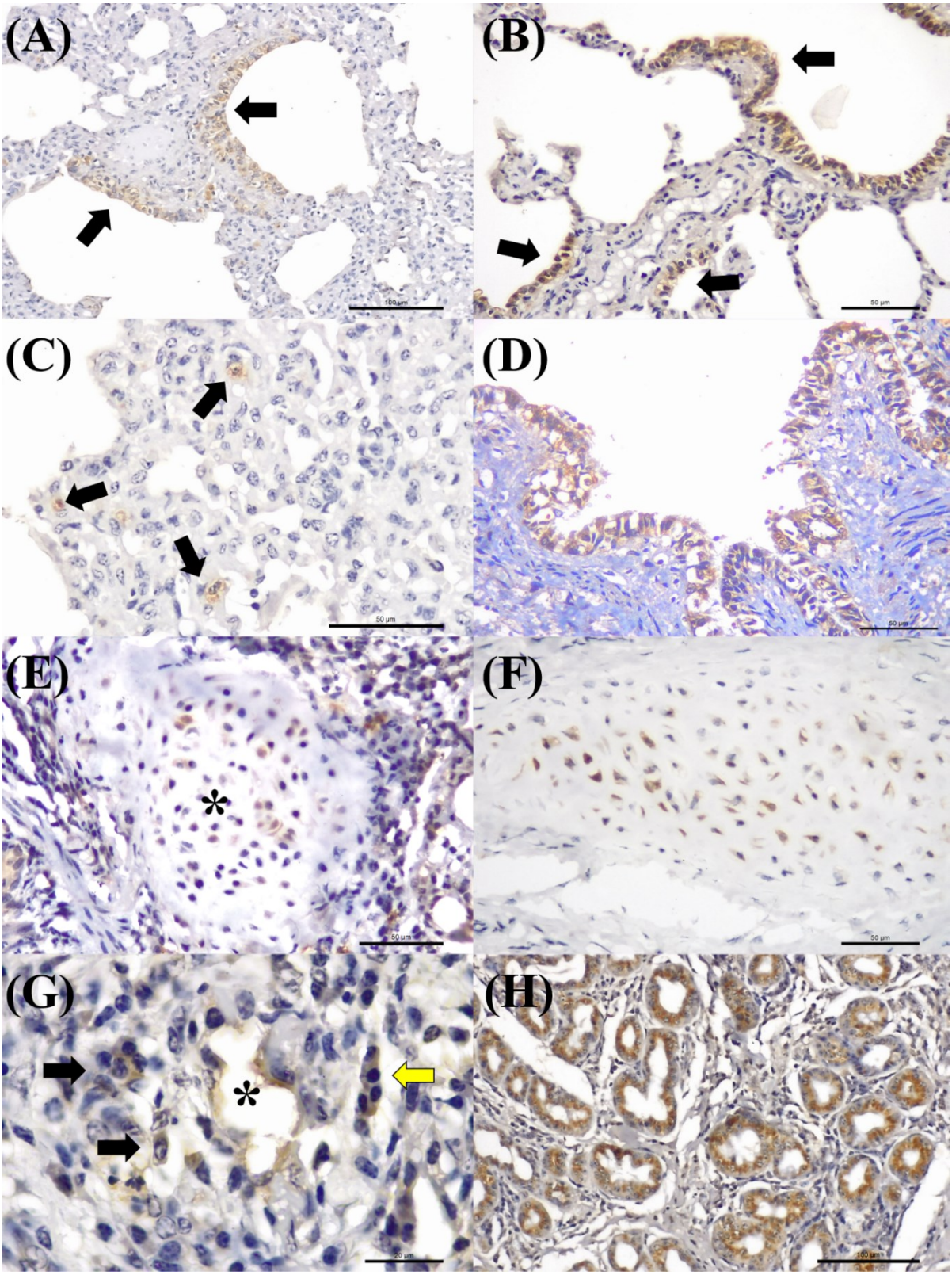
endothelium cells, mixed peribronchial glands (Figure 4D), and in hyperplastic type II pneumocytes.

Figure 4 - Immunohistochemical detection of BVDV antigens in cattle with bovine respiratory disease. There is positive immunoreactivity to BVDV in the epithelial cells of the (A) terminal and (B) respiratory bronchiole of a cow with bronchopneumonia. (C) Observe positive immunolabeling at chondrocytes of the hyaline cartilage (asterisk), epithelial cells of the bronchus (arrow), (D) mixed peribronchial glands (arrows), and bronchial epithelial cells (asterisk); (E) closer view showing positive immunoreactivity within epithelial cells of the mixed peribronchial glands. (F) BVDV antigens within alveolar macrophages (yellow arrow), the endothelia of a capillary (black arrows) and venule (asterisk) of the lung. Immunoperoxidase counterstained with hematoxylin. Bar: A-C 50 µm; D 200 µm; E and F 20 µm.



MCFV positive intracytoplasmic immunoreactivity (Figure 2C) was identified primarily within bronchiolar epithelial cells (Figure 5A-B), alveolar macrophages (Figure 5C), type I pneumocytes, degenerated bronchial epithelial cells (Figure 5D), chondrocytes of the bronchial hyaline cartilage (Figure 5E-F), endothelial cells of pulmonary venule (Figure 5G), hyperplasic type II pneumocytes, mixed peribronchial glands (Figure 5H), and alveolar lymphocytes. Positive immunoreactivity to antigens of MCFV was restricted to the pulmonary lesions within the categories classified as cellular and vascular alterations and interstitial pneumonia, without being observed in tissues diagnosed as bronchopneumonia. Additionally, immunoreactivity to MCFV antigens was patchy within the pneumocytes of cows with interstitial pneumonia and was comparatively more predominant in type 1 relative to type 2 cells.

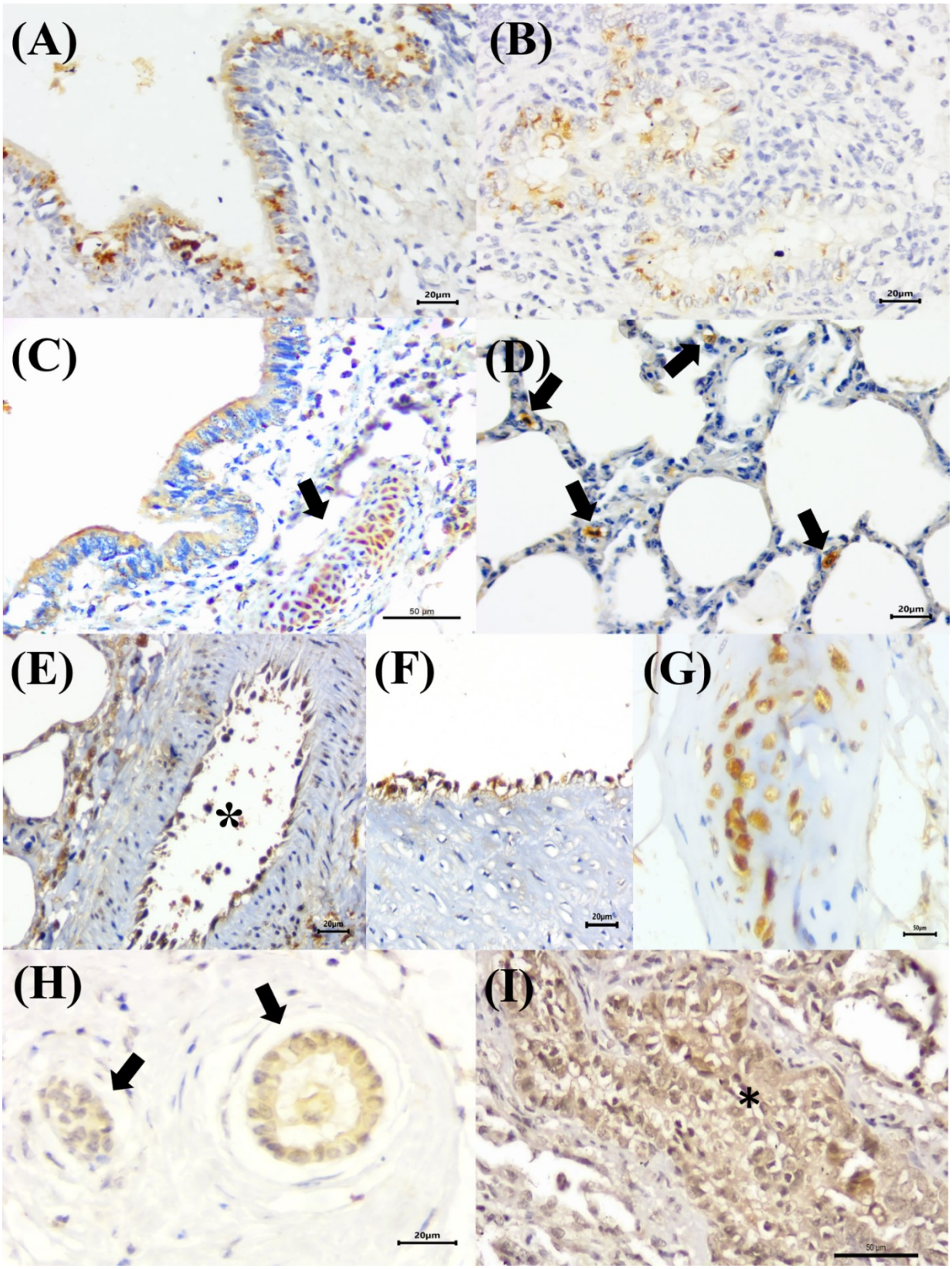
Figure 5 – Immunohistochemical demonstration of immunoreactivity to MCFV in cattle with bovine respiratory disease. There is positive intracytoplasmic immunoreactivity to antigens of MCFV within the cytoplasm of epithelial cells of the (A-B) terminal bronchiole and the patchy immunoreactivity within (C) alveolar epithelium (arrows) in a case of interstitial pneumonia. (D) Observe intracytoplasmic immunoreactivity to antigens of MCFV within (E-F) degenerated bronchial epithelial cells, (E) chondrocytes of the hyaline cartilage (asterisk), (G) endothelium cells of a pulmonary venule (asterisk), macrophages (black arrows), lymphocytes (yellow arrow), and (H) within epithelial cells of the mixed peribronchial glands. Immunoperoxidase counterstained with hematoxylin. Bar; A, H 100 µm; B-F 50 µm and G 20 µm.



Immunoreactivity to BRSV (Figure 2D), with positive intracytoplasmic immunoreactivity, occurred within bronchial and bronchiolar epithelial cells (Figure 6A-B), chondrocytes of the bronchial hyaline cartilage (Figure 6C), mixed peribronchial

glands, and alveolar macrophages (Figure 6D). Like MCFV, BRSV antigens were observed only in the categories classified as circulatory changes and interstitial pneumonia.

Figure 6 – Immunohistochemical identification of BRSV, BoAHV1, and BPIV-3 antigens in singular infections of cattle with bovine respiratory disease. (A) There is positive intracytoplasmic immunoreactivity to BRSV antigens within bronchial and (B) bronchiolar epithelial cells, (C) chondrocytes of the hyaline cartilage (arrow), and (D) alveolar macrophages (arrows) (E,F). Observe positive intracytoplasmic immunolabeling for BoAHV1 antigens within endothelial cells [(E), asterisk] and (G) chondrocytes of the hyaline cartilage. (H) There is positive intracytoplasmic immunoreactivity to BPIV-3 antigens within epithelial cells of the normal (arrows) and (I) vacuolized bronchiolar cells (asterisk). Immunoperoxidase counterstained with hematoxylin. Bar: (A,B,D–F,H) 20 μm; (C,G,I) 50 μm.



BoAHV1 antigens (Figure 2E) were only observed in cases of interstitial pneumonia and bronchopneumonia, with intracytoplasmic immunoreactivity within bronchial and bronchiolar epithelial cells, chondrocytes of the bronchial hyaline

cartilage (Figure 6E), pulmonary endothelial cells (Figure 6F-G), necrotic bronchial epithelial cells, and type I pneumocytes.

BPIV-3 antigens were observed only in one animal with circulatory changes and revealed cytoplasmic immunoreactivity within the cells of the normal (Figure 6H) and degenerated (Figure 6I) bronchiolar epithelium.

Figure 7 illustrates an interesting feature identified in mixed infections due to BVDV and MCFV in 12 animals. Concomitant infections were observed predominantly in bronchopneumonia (16.7%; 2/12) and interstitial pneumonia (75%; 9/12) and were also associated with the development of cellular and vascular alterations (8.3%; 1/12). More interestingly, with simultaneous immunolabeling in bronchopneumonia, antigens of both viruses were identified within bronchial and bronchiole epithelium (Figure 8A-B), necrotic bronchiolar cells (Figure 8C-D), and alveolar macrophages (Figure 8E). Additionally, there was positive immunoreactivity to MCFV within goblet cells (Figure 8F), chondrocytes of the bronchial hyaline cartilage, pulmonary endothelium, mixed peribronchial glands, alveolar lymphocytic infiltrate, and degenerated bronchial epithelium (Figure 8G-H).

Figure 7 – Comparative demonstration of positive immunolabeling for malignant catarrhal fever virus and bovine viral diarrhea virus antigens within histologic elements of lungs of cattle with BRD

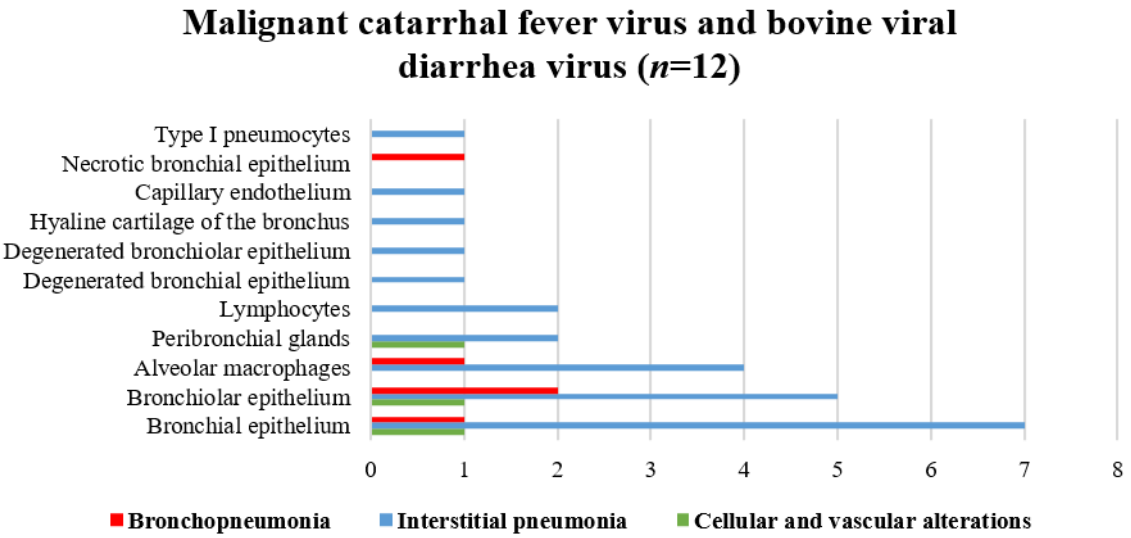
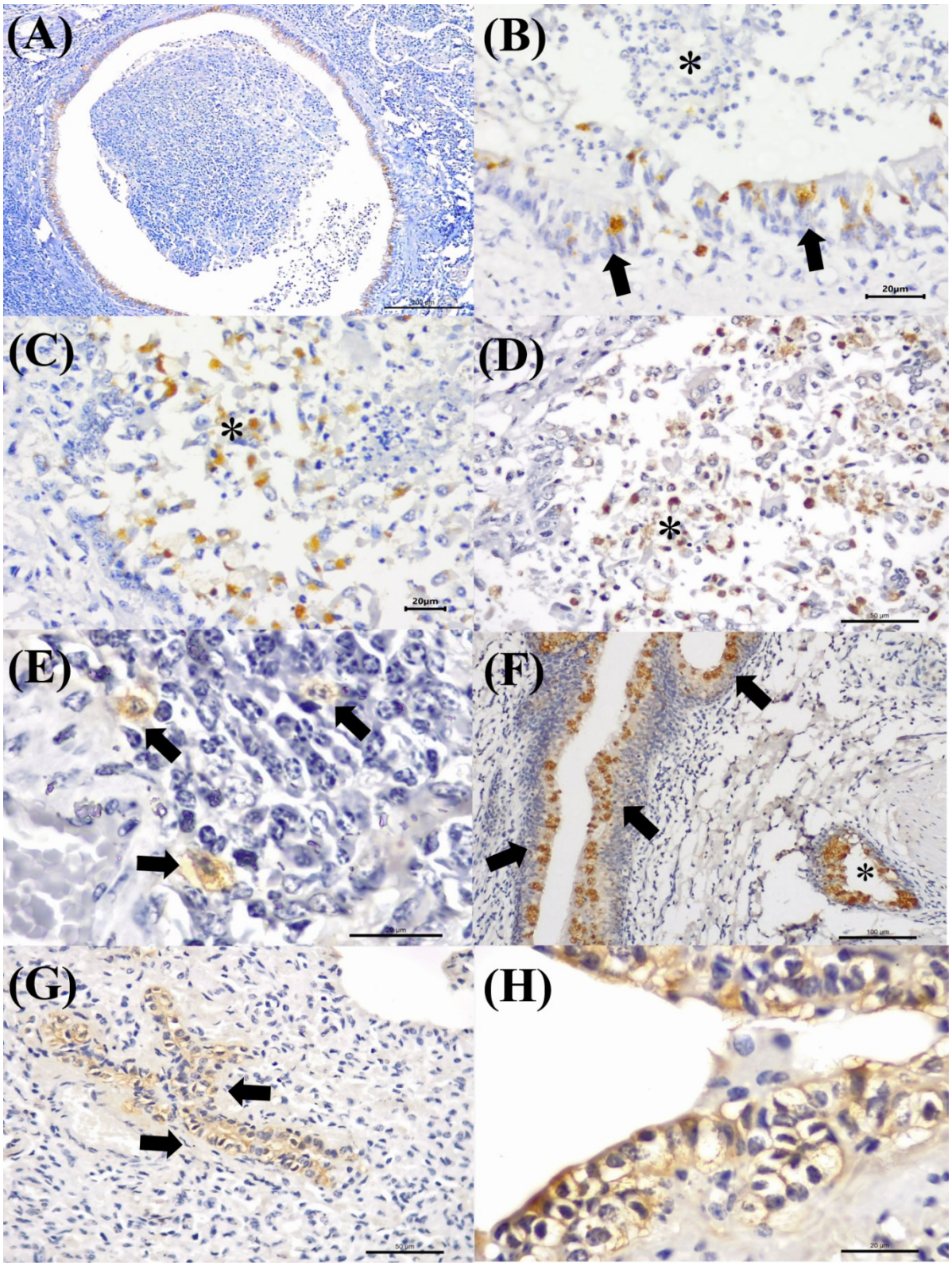


Figure 8 – Immunohistochemical identification of BVDV, and MCFV antigens in cattle with bronchopneumonia with bovine respiratory disease. **(A)** There is positive intracytoplasmic immunoreactivity to BVDV in the bronchial cells of an ecstatic

bronchiole, **(B)** closer view of epithelia cells (arrows), and negative immunoreactivity in neutrophils (asterisk). **(C-D)** Immunoreactivity to MCFV antigens within necrotic bronchiolar epithelia cells, **(E)** alveolar macrophages degenerated, **(F)** goblet cells (arrows), normal (asterisk), and **(G-H)** degenerated bronchiolar epithelial cells. Immunoperoxidase counterstained with hematoxylin. Bar: **A** 200 µm; **B, C, E** and **H** 20 µm; **D** and **G** 50 µm; **F** 100 µm



7.4.4 Frequency and relationship between the occurrence of infectious disease pathogens and pulmonary disease

A high occurrence of respiratory disease pathogens was observed in the pulmonary tissues evaluated, with antigens of more than one respiratory disease pathogen detected in 82.7% (120/145) of these, while 36.7% (44/120) of the pulmonary tissues contained only one pathogen associated with the development of BRD. When the occurrence of single infections was evaluated (Table 4), *M. bovis* antigens were the most frequently identified (31.8%; 14/44), followed by BVDV (22.7%; 10/44), MCFV (18.2%, 8/44), BRSV (13.6%; 6/44), BoAHV1 (11.4%; 5/44), and BPIV-3 (2.3%; 1/44). However, antigens of infectious disease agents were not identified in 20.8% (25/120) of the pulmonary tissues evaluated. Antigens of MCFV and *M. bovis* were associated with singular (18.2%, 8/44; 22.7%, 10/44), dual (70%, 28/40; 45%, 18/40), triple (76%, 19/25; 68%, 17/25), quadruple (88.9%, 8/9; 66.7%, 6/9), and quintuple (100%, 2/2; 100%, 2/2) infections, respectively, during this study.

Two respiratory disease agents were simultaneously identified within the lungs of 40 cows (Table 4), resulting in mixed infections. Antigens of MCFV were the most frequently observed associated with BVDV (42.9%; 12/28), followed by *M. bovis* (35.7%; 10/28), BRSV (10.7%; 3/28), and BoAHV1 (10.7%; 3/28). Antigens of BVDV were identified in more than half (53.9%; 41/76; Table 4) of these cases, while mixed infections associated with BVDV predominantly included MCFV (40.8%; 31/76) and *M. bovis* (22.4%; 17/76). Additionally, dual infections were more frequently associated with intralesional antigens of MCFV (*n*=28; Table 4), being predominantly associated with antigens of BVDV (42.8%; 12/28) and *M. bovis* (35.7%; 10/28).

In animals infected with a single agent, infection of normal bronchiolar epithelial cells was observed in all *M. bovis* infected cows (100%; 14/14), followed by BRSV (83,3%; 5/6), MCFV (50%; 4/8), BVDV (40%; 4/10), and BoAHV1 (40%; 2/5). Few pulmonary tissues had positive immunoreactivity at the capillary endothelium, being observed for BVDV (30%; 3/10), BoAHV1 (20%; 1/5), MCFV (12.5%; 1/8), and *M. bovis* (7.1%; 1/14; Figure 4D).

Positive immunolabeling at the hyaline cartilage of the bronchus was identified in the pulmonary tissues of 27.3% (12/44) cows; BRSV (66.7%; 4/6) antigens were more frequently identified within chondrocytes of the hyaline cartilage of the bronchus, followed by BoAHV1 (40%; 2/5), BVDV (30%; 3/10), *M. bovis* (14.3%; 2/14),

MCFV (12.5%; 1/8); antigens of BPIV-3 were not observed within chondrocytes. A few cattle had positive immunolabelling at normal peribronchial glands for BRSV (16.7%; 1/6), MCFV (12.5%; 1/8), and *M. bovis* (7.1%; 1/14). Positive immunoreactivity within lymphocytes was observed in 11 cows and was associated with antigens of *M. bovis* (54.5%; 6/11); BVDV (36.4%; 4/11) and MCFV (9.1%; 1/11); antigens of BRSV and BPIV-3 were not observed within lymphocytes. Intralesional pleomorphic organisms, stained by Giemsa, were identified in 35.7% (5/14; Figure 5C) of cows that contained antigens of *M. bovis.*

The most frequent infectious disease pathogen (Table 3) identified in association with BRD was MCFV (53.3%; 64/120), followed by *M. bovis* (47.5%; 57/120), BVDV (42.5%; 51/120), BoAHV1 (28.3%; 34/120), BRSV (24.2%; 29/120), and BPIV-3 (8.3%; 10/120). Furthermore, singular (36.7%; 44/120), dual (33.3%; 40/120), triple (20.8%; 25/120), quadruple (7.5%; 9/120), and quintuple (1.7%; 2/120) infections were identified (Table 4).

Necrosis was observed at the epithelial cells and peribronchial glands in the lungs of 6.8% (3/44) cows. Of these cases, necrosis affecting bronchial epithelia was associated with antigens of BoAHV1 (20%; 1/5) and *M. bovis* (7.1%; 1/14), with *M. bovis* antigens being observed in 7.1% (1/14) of the necrotic peribronchial glands.

Hyperplastic lesions were identified in few cows (9.1%; 4/44); in two of these, there was bronchus-associated lymphoid tissue (BALT) hyperplasia associated with positive immunoreactivity for *M. bovis* antigens. Hyperplasia of type II pneumonocytes was observed in 2 (4.5%; 2/44) cows; with positive immunoreactivity to MCFV (2.3%; 1/44) and BVDV (2.3%; 1/44).

7.5 DISCUSSION

The results of this study demonstrated the multietiological nature of BRD, in which 63.3% (76/120) of the lungs of cattle evaluated were infected by two or more infectious disease agents. The IHC identification of infectious disease pathogens on FFPE tissues is a sensitive method to detect intralesional antigens and was previously used to identify BVDV, BoAHV1, BRSV, *M. bovis* (GERSHWIN *et al.*, 2015; OLIVEIRA *et al.*, 2020a), BPIV-3 (HAINES *et al.*, 1992; OLIVEIRA *et al.*, 2020a) and MCFV/OvHV-2 (HEADLEY *et al.*, 2020c) antigens in tissues of BRD-affected cattle. Additionally, the *in situ* detection of intralesional tissue antigens is an excellent method

for retrospective studies using archival samples. Moreover, this diagnostic method is preferred over molecular testing, to confirm disease association, since the intralesional identification of disease pathogens within affected tissue clearly demonstrates the association between infectious disease agents and histologic alteration and/or pattern of disease (FULTON *et al.*, 2009; MAES *et al.*, 2014).

In a previous study by our group in cattle with neurological manifestation of MCF associated with OvHV-2 but without the classic manifestations of MCF (HEADLEY *et al.*, 2020c), positive immunoreactivity was not observed within the pulmonary tissues available for evaluation. Similarly, in the current study, the cows investigated did not demonstrate the typical clinical manifestations of MCF and were therefore without a clinical diagnosis of MCF. The pulmonary disease associated with MCFV antigens identified during this study can be classified as subacute to chronic interstitial pneumonia due to the accumulated lymphocytes and macrophages (LÓPEZ & MARTINSON, 2017). Moreover, OvHV-2 is known to produce chronic disease in cattle characterized by proliferating arterial lesions (O'TOOLE *et al.*, 1995; O'TOOLE *et al.*, 1997; HEADLEY *et al.*, 2020c); proliferative vascular lesions were observed in the lungs of cattle with interstitial pneumonia associated with intralesional antigens of MCFV and represented 28.3% (34/120) of all interstitial pneumonias identified during this study. These findings may suggest that MCFV produces interstitial pneumonia with vascular proliferating lesions as the prominent histologic feature, which may be useful to distinguish MCFV-induced interstitial pneumonia from other viral pneumonias of cattle. Another interesting feature during this study was the patchy immunoreactivity of pneumocytes in interstitial pneumonia associated with MCFV antigens; similar findings were described in pigs experimentally infected with OvHV-2 (LI *et al.*, 2012). Additionally, experimental studies done in sheep (TAUS *et al.*, 2005; TAUS *et al.*, 2010) and pigs (LI *et al.*, 2012), have suggested that the interstitial pneumonia induced by OvHV-2 results in lytic replication predominantly within type II pneumocytes (TAUS *et al.*, 2010). In the present study, intralesional MCFV antigens were observed within hyperplastic type II pneumocytes, degenerated and necrotic bronchiolar and bronchial epithelial; these findings herein described corroborate with those of experimental studies (TAUS *et al.*, 2005; LI *et al.*, 2012). Consequently, this pathogen may be an innocent bystander, or a primary infectious disease agent, acting individually or in association with other pathogens towards the development of BRD in cattle, and should be considered as a possible infectious agent associated with the development

of BRD. Moreover, a MCFV, more likely OvHV-2, was related with the occurrence of respiratory disease in a calf that was simultaneously infected with BVDV and *Aspergillus fumigatus* (HEADLEY *et al.*, 2020a).

Furthermore, the MAb-15A antibody that detects epitopes of MCF viruses (LI *et al.*, 1994), was shown to effectively identify antigens of OvHV-2 in FFPE of cattle with MCF (HEADLEY *et al.*, 2020c). The IHC findings observed in this study demonstrated the participation of MCFV in the development of respiratory disease in the cows evaluated. Furthermore, positive immunoreactivity for MVFV was observed within epithelial cells of the lungs of cattle with different categories of pulmonary disease, but principally in cases of interstitial pneumonia. In Brazil, only OvHV-2 is known to be associated with the development of MCF in ruminants (HEADLEY *et al.*, 2020b), suggesting that the MCFV identified in these animals was most likely OvHV-2; similar findings were recently described (HEADLEY *et al.*, 2020a; XAVIER, 2021). Collectively, these findings support the hypothesis that OvHV-2 may be an infectious disease agent of BRD (HEADLEY *et al.*, 2020a; XAVIER, 2021). Previous investigations have shown that OvHV-2 induces infiltrative, degenerative (XAVIER, 2021) and necrotic changes in the urinary bladder (LIGGITT & DEMARTINI, 1980; XAVIER, 2021), kidney cells (XAVIER, 2021), salivary gland (LIGGITT & DEMARTINI, 1980), as well as the gastrointestinal and respiratory system of cattle (LIGGITT & DEMARTINI, 1980; HEADLEY *et al.*, 2020a). Furthermore, a review of all published cases of MCF in Brazil revealed that interstitial pneumonia is a frequently diagnosed histologic alteration described in cattle infected with OvHV-2 (HEADLEY *et al.*, 2020b).

Only *M. bovis* antigens were observed in the lungs of cattle with BALT hyperplasia; suggesting that this lesion can be used as an indicator of *M. bovis*-induced pneumonia in cattle as previously described (GAGEA *et al.*, 2006a; HERMEYER *et al.*, 2012). It should be emphasized that in most cases of BALT hyperplasia, the pulmonary tissue evaluated had more than one histologic pattern of *M. bovis*-induced pulmonary disease, while in only two animals cuffing pneumonia was the only histologic alteration observed. Other histologic patterns of *M. bovis*-induced pneumonia identified were caseonecrotic- and suppurative bronchopneumonia; similar results have been described (GAGEA *et al.*, 2006a; CASWELL *et al.*, 2010; HERMEYER *et al.*, 2012). The caseonecrotic bronchopneumonia (also referred to as necrosuppurative bronchopneumonia), is considered the most important diagnostic histologic feature to

differentiate *M. bovis*-induced pneumonia from other bacterial pneumonias of cattle (CASWELL *et al.*, 2010; HERMEYER *et al.*, 2012).

A limitation of this study was the absence of the IHC analyses to identify members of the *Pasteurellaceae* family associated with the development of BRD. Although *P.multocida, M. haemolytica, and H. somni* are the most common bacterial pathogens (GRIFFIN *et al.*, 2010; PANCIERA & CONFER, 2010; CASWELL & WILLIAMS, 2016; LÓPEZ & MARTINSON, 2017), these pathogens were not evaluated during this study due to the lack commercial antibodies for IHC. Additionally, attempts to obtain in-house polyclonal antibodies or hyperimmune serum against these bacterial agents were frustrating. Nevertheless, these organisms are commensals of the bovine nasopharynx which, during periods of stress or viral infection, can overwhelm host defense mechanisms establishing infection in the lower respiratory tract and are associated with the development of fibrinosuppurative or suppurative bronchopneumonia, pulmonary abscesses, and necrosis in cattle (FULTON *et al.*, 2009; PANCIERA & CONFER, 2010; CASWELL & WILLIAMS, 2016; LÓPEZ & MARTINSON, 2017). Consequently, the BALT hyperplasia and caseonecrotic bronchopneumonia associated with intralesional antigens of *M. bovis* can be considered as histologic patterns specific for pulmonary disease by this pathogen as opposed to histologic patterns of pulmonary disease related to *P. multocida, M. haemolytica*, and *H. somni*. We had previously postulated that Giemsa staining may be a cheap and adequate method to identify intralesional *Mycoplasma* organisms (OLIVEIRA *et al.*, 2020a). Similar results were identified in this study, suggesting that this simple histochemical stain may be used to confirm the presence of this organism, principally in cases with the classical histologic presentation of mycoplasma pneumonia.

In this study, 63.3% (76/120) of the pulmonary infections observed were mixed; tissues antigens of MCFV and *M. bovis* were observed simultaneously in two to five infections within the same pulmonary section. These findings suggest that these two organisms can produce pneumonia acting individually or in association with other pathogens of BRD. BVDV is a well-known immunosuppressive agent of cattle (POTGIETER, 1995; WELSH *et al.*, 1995; OLCHOWY *et al.*, 1997; LIEBLER-TENORIO *et al.*, 2003; RISALDE *et al.*, 2011; RISALDE *et al.*, 2013; STRONG *et al.*, 2015; REID *et al.*, 2016), which could have favored concomitant infections, including MCFV and *M. bovis*. We have previously discussed the relationship between BVDV

and *M. bovis*, and the synergism between these two organisms (OLIVEIRA *et al.*, 2020a). In that study there were four singular infections (11.4%; 4/35) associated with *M. bovis* (OLIVEIRA *et al.*, 2020a); in the current study, 11.7% (14/120) of the affected cows were infected with *M. bovis*. These results corroborate with previous studies that have identified *M. bovis*, in single infections, as a primary contributor towards the development of BRD (ARCANGIOLI *et al.*, 2008; NICHOLAS, 2011; GERSHWIN *et al.*, 2015). However, the same does not hold for MCFV or OvHV-2 as described above, since there are few reports associating these pathogens with BRD.

This study demonstrated high frequencies of infections by MCFV (53.3%; 64/120) and *M. bovis* (47.5%; 57/120) in the cattle from geographical locations of Brazil. These elevated occurrences can be related to several conditions, including the absence of a vaccine or specific treatment to effectively control MCFV and the frequently chronic presentation of *M. bovis*-induced pneumonia (CASWELL *et al.*, 2010), which results in a late diagnosis. Consequently, farmers must be educated relative to the existence of these diseases, especially concerning the adoption of adequate control and prophylactic measures (MACLACHLAN & DUBOVI, 2017). Another factor that may have contributed to the elevated occurrence of *M. bovis* during this study is the well-established microbial resistance of this organism to common antibiotic therapy (CASWELL *et al.*, 2010; SULYOK *et al.*, 2017).

7.6 CONCLUSION

These results suggest that most cattle evaluated presented some form of pulmonary lesion associated with BRD. The occurrence of interstitial pneumonia was most frequently related to antigens of MCFV and BVDV, while *M. bovis* was frequently associated with caseonecrotic bronchopneumonia. These findings suggest that MCFV, most likely involving OvHV-2, was associated with the development of pulmonary disease in cattle and should be considered as a primary disease pathogen of BRD, acting innocently, singularly or in association, primarily with BVDV. Moreover, the concomitant occurrence of MCFV and BVDV within the lungs of cattle with pneumonia suggest a possible synergism between these two infectious agents towards the development of BRD. Furthermore, proliferating vascular lesions in the lung may be an important histologic feature to diagnose MCFV-induced interstitial pneumonia.

7.7 REFERENCES

R Core Team. R: A language and environment for statistical computing. 2021. R Foundation for Statistical Computing, Vienna, Austria. URL Acessed: 2020/01/10.

ARCANGIOLI, M.-A.; DUET, A.; MEYER, G.; DERNBURG, A.; BÉZILLE, P.; POUMARAT, F.; LE GRAND, D. The role of *Mycoplasma bovis* in bovine respiratory disease outbreaks in veal calf feedlots. **The Veterinary Journal**, v. 177, n. 1, p. 89-93, 2008.

BAPTISTA, A. L.; REZENDE, A. L.; FONSECA, P. D. A.; NOGUEIRA, G. M.; HEADLEY, S. A.; MENEZES, G. L.; ALFIERI, A. A.; SAUT, J. P. E. Bovine respiratory disease complex associated mortality and morbidity rates in feedlot cattle from southeastern Brazil. **Journal of Infection in Developing Countries**, v. 11, n. 10, p. 791-799, 2017.

BEUTTEMMULLER, E. A.; ALFIERI, A. F.; HEADLEY, S. A.; ALFIERI, A. A. Brazilian strain of bovine respiratory coronavirus is derived from dual enteric and respiratory tropism. **Genetics and Molecular Research**, v. 16, n. 2, 2017.

BRASIL, N. D. A.; HINNAH, F. L.; FISS, L.; SALLIS, E. S. V.; GRECCO, F. B.; LADEIRA, S. R. L.; MARCOLONGO-PEREIRA, C.; SCHILD, A. L. Doenças respiratórias em bezerros na região sul do Rio Grande do Sul: estudo retrospectivo de 33 surtos. **Pesquisa Veterinária Brasileira**, v. 33, p. 745-751, 2013.

CASTRO, M. M.; OLIVEIRA, T. E. S.; HEADLEY, S. A. Bovine respiratory disease in Brasil: a short review. **Semina: Ciências Agrárias**, v. 42, n.

CASWELL, J. L.; BATEMAN, K. G.; CAI, H. Y.; CASTILLO-ALCALA, F. *Mycoplasma bovis* in respiratory disease of feedlot cattle. **Veterinary Clinics of North America: Food Animal Practice**, v. 26, n. 2, p. 365-379, 2010.

CASWELL, J. L.; WILLIAMS, K. J. Respiratory system. In: MAXIE, M. G. (Ed.). **Jubb, Kennedy, and Palmer's Pathology of domestic animals**. 5th. Philadelphia: Saunders/Elsevier, v.2, 2016. cap. 5, p.465-591.

COSTA, É. A.; BASTIANETTO, E.; VASCONCELOS, A. C.; BOMFIM, M. R. Q.; FONSECA, F. G. D.; GOMES, A. D.; LEITE, R. C.; RESENDE, M. An outbreak of malignant catarrhal fever in Murrah buffaloes in Minas Gerais, Brazil. **Pesquisa Veterinária Brasileira**, v. 29, n. 5, p. 395-400, 2009.

CUSACK, P.; MCMENIMAN, N.; LEAN, I. The medicine and epidemiology of bovine respiratory disease in feedlots. **Australian Veterinary Journal**, v. 81, n. 8, p. 480-487, 2003.

DRIEMEIER, D.; GOMES, M. J. P.; MOOJEN, V.; ARNS, C. W.; VOGG, G.; KESSLER, L.; COSTA, U. M. D. Manifestação clínico-patológica de infecção natural pelo vírus respiratório sincicial bovino (BRSV) em bovinos de criação extensiva no Rio Grande do Sul, Brasil. **Pesquisa Veterinária Brasileira**, v. 17, n. 2, p. 77-81, 1997.

FLORES, E. F.; WEIBLEN, R.; MEDEIROS, M.; BOTTON, S. A.; IRIGOYEN, L. F.; DRIEMEIER, D.; SCHUCH, L. F.; MORAES, M. A retrospective search for bovine respiratory syncytial virus (BRSV) antigens in histological specimens by immunofluorescence and immunohistochemistry. **Pesquisa Veterinária Brasileira**, v. 20, n. 4, p. 139-143, 2000.

FRANCOZ, D.; BUCZINSKI, S.; BELANGER, A. M.; FORTE, G.; LABRECQUE, O.; TREMBLAY, D.; WELLEMANS, V.; DUBUC, J. Respiratory pathogens in Quebec dairy calves and their relationship with clinical status, lung consolidation, and average daily gain. **Journal of Veterinary Internal Medicine**, v. 29, n. 1, p. 381-387, 2015.

FULTON, R. W. Chapter 42 - Viral diseases of the bovine respiratory tract. In: ANDERSON, D. E. e RINGS, D. M. (Ed.). **Food Animal Practice** 5th. Saint Louis: W.B. Saundersv. cap. 42, p.171-191.

FULTON, R. W.; BLOOD, K. S.; PANCIERA, R. J.; PAYTON, M. E.; RIDPATH, J. F.; CONFER, A. W.; SALIKI, J. T.; BURGE, L. T.; WELSH, R. D.; JOHNSON, B. J.; RECK, A. Lung pathology and infectious agents in fatal feedlot pneumonias and relationship with mortality, disease onset, and treatments. **Journal of Veterinary Diagnostic Investigation**, v. 21, n. 4, p. 464-477, 2009.

GAETA, N. C.; RIBEIRO, B. L. M.; ALEMÁN, M. A. R.; YOSHIHARA, E.; MARQUES, E. C.; HELLMEISTER, A. N.; PITUCO, E. M.; GREGORY, L. Serological investigation of antibodies against respiratory viruses in calves from Brazilian family farming and their relation to clinical signs of bovine respiratory disease. **Pesquisa Veterinária Brasileira**, v. 38, p. 642-648, 2018.

GAGEA, M. I.; BATEMAN, K. G.; SHANAHAN, R. A.; VAN DREUMEL, T.; MCEWEN, B. J.; CARMAN, S.; ARCHAMBAULT, M.; CASWELL, J. L. Naturally occurring *Mycoplasma bovis*-associated pneumonia and polyarthritis in feedlot beef calves. **Journal of Veterinary Diagnostic Investigation**, v. 18, n. 1, p. 29-40, 2006a.

GAGEA, M. I.; BATEMAN, K. G.; VAN DREUMEL, T.; MCEWEN, B. J.; CARMAN, S.; ARCHAMBAULT, M.; SHANAHAN, R. A.; CASWELL, J. L. Diseases and pathogens associated with mortality in Ontario beef feedlots. **Journal of Veterinary Diagnostic Investigation**, v. 18, n. 1, p. 18-28, 2006b.

GERSHWIN, L. J.; VAN EENENNAAM, A. L.; ANDERSON, M. L.; MCELIGOT, H. A.; SHAO, M. X.; TOAFF-ROSENSTEIN, R.; TAYLOR, J. F.; NEIBERGS, H. L.; WOMACK, J. Single pathogen challenge with agents of the bovine respiratory disease complex. **PLoS One**, v. 10, n. 11, p. e0142479, 2015.

GRIFFIN, D.; CHENGAPPA, M. M.; KUSZAK, J.; MCVEY, D. S. Bacterial pathogens of the bovine respiratory disease complex. **The Veterinary Clinics of North America Food Animal Practice**, v. 26, n. 2, p. 381-394, 2010.

GRISSETT, G. P.; WHITE, B. J.; LARSON, R. L. Structured literature review of responses of cattle to viral and bacterial pathogens causing bovine respiratory disease complex. **Journal of Veterinary Internal Medicine**, v. 29, n. 3, p. 770-780, 2015.

HAINES, D. M.; KENDALL, J. C.; REMENDA, B. W.; BREKER-KLASSEN, M. M.;

CLARK, E. G. Monoclonal and polyclonal antibodies for immunohistochemical detection of bovine parainfluenza type 3 virus in frozen and formalin-fixed paraffin-embedded tissues. **Journal of Veterinary Diagnostic Investigation**, v. 4, n. 4, p. 393-399, 1992.

HAY, K. E.; MORTON, J. M.; MAHONY, T. J.; CLEMENTS, A. C. A.; BARNES, T. S. Associations between animal characteristic and environmental risk factors and bovine respiratory disease in Australian feedlot cattle. **Preventive Veterinary Medicine**, v. 125, p. 66-74, 2016.

HEADLEY, S. A.; BALBO, L. C.; ALFIERI, A. F.; SAUT, J. P. E.; BAPTISTA, A. L.; ALFIERI, A. A. Bovine respiratory disease associated with *Histophilus somni* and bovine respiratory syncytial virus in a beef cattle feedlot from Southeastern Brazil. **Semina: Ciências Agrárias**, v. 38, p. 283-294, 2017.

HEADLEY, S. A.; MÜLLER, M. C.; OLIVEIRA, T. E. S.; DUARTE, C. A. B. G.; PEREIRA, P. F. V.; VIEIRA, M. V.; CUNHA, C. W.; FLORES, E. F.; LISBÔA, J. A. N.; PRETTO-GIORDANO, L. G. Diphtheric aspergillosis tracheitis with gastrointestinal dissemination secondary to viral infections in a dairy calf. **Microbial Pathogenesis**, v. 149, p. 104497, 2020a.

HEADLEY, S. A.; OKANO, W.; BALBO, L. C.; MARCASSO, R. A.; OLIVEIRA, T. E.; ALFIERI, A. F.; NEGRI FILHO, L. C.; MICHELAZZO, M. Z.; RODRIGUES, S. C.; BAPTISTA, A. L.; SAUT, J. P. E.; ALFIERI, A. A. Molecular survey of infectious agents associated with bovine respiratory disease in a beef cattle feedlot in southern Brazil. **Journal of Veterinary Diagnostic Investigation**, v. 30, n. 2, p. 249-251, 2018.

HEADLEY, S. A.; OLIVEIRA, T. E. S.; CUNHA, C. W. A review of the epidemiological, clinical, and pathological aspects of sheep-associated malignant catarrhal fever with emphasis on Brazil. **Brazilian Journal of Microbiology**, v. 51, n. 3, p. 1405-1432, 2020b.

HEADLEY, S. A.; OLIVEIRA, T. E. S.; LI, H.; LISBÔA, J. A. N.; QUEIROZ, G. R.; FRITZEN, J. T. T.; FLORES, E. F.; ALFIERI, A. A.; CUNHA, C. W. Immunohistochemical detection of intralesional antigens of Ovine Gamaherpesvirus-2 in cattle with Sheep-Associated Malignant Catarrhal Fever. **Journal of Comparative Pathology**, v. 174, p. 86-98, 2020c.

HERMEYER, K.; BUCHENAU, I.; THOMASMEYER, A.; BAUM, B.; SPERGSER, J.; ROSENGARTEN, R.; HEWICKER-TRAUTWEIN, M. Chronic pneumonia in calves after experimental infection with *Mycoplasma bovis* strain 1067: characterization of lung pathology, persistence of variable surface protein antigens and local immune response. **Acta Veterinaria Scandinavica**, v. 54, n. 1, p. 9, 2012.

KLIMA, C. L.; HOLMAN, D. B.; RALSTON, B. J.; STANFORD, K.; ZAHEER, R.; ALEXANDER, T. W.; MCALLISTER, T. A. Lower respiratory tract microbiome and resistome of bovine respiratory disease mortalities. **Microbial Ecology**, v. 78, n. 2, p. 446-456, 2019.

KLIMA, C. L.; ZAHEER, R.; COOK, S. R.; BOOKER, C. W.; HENDRICK, S.; ALEXANDER, T. W.; MCALLISTER, T. A. Pathogens of bovine respiratory disease in

North American feedlots conferring multidrug resistance via integrative conjugative elements. **Journal of Clinical Microbiology**, v. 52, n. 2, p. 438-448, 2014.

LI, H.; BROOKING, A.; CUNHA, C. W.; HIGHLAND, M. A.; O'TOOLE, D.; KNOWLES, D. P.; TAUS, N. S. Experimental induction of malignant catarrhal fever in pigs with ovine herpesvirus 2 by intranasal nebulization. **Veterinary Microbiology**, v. 159, n. 3, p. 485-489, 2012.

LI, H.; SHEN, D. T.; KNOWLES, D. P.; GORHAM, J. R.; CRAWFORD, T. B. Competitive inhibition enzyme-linked immunosorbent assay for antibody in sheep and other ruminants to a conserved epitope of malignant catarrhal fever virus. **Journal of Clinical Microbiology**, v. 32, n. 7, p. 1674-1679, 1994.

LIEBLER-TENORIO, E. M.; RIDPATH, J. F.; NEILL, J. D. Lesions and tissue distribution of viral antigen in severe acute versus subclinical acute infection with BVDV2. **Biologicals: Journal of the International Association of Biological Standardization**, v. 31, n. 2, p. 119-122, 2003.

LIGGITT, H. D.; DEMARTINI, J. C. The pathomorphology of malignant catarrhal fever :1. Multisystemic epithelial lesions. **Veterinary Pathology**, v. 17, n. 1, p. 58-72, 1980.

LÓPEZ, A.; MARTINSON, S. A. Respiratory system, mediastinum, and pleurae. In: ZACHARY, J. F. (Ed.). **Pathologic basis of veterinary disease**. 6th. St. Louis, Missouri: Elsevier, cap. 9, p.471-560.

LUNA, L. G. Manual of histologic staining methods of the Armed Forces Institute of Pathology. 3rd. New York: McGraw-Hill, 1968.

MACLACHLAN, N. J.; DUBOVI, E. J. Chapter 9 - Herpesvirales. In: MACLACHLAN, N. J. e DUBOVI, E. J. (Ed.). **Fenner's Veterinary Virology**. Boston: Academic Pressv. p.189-216.

MAES, R. K.; LANGOHR, I. M.; WISE, A. G.; SMEDLEY, R. C.; THAIWONG, T.; KIUPEL, M. Beyond H&E: integration of nucleic acid-based analyses into diagnostic pathology. **Veterinary Pathology**, v. 51, n. 1, p. 238-256, 2014.

MAGALHÃES, L. Q.; BAPTISTA, A. L.; FONSECA, P. D. A.; MENEZES, G. L.; NOGUEIRA, G. M.; HEADLEY, S. A.; FRITZEN, J. T. T.; ALFIERI, A. A.; SAUT, J. P. E. Use of metaphylactic protocols based on the risk to develop bovine respiratory diseases in feedlot cattle. **Ciência Rural**, v. 47, n. 8, p. e20161110, 2017.

MARGINEDA, C. A.; ZIELINSKI, G. O.; JURADO, S.; ALEJANDRA, F.; MOZGOVOJ, M.; ALCARAZ, A. C.; LÓPEZ, A. *Mycoplasma bovis* pneumonia in feedlot cattle and dairy calves in Argentina. **Brazilian Journal of Veterinary Pathology**, v. 10, n. 2, p. 79 – 86, 2017.

NICHOLAS, R. A. Bovine mycoplasmosis: silent and deadly. **The Veterinary Record**, v. 168, n. 17, p. 459-462, 2011.

NICHOLAS, R. A.; AYLING, R. D. *Mycoplasma bovis*: disease, diagnosis, and control. **Research in Veterinary Science**, v. 74, n. 2, p. 105-112, 2003.

2787 O'NEILL, R.; MOONEY, J.; CONNAGHAN, E.; FURPHY, C.; GRAHAM, D. A. Patterns
2788 of detection of respiratory viruses in nasal swabs from calves in Ireland: a retrospective
2789 study. **The Veterinary Record**, v. 175, n. 14, p. 351, 2014.

2790 O'TOOLE, D.; LI, H. The pathology of malignant catarrhal fever, with an emphasis on
2791 ovine herpesvirus 2. **Veterinary Pathology**, v. 51, n. 2, p. 437-452, 2014.

2792 O'TOOLE, D.; LI, H.; MILLER, D.; WILLIAMS, W. R.; CRAWFORD, T. B. Chronic and
2793 recovered cases of sheep-associated malignant catarrhal fever in cattle. **The**
2794 **Veterinary Record**, v. 140, n. 20, p. 519-524, 1997.

2795 O'TOOLE, D.; LI, H.; ROBERTS, S.; ROVNAK, J.; DEMARTINI, J.; CAVENDER, J.;
2796 WILLIAMS, B.; CRAWFORD, T. Chronic generalized obliterative arteriopathy in cattle:
2797 a sequel to sheep-associated malignant catarrhal fever. **Journal of Veterinary**
2798 **Diagnostic Investigation**, v. 7, n. 1, p. 108-121, 1995.

2799 O'TOOLE, D.; LI, H.; SOURK, C.; MONTGOMERY, D. L.; CRAWFORD, T. B.
2800 Malignant catarrhal fever in a bison (*Bison bison*) feedlot, 1993–2000. **Journal of**
2801 **Veterinary Diagnostic Investigation**, v. 14, n. 3, p. 183-193, 2002.

2802 OLCHOWY, T. W. J.; SLAUSON, D. O.; BOCHSLER, P. N. Induction of procoagulant
2803 activity in virus infected bovine alveolar macrophages and the effect of
2804 lipopolysaccharide. **Veterinary Immunology and Immunopathology**, v. 58, n. 1, p.
2805 27-37, 1997.

2806 OLIVEIRA, T. E. S.; PELAQUIM, I. F.; FLORES, E. F.; MASSI, R. P.; VALDIVIEZO,
2807 M. J. J.; PRETTO-GIORDANO, L. G.; ALFIERI, A. A.; SAUT, J. P. E.; HEADLEY, S.
2808 A. *Mycoplasma bovis* and viral agents associated with the development of bovine
2809 respiratory disease in adult dairy cows. **Transboundary and Emerging Diseases**, v.
2810 67, n. Suppl. 2, p. 82–93, 2020a.

2811 OLIVEIRA, V. H. S.; DALL AGNOL, A. M.; FRITZEN, J. T. T.; LORENZETTI, E.;
2812 ALFIERI, A. A.; ALFIERI, A. F. Microbial diversity involved in the etiology of a bovine
2813 respiratory disease outbreak in a dairy calf rearing unit. **Comparative Immunology,**
2814 **Microbiology and Infectious Diseases**, v. 71, p. 101494, 2020b.

2815 PANCIERA, R. J.; CONFER, A. W. Pathogenesis and pathology of bovine pneumonia.
2816 **The Veterinary Clinics of North America Food Animal Practice**, v. 26, n. 2, p. 191-
2817 214, 2010.

2818 PEIXOTO, P. V.; MOTA, R. A.; BRITO, M. F.; CORBELLINI, L. G.; DRIEMEIER, D.;
2819 SOUZA, M. I. Infecção natural pelo vírus sincicial respiratório bovino (BRSV) no estado
2820 de Alagoas. **Pesquisa Veterinária Brasileira**, v. 20, n. 4, p. 171-175, 2000.

2821 POTGIETER, L. N. Immunology of bovine viral diarrhea virus. **The Veterinary Clinics**
2822 **of North America Food Animal Practice**, v. 11, n. 3, p. 501-520, 1995.

2823 PRETTO, L. G.; MÜLLER, E. E.; FREITAS, J. C.; METTIFOGA, E.; BUZINHANI, M.;
2824 YAMAGUTI, M. Isolamento de *Mycoplasma bovis* em bezerros com pneumonia.
2825 **Veterinária Notícias**. 7**:** 69-73 p. 2001.

2826 QUEIROZ, G. R.; OLIVEIRA, R. A. M. D.; FLAIBAN, K. K. M. C.; SANTIS, G. W. D.;

BRACARENSE, A. P. F. R. L.; HEADLEY, S. A.; ALFIERI, A. A.; LISBÔA, J. A. N. Diagnóstico diferencial das doenças neurológicas dos bovinos no estado do Paraná. **Pesquisa Veterinária Brasileira**, v. 38, p. 1264-1277, 2018.

REID, E.; JULEFF, N.; WINDSOR, M.; GUBBINS, S.; ROBERTS, L.; MORGAN, S.; MEYERS, G.; PEREZ-MARTIN, E.; TCHILIAN, E.; CHARLESTON, B.; SEAGO, J. Type I and III IFNs produced by plasmacytoid dendritic cells in response to a member of the *Flaviviridae* suppress cellular immune responses. **Journal of Immunology**, v. 196, n. 10, p. 4214-4226, 2016.

RISALDE, M. A.; MOLINA, V.; SANCHEZ-CORDON, P. J.; PEDRERA, M.; PANADERO, R.; ROMERO-PALOMO, F.; GOMEZ-VILLAMANDOS, J. C. Response of proinflammatory and anti-inflammatory cytokines in calves with subclinical bovine viral diarrhea challenged with bovine herpesvirus-1. **Veterinary Immunology and Immunopathology**, v. 144, n. 1-2, p. 135-143, 2011.

RISALDE, M. A.; MOLINA, V.; SONCHEZ-CORDON, P. J.; PEDRERA, M.; ROMERO-PALOMO, F.; BAUTISTA, M. J.; MORENO, A.; GOMEZ-VILLAMANDOS, J. C. Comparison of pathological changes and viral antigen distribution in tissues of calves with and without preexisting bovine viral diarrhea virus infection following challenge with bovine herpesvirus-1. **American Journal of Veterinary Research**, v. 74, n. 4, p. 598-610, 2013.

RODRIGUEZ, F.; BRYSON, D. G.; BALL, H. J.; FORSTER, F. Pathological and immunohistochemical studies of natural and experimental *Mycoplasma bovis* pneumonia in calves. **Journal of Comparative Pathology**, v. 115, n. 2, p. 151-162, 1996.

SILVA, M. S.; BRUM, M. C. S.; WEIBLEN, R.; FLORES, E. F. Identificação e diferenciação de herpesvírus bovino tipos 1 e 5 isolados de amostras clínicas no Centro-Sul do Brasil, Argentina e Uruguai (1987-2006). **Pesquisa Veterinária Brasileira**, v. 27, n. 10, p. 403-408, 2007.

STRONG, R.; LA ROCCA, S. A.; PATON, D.; BENSAUDE, E.; SANDVIK, T.; DAVIS, L.; TURNER, J.; DREW, T.; RAUE, R.; VANGEEL, I.; STEINBACH, F. Viral dose and immunosuppression modulate the progression of acute BVDV-1 infection in calves: Evidence of long term persistence after intra-nasal infection. **PLoS One**, v. 10, n. 5, p. e0124689-e0124689, 2015.

SULYOK, K. M.; KREIZINGER, Z.; WEHMANN, E.; LYSNYANSKY, I.; BÁNYAI, K.; MARTON, S.; JERZSELE, Á.; RÓNAI, Z.; TURCSÁNYI, I.; MAKRAI, L.; JÁNOSI, S.; NAGY, S. Á.; GYURANECZ, M. Mutations associated with decreased susceptibility to seven antimicrobial families in field and laboratory-derived *Mycoplasma bovis* strains. **Antimicrobial Agents and Chemotherapy**, v. 61, n. 2, p. e01983-01916, 2017.

SZEREDI, L.; JANOSI, S.; PALFI, V. Microbiological and pathological examination of fatal calf pneumonia cases induced by bacterial and viral respiratory pathogens. **Acta Veterinaria Hungarica**, v. 58, n. 3, p. 341-356, 2010.

TAUS, N. S.; SCHNEIDER, D. A.; OAKS, J. L.; YAN, H.; GAILBREATH, K. L.; KNOWLES, D. P.; LI, H. Sheep (*Ovis aries*) airway epithelial cells support ovine

herpesvirus 2 lytic replication in vivo. **Veterinary Microbiology**, v. 145, n. 1-2, p. 47-53, 2010.

TAUS, N. S.; TRAUL, D. L.; OAKS, J. L.; CRAWFORD, T. B.; LEWIS, G. S.; LI, H. Experimental infection of sheep with ovine herpesvirus 2 via aerosolization of nasal secretions. **The Journal of General Virology**, v. 86, n. Pt 3, p. 575-579, 2005.

TAYLOR, J. D.; FULTON, R. W.; LEHENBAUER, T. W.; STEP, D. L.; CONFER, A. W. **The epidemiology of bovine respiratory disease: What is the evidence for predisposing factors?** The Canadian Veterinary Journal. 51**:** 1095-1102 p. 2010.

TORTORELLI, G.; CARRILLO GAETA, N.; MENDONÇA RIBEIRO, B. L.; MIRANDA MARQUES, L.; TIMENETSKY, J.; GREGORY, L. Evaluation of *Mollicutes* microorganisms in respiratory disease of cattle and their relationship to clinical signs. **Journal of Veterinary Internal Medicine**, v. 31, n. 4, p. 1215-1220, 2017.

VAZ, R. Z.; LOBATO, J. F. P.; PASCOAL, L. L. Desenvolvimento de bezerros de corte desmamados aos 80 ou 152 dias até os 15-16 meses de idade. **Revista Brasileira de Zootecnia**, v. 40, n. 1, p. 221-229, 2011.

WELSH, M. D.; ADAIR, B. M.; FOSTER, J. C. Effect of BVD virus infection on alveolar macrophage functions. **Veterinary Immunology and Immunopathology**, v. 46, n. 3, p. 195-210, 1995.

XAVIER, A. A. C. Ocorrência do vírus da febre catarral maligna em bovinos do estado do Paraná, Sul do Brasil: um estudo imuno-histoquímico retrospectivo. 2021. master's thesis Ciência Animal, Universidade Estadual de Londrina, Londrina.

ZHANG, M.; HILL, J. E.; GODSON, D. L.; NGELEKA, M.; FERNANDO, C.; HUANG, Y. The pulmonary virome, bacteriological and histopathological findings in bovine respiratory disease from western Canada. **Transboundary and Emerging Diseases**, v. 67, n. 2, p. 924-934, 2020.

8 ARTIGO 4 - INFECTIOUS DISEASE AGENTS ASSOCIATED WITH PULMONARY ALTERATIONS IN ABORTED BOVINE FETUSES

Thalita Evani Silva de Oliveira, Gabriela Sanches Scuisato, Juliana Torres Tomazi Fritzen, Denise Correia Silva, Rodrigo Pelisson Massi, Isadora Fernanda Pelaquim, Luara Evangelista Silva, Eduardo Furtado Flores, Renato de Lima Santos, Lucienne Garcia Pretto-Giordano, Júlio Augusto Naylor Lisbôa, Amauri Alcindo Alfieri, Selwyn Arlington Headley. **Animals**. 2022, 12(13), 1596. https://doi.org/10.3390/ani12131596

8.1 ABSTRACT

This study investigated the occurrence of selected pathogens of bovine respiratory disease in fetal pulmonary tissue of cattle and associated these with patterns of disease. Fetal pulmonary (*n*=37) tissues were evaluated by histopathology; immunohistochemical assays identified intralesional antigens of bovine alphaherpesvirus 1 (BoAHV1), bovine viral diarrhea virus (BVDV), bovine parainfluenza virus 3 (BPIV-3), bovine respiratory syncytial virus (BRSV), and *Mycoplasma bovis*. Molecular assays were performed to amplify reproductive disease pathogens and bovine gamma- herpesvirus 6 (BoGHV6) from 12 lungs. The 2 patterns of pulmonary diseases were interstitial pneumonia (12/37) and suppurative bronchopneumonia (1/37). The frequency of the intralesional antigens identified was BRSV (16.2%; 6/37), BVDV (13.5%; 5/37), BoAHV1 (8.1%; 3/37), *M. bovis* (5.4%; 2/37), and BPIV-3 (2.7%; 1/37). Interstitial pneumonia was associated with BRSV (*n*=3), BoAHV1 (*n*=3), and BVDV (*n*=2); suppurative bronchopneumonia contained a Gram-positive bacterium and BVDV and BRSV. Reproductive pathogens detected included *Leptospira* spp., (*n*=3), BVDV, *Neospora caninum*, and *Brucella abortus* (*n*=2). BoGHV6 DNA was identified in the lungs of two fetuses with interstitial pneumonia. These findings suggest that these fetuses were infected transplacentally by several pathogens. The role of some of these pathogens herein identified must be further elucidated in the possible participation of fetal disease.

Keywords: abortion; BoGHV6; BVDV; BRSV; fetopathy; interstitial pneumonia; transplacental infection.

8.2 Introduction

The bovine respiratory disease (BRD) complex is a multifactorial and multi-etiological disease associated with several bacterial and viral agents, together with risk factors or stressors, that favor the development of pneumonic conditions, resulting in varying rates of morbidity and mortality in cattle of all age groups [1–3]. Frequent stressors of BRD include weaning, comingling, transportation, abrupt dietary alterations [1,3], and several management factors at feedlots [1]. In Brazil, information relative to the occurrence of infectious agents associated with BRD is scarce and insipient [4] when compared with the data existing in North America [1,2,5,6] and Australia [5]. Consequently, it is difficult to correlate productive losses due to the BRD in feedlot cattle since the available data may not reflect the real situation of cattle health, as well as morbidity and mortality indices in Brazil [6].

The viral agents frequently associated with BRD include bovine alphaherpesvirus 1 (BoAHV1), bovine viral diarrhea virus (BVDV), bovine parainfluenza virus 3 (BPIV-3), bovine respiratory syncytial virus (BRSV), and bovine coronavirus (BCoV) [7–9]. Bacterial agents associated with BRD include *Histophilus somni, Mannheimia haemolytica, Mycoplasma bovis*, and *Pasteurella multocida* [4,8,10,11]. Our group has identified all these agents in feedlot and dairy cattle with BRD from several geographical regions of Brazil [11,13–16] and has contributed to the understanding of disease patterns associated with the development of BRD [10,15].

Although numerous reports have investigated the infectious agents associated with BRD in feedlot cattle worldwide [4,7,8,15], there are comparatively fewer studies with histologic details involving fetal lungs of cattle [16–18] as compared with the innumerous studies describing the lesions observed in several fetal organs. Infectious agents previously associated with fetal lungs and/or pneumonia in cattle include *Brucella abortus* [19,20],

M. bovis [17], BPIV-3 [18], BoAHV1 [16,21], and BVDV [10]. Most of these studies have identified the associated agents by in situ diagnostic methods, such as immunohistochemistry (IHC) [10,16,17], in situ hybridization (ISH) [17], as well as molecular identification [16,18] and culture and isolation [19,21] in conjunction with histopathologic evidence of pulmonary disease. The IHC and ISH diagnostic strategies demonstrate the intralesional presence of agent-specific antigens associated with histopathological evidence of lesions [22,23], with the obtained results being a strong

indication of an associated disease process within the affected tissues [23], thereby providing evidence of the related disease agent with the pat- tern of pulmonary disease [15]. Furthermore, diagnostic IHC is recommended to identify a wide range of infectious reproductive agents in cattle [24].

This study investigated the presence of selected infectious agents of BRD in aborted bovine fetal lungs to determine whether these pathogens were associated with pneumonia and/or other pulmonary alterations.

8.3 MATERIALS AND METHODS

8.3.1 Sample Collection, Study Location, and Inclusion Criteria

Retrospective studies were performed on aborted bovine fetuses submitted for histopathologic diagnosis at the Laboratory of Animal of Pathology, Universidade Estadual de Londrina (UEL), Paraná, southern Brazil, and at the Veterinary Diagnostic Laboratory, Universidade Federal de Minas Gerais (UFMG), midwestern Brazil, from 2009 to 2019.

All files within the registry were reviewed to identify fetal bovine tissues submitted for diagnosis. Subsequently, only cases that contained the pathologic data and the correlated paraffin blocks and/or glass slides of fetuses with pulmonary tissue were included in this study. Additionally, when necessary, histological slides were redone by using the Hematoxylin and eosin staining technique. Furthermore, the Giemsa and Gram Brown–Brenn histochemical stains were performed on selected pulmonary tissues to identify intralesional organisms.

8.3.2 Histopathology and immunohistochemistry

All pulmonary tissues were initially screened to identify the typical histopathological patterns of interstitial pneumonia, bronchopneumonia, granulomatous pneumonia, and embolic pneumonia [25]. Thereafter, the selected pulmonary tissues were reviewed for predetermined histopathologic patterns of pulmonary disease or histological alterations and categorized as: 0, normal lung; 1, circulatory, reversible, and irreversible cellular alterations; 2, interstitial pneumonia; and 3, suppurative bronchopneumonia, as previously described [15]. Additionally, the

histological elements identified in each pulmonary alteration were observed and tabulated.

Subsequently, pulmonary tissues were submitted to IHC assays designed to identify specific agents known to be associated with BRD: BVDV, BoAHV1, BRSV, BPIV-3, and *M. bovis* [4,10], as previously described [10]. These agents were selected due to the avail- ability of monoclonal and/or polyclonal antibodies; a list of the antibodies used in this study with the respective dilutions and methods of antigen retrieval is provided (Table 1)

Table 1 – Antibodies, dilutions and antigen retrieval methods used in immunohistochemical assays

Antibody (clone)	Antigen retrieval	Dilution	Distilled water (mL)	Hydrogen peroxide (6%; mL)	Source
BoAHV1 (Mab 9E7)	Citrate buffer (pH 6,0)	1:700	50	100	VMRD, Pullman, WA, USA
BPIV-3	TRIS+EDTA buffer (pH 9)	1:30	110	40	Dr. Eduardo F. Flores
BRSV (15c7)	Citrate buffer (pH 6)	1:300	50	100	Dr. Eduardo F. Flores
BVDV (15c5)	Citrate buffer (pH 6)	1:1500	50	100	Dr. Eduardo F. Flores
Mycoplasma bovis	Citrate buffer (pH 6)	1:10	110	40	Dr. Lucienne G. Pretto-Giordano

BoAHV1: bovine alphaherpesvirus 1; BPIV-3: bovine parainfluenza virus 3; BRSV: bovine respiratory syncytial virus; BVDV: bovine viral diarrhea virus; MAb: monoclonal antibody; TRIS: tris (hydroxymethyl) aminomethane; EDTA: ethylenediaminetetraacetic acid.

Positive controls consisted of pulmonary fragments known to contain antigens of BVDV, BoAHV1, BRSV, BPIV-3, and *M. bovis* [10]. Negative control was performed by replacing the primary antibody with diluent; positive and negative controls were included in each IHC assay. The data obtained were tabulated and analyzed.

8.3.3 Molecular detection of agents associated with reproductive diseases in cattle

The extracted nucleic acids from the lungs of fetuses' number 9, 12, 17, 19, 23, 24, and 27–32 were used in molecular assays designed to identify a panel

of infectious agents associated with reproductive disease in cattle, using PCR and/or RT-PCR assays as previously described [27] in a thermocycler (Proflex PCR System, Applied Biosystems; Marsiling Ind Estate Road 3, Singapore). These included PCR/RT-PCR assays to detect BoAHV1 [27], BVDV [28], *Listeria monocytogenes* [29], *Histophilus somni* [30], *Neospora caninum* [31], *Leptospira* spp. [32], and *Brucella abortus* [33]. Additionally, the extracted nucleic acids of these fetuses maintained at −80 ◦C (except numbers 9 and 12) were used in nested-PCR (nPCR) assays designed to amplify the bovine gammaherpesvirus 6 (BoGHV6) polymerase gene [34], since there is emerging evidence that BoGHV6 may be a potential pathogen of bovine fetuses [35]. Only fetuses submitted frozen and/or refrigerated were used for molecular detection; all other fetuses were submitted in formalin solution for histopathologic evaluation and, thus, were not used for molecular identification.

Positive controls consisted of nucleic acids extracted from Madin–Darby bovine kidney cell culture inoculated with BVDV (NADL strain) and BoAHV1 (Los Angeles strain) and field strains of previous cases of OvGHV2 [36], *L. monocytogenes* [37], *H. somni* [38], *N. caninum* [31], *Leptospira* spp., *B. abortus* [39], and BoGHV6 [35]. Nuclease-free water (Invitrogen Corp., Carlsbad, CA, USA) was used as negative control in all PCR and RT-PCR assays; positive and negative controls were included in all molecular assays. PCR/RT- PCR products were resolved by electrophoresis in 2% agarose gels, stained with ethidium bromide, and examined under ultraviolet light.

8.3.4 Sequencing and phylogenetic analysis of BoHV-6 polymerase gene

The nPCR products of BoGHV6 were 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 software (http://asparagin.cenargen.embrapa.br/phph/, accessed on 21 April 2022), respectively. Similarity searches of the BoGHV6

polymerase gene were performed with nucleotide (nt) sequences deposited in GenBank using the Basic Local Alignment Search Tool software (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 21 April 2022). Sequencing was done only for BoGHV6 since there is still controversy as to the role of this pathogen in infections [35].

8.3.5 Animal ethics

This study followed the animal use rules of the National Council for Animal Control in Experiments and was approved by the Ethics Committee on Animal Usage, Universidade Estadual de Londrina (CEUA/UEL; protocol, 835.2019.45).

8.4 RESULTS

8.4.1 Histopathological findings and pulmonary patterns

During the period (2009–2019), 45 fetuses were submitted for routine post-mortem evaluations at UEL, southern Brazil, and three at UFMG, midwestern Brazil. However, only 34 fetuses from UEL, Paraná, and those from UFMG (*n*=3), fulfilled the selection criteria and were included since they contained paraffin blocks, pulmonary tissues, and the biological data of the submitted animal.

Normal pulmonary tissue was observed in 48.8% (18/37) of the cases, circulatory and/or cellular alterations were diagnosed in 18.9% (7/37), 29.7% (11/37) had interstitial (Figure 1A), with one case (1/37; 2.7%) of fetal suppurative bronchopneumonia being identified (Table 2). Accordingly, pneumonia was identified in 35.1% (12/37) of the fetal tissues, while 64.9% (25/37) of the fetal lungs did not show histologic evidence of pneumonia.

Figure 1 – Principal histopathologic and immunohistochemical findings observed in fetal lungs of cattle. There is interstitial pneumonia (A) and degeneration (arrows) of bronchial epithelium (B). Observe positive intracytoplasmic immunoreactivity to antigens of BRSV (C), BVDV within alveolar epithelium (D), and within a region of suppurative bronchopneumonia (E); BVDV immunoreactivity is highlighted at the insert. There is positive intracytoplasmic immunoreactivity to BoAHV1 within degenerated bronchial epithelium (F).

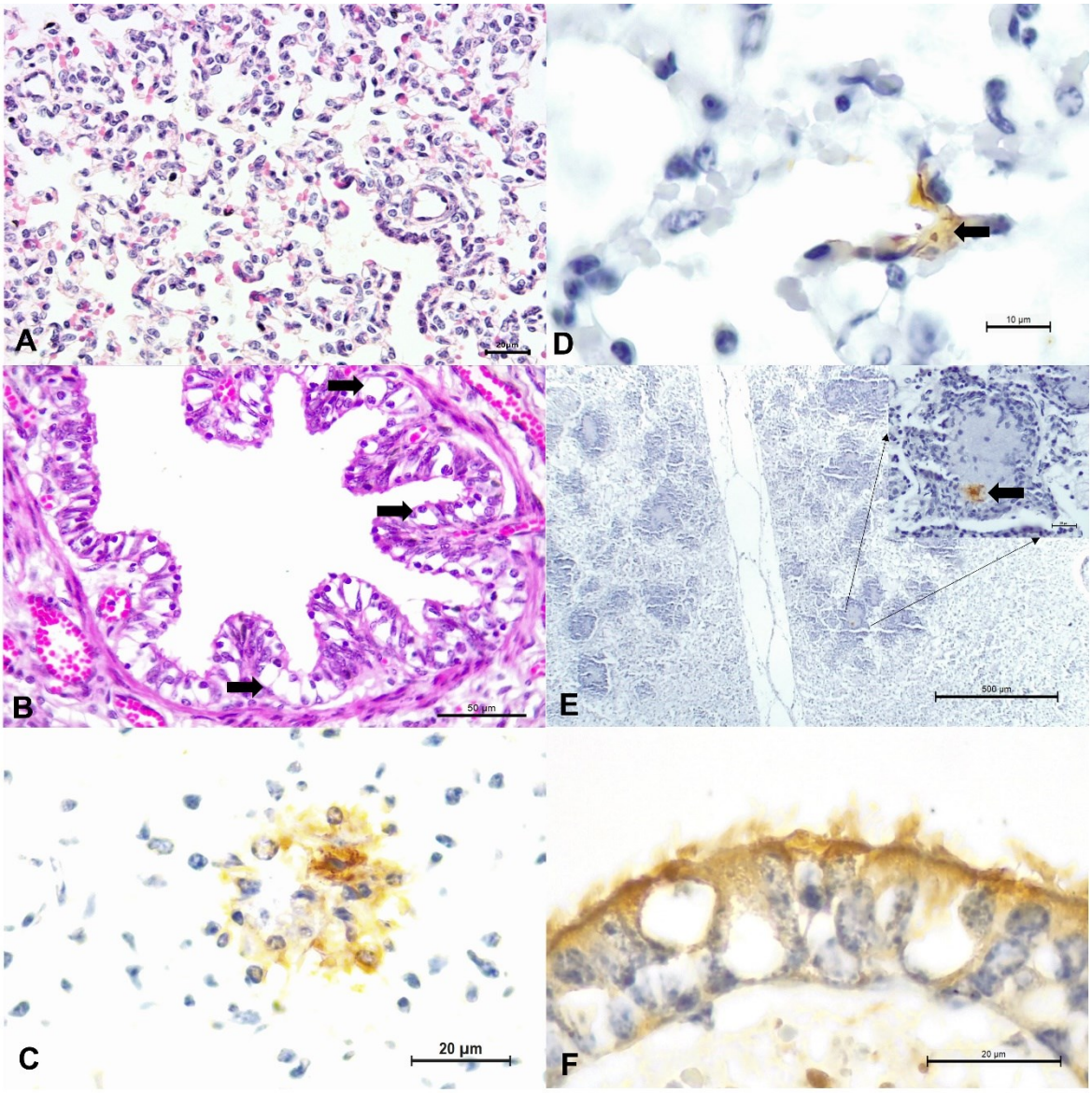


Table 2 – Patterns of pulmonary disease observed in fetuses from southern and midwestern Brazil

Histological pattern of pneumonia	Number of fetuses	Frequency (%)
Interstitial pneumonia	11	29.7
Suppurative bronchopneumonia	1	2.7
Without pneumonia	25	67.6
Total	37	100

The histological changes (*n*=52) observed in the lungs evaluated with and without pneumonia are presented in Table 3; since some of these alterations occurred simultaneously in the same fetal lung. The most frequent histological change was pulmonary congestion (27%; 14/52), followed by ballooning degeneration (Figure

1B) of the bronchial epithelium (19.2%; 10/52) and bronchiolar (13.5%; 7/52). Additionally, the fetus with suppurative bronchopneumonia contained accumulations of an intralesional Gram-positive coccoid bacteria.

Table 3 – Histological findings observed within the patterns of pulmonary lesions observed in 37 fetuses

Histological findings	Number of Fetuses	Frequency (%)
Pulmonary congestion	14	27
Normal lung	14	27
Ballooning degeneration of the bronchial epithelium	10	19.2
Ballooning degeneration of the bronchiolar epithelium	7	13.5
Alveolar edema	3	5.8
Bronchial epithelial necrosis	2	3.8
Bronchiolar epithelial necrosis	1	1.9
Fibrinoid alteration	1	1.9
Total	52	100

8.4.2 Relationship between immunohistochemical identification of infectious agents and pulmonary alterations

Table 4 demonstrates the relationship between pulmonary changes and IHC detection of intralesional agents. Positive immunoreactivity to antigens associated with BRD were observed in 29.7% (11/37) of the fetal lungs in the above-mentioned categories: 0, normal lung (*n*=0); 1, pulmonary tissue with circulatory alterations (*n*=5); 2, interstitial pneumonia (*n*=6); and 3, suppurative bronchopneumonia (*n*=1); 70.3% (26/37) of the fetal pulmonary fragments did not contain any of the analyzed agents. The most frequent intralesional agents identified was BRSV (16.2%; 6/37), followed by BVDV (13.5%; 5/37), BoAHV1 (8.1%; 3/37), *M. bovis* (5.4%; 2/37), and BPIV-3 (2.7%; 1/37).

3117 **Table 4** – Principal histopathological findings, patterns of pneumonia, and infectious agents observed in the lungs of bovine fetuses
3118 by immunohistochemistry and molecular detection

Fetuses	Principal histopathologic alteration / Pattern of pulmonary disease	Immunohistochemistry	Molecular detection		Type of infection
			Reproductive panel	BoGHV6	
Lungs without any infectious disease agent by IHC					
#1	Normal	-ve	ND	ND	None
#2	Normal	-ve	ND	ND	None
#3	Normal	-ve	ND	ND	None
#4	Normal	-ve	ND	ND	None
#5	Normal	-ve	ND	ND	None
#6	Normal	-ve	ND	ND	None
#7	Normal	-ve	ND	ND	None
#8	Normal	-ve	ND	ND	None
#9*	Normal	-ve	*Leptospira* spp.	-ve	Single
#10	Normal	-ve	ND	ND	None
#11	Normal	-ve	ND	ND	None
#12*	Normal	-ve	-ve	-ve	None
#13	Normal	-ve	ND	ND	None
#14	Normal	-ve	ND	ND	None
#15	Congestion	-ve	ND	ND	None
#16	Congestion	-ve	ND	ND	None
#17*	Congestion	-ve	BVDV	-ve	Single
#18	Congestion	-ve	ND	ND	None
#19*	Congestion Ballooning degeneration (bronchial epithelium)	-ve	-ve	-ve	None
#20	Congestion Edema Ballooning degeneration (bronchial and bronchiolar epithelium)	-ve	ND	ND	None
#21	Congestion Interstitial pneumonia	-ve	ND	ND	None
#22	Congestion Edema Interstitial pneumonia	-ve	ND	ND	None
#23*	Interstitial pneumonia	-ve	*Leptospira* spp. *B. abortus*	-ve	Dual
#24*	Interstitial pneumonia	-ve	-ve	+ve	Single
#25	Interstitial pneumonia	-ve	ND	ND	None

continue…

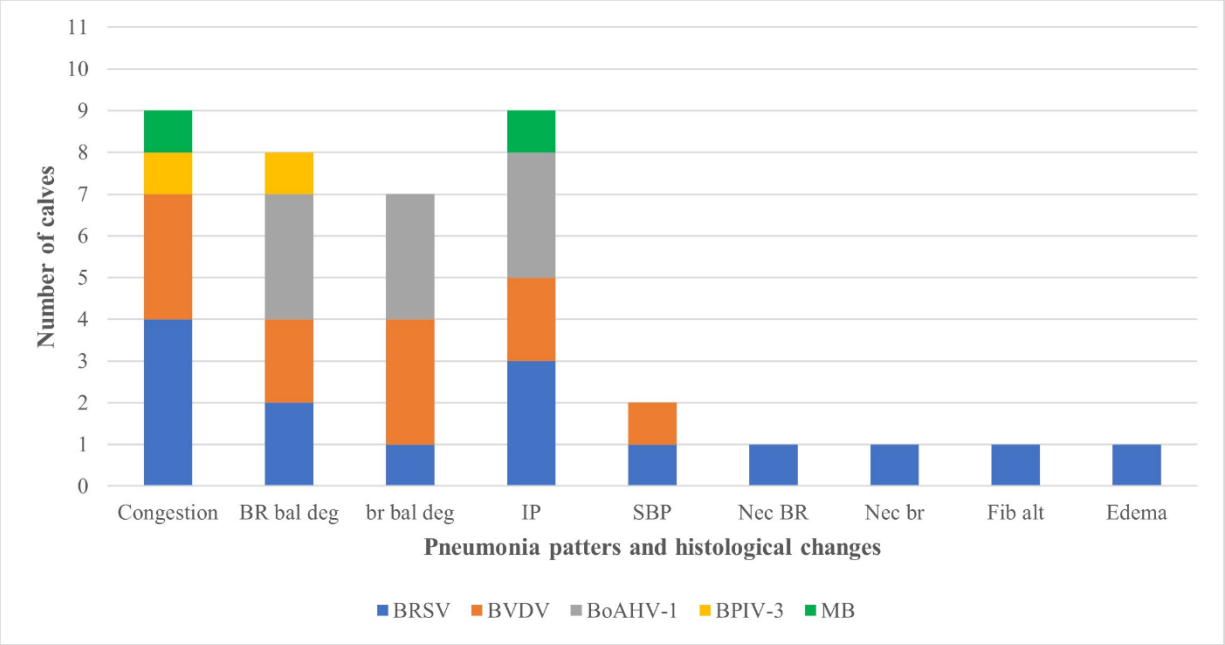
Continue…

Fetuses	Principal histopathologic alteration / Pattern of pulmonary disease	Immunohistochemistry	Molecular detection		Type of Infection
			Reproductive Panel BoGHV6	Reproductive Panel BoGHV6	
Lungs without any infectious disease agent by IHC					
#26	Interstitial pneumonia Ballooning degeneration (bronchial epithelium) Necrosis of bronchial epithelium	-ve	ND	ND	None
Lungs with infectious agent(s) identified by IHC					
#27*	Congestion	BRSV, *M. bovis*	*N. caninum*	+ve	Quadruple
#28*	Congestion Ballooning degeneration (bronchial epithelium)	BRSV, BPIV-3	*N. caninum*	-ve	Triple
#29*	Congestion Ballooning degeneration (bronchial and bronchiolar epithelium)	BVDV	*B. abortus*, BVDV	+ve	Triple
#30*	Congestion Ballooning degeneration (bronchial and bronchiolar epithelium)	BVDV	-ve	-ve	Single
#31*	Interstitial pneumonia Ballooning degeneration (bronchial and bronchiolar epithelium)	BVDV	*Leptospira* spp.	-ve	Dual
#32*	Interstitial pneumonia Ballooning degeneration (bronchial and bronchiolar epithelium)	BRSV, BoAHV1	-ve	+ve	Triple
#33	Interstitial pneumonia Ballooning degeneration (bronchial and bronchiolar epithelium)	BoAHV1	ND	ND	Single
#34	Interstitial pneumonia Ballooning degeneration (bronchial and bronchiolar epithelium)	BVDV, BoAHV1, *M. bovis*	ND	ND	Triple
#35	Interstitial pneumonia Fibrinoid alteration Necrosis of bronchial and bronchiolar epithelium	BRSV	ND	ND	Single
#36	Interstitial pneumonia Edema Congestion	BRSV	ND	ND	Single
#37	Congestion Suppurative bronchopneumonia Accumulation of bacteria	BVDV, BRSV	ND	ND	Dual

Footnote: -ve, negative; +ve, positive; ND, not done. *, fetuses evaluated for the molecular identification of a panel of reproductive disease agents (BoAHV1, BVDV, *Leptospira* spp., *Histophilus somni*, *Brucella abortus*, *Neospora caninum*, and *M. bovis*). BoAHV1: bovine alphaherpesvirus 1; BPIV-3: bovine parainfluenza virus 3; BRSV: bovine respiratory syncytial virus; BVDV: bovine viral diarrhea virus; *M. bovis*: *Mycoplasma bovis*.

The association between intralesional accumulation of tissue antigens and the categories of pulmonary alterations identified in the fetal lungs by IHC is graphically presented (Figure 2). All antibodies used showed patchy, intracytoplasmic immunoreactivity within several histologic pulmonary elements. Interstitial pneumonia was associated with intralesional antigens of BRSV (*n*=3), BoAHV1 (*n*=3), BVDV (*n*=2), and *M. bovis* (*n*=1). However, in 54.5% (6/11) of lungs with interstitial pneumonia, none of the agents investigated was identified. The suppurative bronchopneumonia observed in fetus #37 contained intralesional antigens of BVDV and BRSV, with accumulations of Gram-positive bacteria. Circulatory (category 1) alterations were associated with the intralesional accumulations of antigens of BRSV (*n*=3), BVDV (*n*=2), BPIV-3 (*n*=1), and *M. bovis* (*n*=1).

Figure 2 – Relationship between pneumonic patterns and histological changes with the associated infected agent identified by immunohistochemistry in the lungs of aborted bovine fetuses.



BoAHV1: bovine alphaherpesvirus 1; BPIV-3: bovine parainfluenza virus 3; BRSV: bovine respiratory syncytial virus; BVDV: bovine viral diarrhea virus; *M. bovis: Mycoplasma bovis*. IP: interstitial pneumonia; BR bal deg, bronchial epithelial ballooning degeneration; SBP: suppurative bronchopneumonia; br bal deg: bronchiolar epithelial ballooning degeneration; Nec BR: bronchial epithelial necrosis; Nec br: bronchiolar epithelial necrosis; Fib Alt: fibrinoid alteration.

Positive immunoreactivity to BRSV antigens was observed within degenerated and normal bronchial and bronchiolar epithelial cells, alveolar epithelium, and epithelial cells of the mixed bronchial gland (Figure 1C). Positive immunoreactivity to BVDV was observed within normal and degenerated bronchial epithelial cells and

also within the suppurative exudate of a fetus with bronchopneumonia (Figure 1D,E). Intralesional BoAHV1 antigens were detected within normal bronchial and bronchiolar epithelial cells, chondrocytes of hyaline cartilage, and capillary endothelium (Figure 1F). Infections due to *M. bovis* resulted in positive immunolabelling within normal, degenerated, and necrotic bronchial and bronchiolar epithelial cells, necrotic epithelial cells of the mixed bronchial glands, endothelial cells, and alveolar macrophages. BPIV-3 antigens revealed positive intracytoplasmic immunoreactivity within normal bronchiolar epithelial cells and alveolar macrophages.

8.4.3 Molecular identification of pathogens and relationship with histologic pulmonary alterations

The results of the molecular detection of a panel of reproductive disease agents and BoGHV6 from the lungs of the fetuses are presented in Table 4, with at least one infectious agent being amplified from most (75%; 9/12) of the fetuses evaluated. The agents detected were Leptospira spp., (*n*=3), BVDV (*n*=2), *N. caninum* (*n*=2), and B. abortus (*n*=2), while BoGHV6 was identified in the lungs of 4 fetuses. Sequence analysis of the amplicons confirmed the identity of the products obtained from the BoGHV6 nPCR assay.

Furthermore, Leptospira spp. was the only agent identified in a lung (#9) without pneumonia and was also observed in a fetal lung with interstitial pneumonia that was concomitantly infected by *B. abortus*. Additionally, BVDV, *N. caninum*, and B. abortus were associated with pulmonary congestion (Table 4). Additionally, 2 (#24 and 32) of the 4 fetal lungs infected by BoGHV6 had a histologic diagnosis of interstitial pneumonia, with 1 lung (#32) being simultaneously infected by BRSV and BoAHV1 as demonstrated by IHC, while the other fetal lung (#24) was only infected by BoGHV6, suggesting a possible association between interstitial fetal pneumonia and BoGHV6.

Moreover, when all IHC and molecular analyses were considered, single (*n*=7), dual (*n*=3), triple (*n*=4), and quadruple (*n*=1) infections were identified. Alternatively, tissue antigens and/or nucleic acids were not identified in 59.5% (22/37) of the fetal lungs. Single fetal infections were associated with BRSV, BVDV (*n*=2), Leptospira spp., BoAHV1, and BoGHV6 (*n*=1). The only quadruple infection identified was associated with the simultaneous identification of antigens of BRSV and *M. bovis*

by IHC and the nucleic acids of *N. caninum* and BoGHV6 via PCR in a congested fetal lung (#27).

8.5 DISCUSSION

This study demonstrates the presence of pathogens known to produce respiratory diseases in cattle within the lungs of aborted bovine fetuses, amplified BoGHV6 DNA from some of these, and associates these pathogens with or without pulmonary alterations in bovine fetuses, demonstrating clear evidence that these fetuses were infected [24]. Furthermore, some well-known pathogens of reproductive disease in cattle, such as BVDV, BoAHV1, Leptospira spp., B. abortus, and *M. bovis*, were also identified and associated with pulmonary alteration(s), collaborating with the results of previous studies that have demonstrated B. abortus [19,20], *M. bovis* [17], BoAHV1 [16,21], BVDV [10] in the fetal lungs of cattle. Collectively, fetal infection by these pathogens was probably due to intrauterine infection resulting from transplacental transmission [40,41]. Consequently, most of the disease agents herein identified may be classified either as primary or secondary fetopathic agents of cattle [24,40] and could have produced direct or indirect effects on fetal survival [41]. The primary fetopathy agents herein identified (BVDV, BoAHV1, *N. caninum*, B. abortus, and Leptospira spp.) can cross the placental barrier, producing fetal disease in healthy cows, whereas the secondary or opportunistic agent (H. somni) crosses the placental barrier when there is placental damage, alteration to the microflora of the reproductive tract, or in immunocompromised cows [40].

The novelty of this study is the identification of antigens of two classical respiratory pathogens (BRSV and BPIV-3) within fetal lungs, the association of BRSV with interstitial pneumonia in two fetuses, while there was double infection by these viruses in one congested fetal lung. As far as the authors are aware, this is a novel description of BRSV associated lesions in bovine fetuses, while there is a previous report of the participation of BPIV-3 in the development of fetal interstitial pneumonia in cattle [18].

Furthermore, the amplification of BoGHV6 DNA from multiple fetuses with interstitial pneumonia and other pulmonary alterations supports the theory that this pathogen should be considered an agent of fetal disease in cattle [35]. This is supported by previous studies which have identified this virus in an aborted fetus from Canada

[42], within uterine secretions of a cow from Belgium [43], and in cows with endometritis from the UK [44]. Recently, we have identified BoGHV6 in several bovine fetuses that were simultaneously infected with *H. somni* and other well-known pathogenic abortive agents of cattle, while one fetus with myocarditis contained only BoGHV6 DNA [36]. Collectively, these are adequate emerging evidence to suggest a possible association of BoGHV6 with fetal disease and possible fetal death in cattle [35]. Furthermore, BoGHV6 and BRSV should be added to the list of secondary fetopathic agents of cattle [40] until the direct and/or indirect effects of these infections on fetal survival [41] and the associated pathogenesis with possible disease manifestations are fully investigated. Nevertheless, the IHC detection of tissue antigens of BoGHV6, using monoclonal or polyclonal antibodies, would determine the participation of this pathogen in the development of fetal pneumonia. Consequently, it would be interesting to determine whether BoGHV6 is just an innocent bystander or an inductor of fetal disease in cattle [35] since this pathogen was frequently identified in mixed infections during this investigation. However, the current role of BoGHV6 in the development of disease is unknown, so caution must be used in the interpretation of these results until experimental studies have demonstrated the participation of this pathogen in the development of disease processes.

This study used two methods to associate the pathogens with infection: molecular and IHC detection/identification. Although molecular detection by PCR/RT-PCR is more sensitive than IHC, intralesional detection of these pathogens by IHC is strong evidence of their association with the disease process [23]. Molecular detection of an infectious agent should not be definitively interpreted as the cause of a specific disease process but is fundamental to differentiate between vaccine and field strain of disease agents [22]. Additionally, the IHC identification and molecular detection of tissue antigens and nucleic acids, respectively, with related histologic evidence of disease in fetal tissues, are suggestive of causal association [40]. Diagnostic IHC was used in previous studies to effectively associate intralesional organisms within fetal lungs of cattle [10,16,17], and as indicated previously, would be needed to associate BoGHV6 with fetal pathology.

Most of the immunohistochemical findings associated with BRD pathogens herein identified in the fetal lungs were previously observed in the lungs of feedlot and dairy cattle with histological evidence of several patterns of pulmonary disease [10,15]. Intralesional immunoreactivity for *M. bovis* was observed within several

epithelial cells of the lung; a previous investigation using IHC demonstrated positive immunoreactivity with the epithelial cells of the alveolar wall but with multifocal identification of *M. bovis* proteins by ISH [17]. Collectively, these results suggest that the distribution of *M. bovis* antigens and/or proteins within the lungs of bovine fetuses seems to be multifocal and not restricted to a specific histologic element of the lung. However, the classical pulmonary pattern of necrosuppurative or suppurative bronchopneumonia associated with pulmonary infections due to *M. bovis* [10] was not observed during this study when compared to a previous report of *M. bovis*-associated pulmonary diseases in a fetus [17]. The fetuses herein infected by *M. bovis* had histologic evidence of interstitial pneumonia and pulmonary congestion and contained other disease pathogens, including *N. caninum*, BRSV, BVDV, and BoAHV1, suggesting that *M. bovis* may not have been directly related to the development of the principal pattern of pulmonary disease identified in these fetuses.

During this study, single, double, triple, and quadruple infections were observed by the identification of intralesional antigens and/or nucleic acids of primary and secondary agents. Single infections were predominant, as frequently described in fetal deaths related to infectious diseases [40] and occurred in other studies investigating fetal pulmonary diseases of cattle [17–19]. Alternatively, the multiple concomitant infections identified in this study are not frequently identified in fetal pathology [40], with only one previous description of spontaneous dual infections in bovine fetuses [16]. Although the reason for the identification of mixed infections in bovine fetuses is unclear, high environmental infectious challenges, such as the endemicity of a disease pathogen, were proposed [40]. Pathogen endemicity seems plausible to the dynamics of reproductive disease pathogens in Brazil, considering that BVDV, BoAHV1, *Leptospira* spp., *B. abortus* [26,45,46], and to some extent *H. somni* [26,47], are frequently associated with fetal pathology and/or abortions in cattle from this country. Additionally, the frequency of concomitant fetal pulmonary infections herein identified may suggest that simultaneous infections in bovine fetuses may be more common than previously described [40].

8.6 Conclusions

In conclusion, molecular and IHC detection confirmed the presence of several agents associated with pulmonary and reproductive diseases of cattle within

fetal lungs that had histologic evidence of interstitial pneumonia and/or pulmonary alterations. Collectively, these assays have demonstrated the occurrence of primary and secondary fetopathy agents in these fetuses and indicate intrauterine/transplacental infection. The amplification of BoGHV6, BRSV, and BPIV-3 from the lungs of several fetuses with histologic evidence of pulmonary alteration, particularly with interstitial pneumonia, suggests that these pathogens should be considered as putative fetopathy agents of cattle.

Nevertheless, the role of BoGHV6 in the development of disease processes is not fully known and must be confirmed by experimental and in situ studies. Finally, the relative frequency of simultaneous infections herein identified may indicate that concomitant fetal infectious may be more frequent than previously diagnosed.

8.7 REFERENCES

1. Taylor, J.D.; Fulton, R.W.; Lehenbauer, T.W.; Step, D.L.; Confer, A.W. The epidemiology of bovine respiratory disease: What is the evidence for predisposing factors? Can. Vet. J. 2010, 51, 1095–1102.

2. Griffin, D.; Chengappa, M.M.; Kuszak, J.; McVey, D.S. Bacterial Pathogens of the Bovine Respiratory Disease Complex. Vet. Clin. N. Am. Food Anim. Pract. 2010, 26, 381–394.

3. Chai, J.; Capik, S.F.; Kegley, B.; Richeson, J.T.; Powell, J.G.; Zhao, J. Bovine respiratory microbiota of feedlot cattle and its association with disease. Vet. Res. 2022, 53, 4.

4. Headley, S.A.; Okano, W.; Balbo, L.C.; Marcasso, R.A.; Oliveira, T.E.; Alfieri, A.F.; Filho, L.C.N.; Michelazzo, M.Z.; Rodrigues, S.C.; Baptista, A.L.; et al. Molecular survey of infectious agents associated with bovine respiratory disease in a beef cattle feedlot in southern Brazil. J. Vet. Diagn. Investig. 2018, 30, 249–251.

5. Gagea, M.I.; Bateman, K.G.; van Dreumel, T.; McEwen, B.J.; Carman, S.; Archambault, M.; Shanahan, R.A.; Caswell, J.L. Diseases and pathogens associated with mortality in Ontario beef feedlots. J. Vet. Diagn. Investig. 2006, 18, 18–28.

6. Francoz, D.; Buczinski, S.; Bélanger, A.M.; Forté, G.; Labrecque, O.; Tremblay, D.; Wellemans, V.; Dubuc, J. Respiratory Pathogens in Québec Dairy Calves and Their Relationship with Clinical Status, Lung Consolidation, and Average Daily Gain. J. Vet. Intern. Med. 2015, 29, 381–387.

7. Cusack, P.M.V.; McMeniman, N.; Lean, I.J. The medicine and epidemiology of bovine respiratory disease in feedlots. Aust. Vet. J. 2003, 81, 480–487.

8. Baptista, A.L.; Rezende, A.L.; de Almeida Fonseca, P.; Massi, R.P.; Nogueira, G.M.; Magalhães, L.Q.; Headley, S.A.; Menezes, G.L.; Alfieri, A.A.; Saut, J.P.E. Bovine respiratory disease complex associated mortality and morbidity rates in feedlot cattle from southeastern Brazil. J. Infect. Dev. Ctries. 2017, 11, 791–799.

9. Fulton, R.W.; Blood, K.S.; Panciera, R.J.; Payton, M.E.; Ridpath, J.F.; Confer, A.W.; Saliki, J.T.; Burge, L.T.;Welsh, R.D.; Johnson, B.J.; et al. Lung pathology and infectious agents in fatal feedlot pneumonias and relationship with mortality, disease onset, and treatments. J. Vet. Diagn. Investig. 2009, 21, 464–477.

10. Fulton, R.W.; Purdy, C.; Confer, A.W.; Saliki, J.; Loan, R.W.; Briggs, R.E.; Burge, L.J. Bovine viral diarrhea viral infections in feeder calves with respiratory disease: Interactions with Pasteurella spp., parainfluenza-3 virus, and bovine respiratory syncytial virus. Can. J. Vet. Res. 2000, 64, 151.

11. Oliveira, T.E.S.; Pelaquim, I.F.; Flores, E.F.; Massi, R.P.; Valdiviezo, M.J.J.; Pretto-Giordano, L.G.; Alfieri, A.A.; Saut, J.P.E.; Headley, S.A. *Mycoplasma bovis* and viral agents associated with the development of bovine respiratory disease in adult dairy cows. Transbound. Emerg. Dis. 2019, 67, 82–93.

12. Tortorelli, G.; Carrillo Gaeta, N.; Mendonça Ribeiro, B.; Miranda Marques, L.; Timenetsky, J.; Gregory, L. Evaluation of mollicutes microorganisms in respiratory disease of cattle and their relationship to clinical signs. J. Vet. Intern. Med. 2017, 31, 1215–1220.

13. Headley, S.A.; Alfieri, A.F.; Oliveira, V.H.; Beuttemmuller, E.A.; Alfieri, A.A. *Histophilus somni* is a potential threat to beef cattle feedlots in Brazil. Vet. Rec. 2014, 175, 249.

14. Headley, S.A.; Balbo, L.C.; Alfieri, A.F.; Saut, J.P.E.; Baptista, A.L.; Alfieri, A.A. Bovine respiratory disease associated with *Histophilus somni* and bovine respiratory syncytial virus in a beef cattle feedlot from Southeastern Brazil. Semin. Ciênc. Agrár. 2017, 38, 283–294.

15. Castro, M.M.; Oliveira, T.E.S.; Headley, S.A. Bovine respiratory disease in Brasil: A short review. Semin. Ciênc. Agrár. 2021, 42, 2081–2110.

16. Oliveira, T.E.S.; Scuisato, G.S.; Pelaquim, I.F.; Cunha, C.W.; Cunha, L.S.; Flores, E.F.; Pretto-Giordano, L.G.; Lisbôa, J.A.N.; Alfieri, A.A.; Saut, J.P.E.; et al. The participation of a Malignant Catarrhal Fever Virus and *Mycoplasma bovis* in the development of single and mixed infections in beef and dairy cattle with bovine respiratory disease. Front. Vet. Sci. 2021, 8, 691448.

17. Crook, T.; Benavides, J.; Russell, G.; Gilray, J.; Maley, M.; Willoughby, K. Bovine herpesvirus 1 abortion: Current prevalence in the United Kingdom and evidence of hematogenous spread within the fetus in natural cases. J. Vet. Diagn. Investig. 2012, 24, 662–670.

18. Hermeyer, K.; Peters, M.; Brügmann, M.; Jacobsen, B.; Hewicker-Trautwein, M. Demonstration of *Mycoplasma bovis* by immunohistochemistry and in situ hybridization in an aborted bovine fetus and neonatal calf. J. Vet. Diagn. Investig. 2012, 24, 364–369.

19. Macías-Rioseco, M.; Mirazo, S.; Uzal, F.A.; Fraga, M.; Silveira, C.; Maya, L.; Riet-Correa, F.; Arbiza, J.; Colina, R.; Anderson, M.L.; et al. Fetal pathology in an aborted Holstein fetus infected with Bovine Parainfluenza Virus-3 Genotype A. Vet. Pathol. 2019, 56, 277–281.

20. López, A.; Hitos, F.; Pérez, A.; Navarro-Fierro, R.R. Lung lesions in bovine fetuses aborted by *Brucella abortus*. Can. J. Comp. Med. 1984, 48, 275–277.

21. Xavier, M.N.; Paixão, T.A.; Poester, F.P.; Lage, A.P.; Santos, R.L. Pathological, immunohistochemical and bacteriological study of tissues and milk of cows and fetuses experimentally infected with *Brucella abortus*. J. Comp. Pathol. 2009, 140, 149–157.

22. Miller, R.B.; Quinn, P.J. Observations on abortions in cattle: A comparison of pathological, microbiological and immunological findings in aborted foetuses and foetuses collected at abattoirs. Can. J. Comp. Med. 1975, 39, 270–290.

23. Maes, R.K.; Langohr, I.M.; Wise, A.G.; Smedley, R.C.; Thaiwong, T.; Kiupel, M. Beyond H&E: Integration of Nucleic Acid–Based analyses into diagnostic pathology. Vet. Pathol. 2013, 51, 238–256.

24. Fulton, R.W.; Confer, A.W. Laboratory test descriptions for bovine respiratory disease diagnosis and their strengths and weaknesses: Gold standards for diagnosis, do they exist? Can. Vet. J. 2012, 53, 754–761.

25. Anderson, M.L. Infectious causes of bovine abortion during mid- to late-gestation. Theriogenology 2007, 68, 474–486.

26. Panciera, R.J.; Confer, A.W. Pathogenesis and pathology of bovine pneumonia. Vet. Clin. Food Anim. Pract. 2010, 26, 191–214.

27. Headley, S.A.; Voltarelli, D.; de Oliveira, V.H.; Bronkhorst, D.E.; Alfieri, A.F.; Filho, L.C.; Okano, W.; Alfieri, A.A. Association of *Histophilus somni* with spontaneous abortions in dairy cattle herds from Brazil. Trop. Anim. Health Prod. 2015, 47, 403–413.

28. Claus, M.P.; Alfieri, A.F.; Folgueras-Flatschart, A.V.; Wosiacki, S.R.; Medici, K.C.; Alfieri, A.A. Rapid detection and differentiation of bovine herpesvirus 1 and 5 glycoprotein C gene in clinical specimens by multiplex-PCR. J. Virol. Methods 2005, 128, 183–188.

29. Vilˇcek, Š.; Herring, A.J.; Herring, J.A.; Nettleton, P.F.; Lowings, J.P.; Paton, D.J. 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. 1994, 136, 309–323.

30. Wesley, I.V.; Larson, D.J.; Harmon, K.M.; Luchansky, J.B.; Schwartz, A.R. A case report of sporadic ovine listerial meningoencephalitis in Iowa with an overview of livestock and human cases. J. Vet. Diagn. Investig. 2002, 14, 314–321.

31. Angen, O.; Ahrens, P.; Tegtmeier, C. Development of a PCR test for identification of *Haemophilus somnus* in pure and mixed cultures. Vet. Microbiol. 1998, 63, 39–48.

3398 32. Marques, F.A.; Headley, A.S.; Figueredo-Pereira, V.; Taroda, A.; Barros, L.D.;
3399 Cunha, I.A.; Munhoz, K.; Bugni, F.M.; Zulpo, D.L.; Igarashi, M.; et al. Neospora caninum:
3400 Evaluation of vertical transmission in slaughtered beef cows (*Bos indicus*). Parasitol.
3401 Res. 2011, 108, 1015–1019.

3402 33. Merien, F.; Amouriaux, P.; Perolat, P.; Baranton, G.; Saint Girons, I. Polymerase
3403 chain reaction for detection of *Leptospira* spp. In clinical samples. J. Clin. Microbiol.
3404 1992, 30, 2219–2224.

3405 34. Baily, G.G.; Krahn, J.B.; Drasar, B.S.; Stoker, N.G. Detection of *Brucella melitensis*
3406 and *Brucella abortus* by DNA amplification. J. Trop. Med. Hyg. 1992, 95, 271–275.

3407 35. Oliveira, C.H.; Oliveira, F.G.; Gasparini, M.R.; Galinari, G.C.; Lima, G.K.; Fonseca,
3408 A.A., Jr.; Barbosa, J.D.; Barbosa-Stancioli, E.F.; Leite, R.C.; Dos Reis, J.K.P. Bovine
3409 herpesvirus 6 in buffaloes (*Bubalus bulalis*) from the Amazon region, Brazil. Trop. Anim.
3410 Health Prod. 2015, 47, 465–468.

3411 36. Headley, S.A.; Fritzen, J.T.T.; Bon, V.R.; Xavier, A.A.C.; Dall Agnol, A.M.; Zucoloto,
3412 N.Z.; Silva, F.H.P.; Figueiredo, J.R.X.; Alfieri, A.F.; Okano,W.; et al. Detection of bovine
3413 gammaherpesvirus 6 in tissues of aborted fetuses from dairy cows concomitantly
3414 infected by *Histophilus somni*. Microb. Pathog. 2022, 169, 105621.

3415 37. Headley, S.; Oliveira, T.; Li, H.; Lisbôa, J.; Queiroz, G.; Fritzen, J.; Flores, E.; Alfieri,
3416 A.; Cunha, C. Immunohistochemical detection of intralesional antigens of Ovine
3417 Gammaherpesvirus-2 in cattle with Sheep-associated Malignant Catarrhal Fever. J.
3418 Comp. Pathol.2020, 174, 86–98.

3419 38. Headley, S.A.; Fritzen, J.T.T.; Queiroz, G.R.; Oliveira, R.A.M.; Alfieri, A.F.; Santis,
3420 G.W.D.; Lisbôa, J.A.N.; Alfieri, A.A. Molecular characterization of encephalitic bovine
3421 listeriosis from southern Brazil. Trop. Anim. Health Prod. 2014, 46, 19–25.

3422 39. Headley, S.A.; Oliveira, V.H.; Figueira, G.F.; Bronkhorst, D.E.; Alfieri, A.F.;
3423 Okano,W.; Alfieri, A.A. *Histophilus somni*-induced infections in cattle from southern
3424 Brazil. Trop. Anim. Health Prod. 2013, 45, 1579–1588.

3425 40. Headley, S.A.; Pereira, A.H.T.; Balbo, L.C.; Di Santia, G.W.; Bracarense, A.P.F.R.L.;
3426 Filho, L.F.C.C.; Schade, J.; Okano, W.; Pereira, P.F.; Morotti, F.; et al. *Histophilus*
3427 *somni*-associated syndromes in sheep from Southern Brazil. Braz. J. Microbiol. 2018,
3428 49, 591–600.

3429 41. Mee, J.F.; Jawor, P.; Stefaniak, T. Role of infection and immunity in bovine perinatal
3430 mortality: Part 1. Causes and current diagnostic approaches. Animals 2021, 11, 1033.

3431 42. Parkinson, T.J. 24—Specific Infectious Diseases Causing Infertility and Subfertility
3432 in Cattle. In Veterinary Reproduction and Obstetrics, 10th ed.; Noakes, D.E., Parkinson,
3433 T.J., England, G.C.W., Eds.;W.B. Saunders: St. Louis, MO, USA, 2019; pp. 434–466.

3434 43. Gagnon, C.A.; Allam, O.; Drolet, R.; Tremblay, D. Quebec: Detection of bovine
3435 lymphotropic herpesvirus DNA in tissues of a bovine aborted fetus. Can. Vet. J. 2010,
3436 51, 1021–1022.

3437 44. Garigliany, M.M.; Bayrou, C.; Cassart, D.; Jolly, S.; Desmecht, D. Bovine
3438 lymphotropic herpesvirus detected in Belgium. Vet. Rec. 2013, 172, 535–536.

3439 45. Cobb, S.P.; Banks, M.; Russell, C.; Thorne, M. Bovine lymphotrophic herpesvirus in
3440 a UK dairy herd. Vet. Rec. 2006, 158, 807–808.

3441 46. Antoniassi, N.A.B.; Juffo, G.D.; Santos, A.S.; Pescador, C.A.; Corbellini, L.G.;
3442 Driemeier, D. Causas de aborto bovino diagnosticadas no Setor de Patologia
3443 Veterinária da UFRGS de 2003 a 2011. Pesqui. Vet. Barsileira 2013, 33, 155–160.

3444 47. Silva, T.M.A.; Oliveira, R.G.; MolI, J.P.D.S.; Xavier, M.N.; Paixão, T.A.; Cortez, A.;
3445 Heinemann, M.B.; Richtzenhain, L.J.; Santos, A.P.L.R.D. Etiologic diagnosis of bovine
3446 infectious abortion by PCR. Ciênc. Rural. 2009, 39, 2563–2570.

9 CONCLUSÕES

A padronização de diferentes anticorpos comerciais e não comerciais para o diagnóstico de DRB foi eficaz, com imunorreatividade positiva intracitoplasmática para todos os antígenos avaliados.

Pneumonia intersticial, broncopneumonia supurativa e broncopneumonia necrosupurativa foram os padrões de pneumonia mais frequentemente observados.

Broncopneumonia necrosupurativa, bronquiolite obliterante e hiperplasia de tecido linfoide associado ao brônquio foram as características histopatológicas associadas à pneumonia por *M. bovis.*

A maior ocorrência de pneumonia intersticial foi relacionada à infecções por BVDV.

A bronquite e bronquiolite necrotizante foram alterações histológicas associadas aos agentes BVDV, BoAHV1 e BRSV.

Degeneração vacuolar do epitélio brônquico e bronquiolar estiveram associadas aos agentes BVDV, BoAHV1, MCFV

BPIV-3 demonstrou baixa ocorrência e, quando presente esteve associado a outro agente infeccioso.

A detecção de DNA do BoGHV6, BRSV e BPIV-3 nos pulmões fetais demonstra infecção transplacentária. No entanto, a participação desses agentes em fetopatia é incerta e merece ser investigada.

M. bovis e BVDV foram os principais agentes infecciosos associados a DRB.

M. bovis foi associado ao desenvolvimento de doença pulmonar em bovinos e deve ser considerado como um patógeno de doença primária no desenvolvimento da DRB, agindo isoladamente ou em sinergismo, principalmente com BVDV.

10. PESPECTIVAS

Com os resultados adquiridos nesta tese, pode ser proposto:

O sequenciamento completo do genoma de OvGHV2 para que possa compreender possíveis genes patogênicos que poderiam explicar as manifestações clínicas atípicas observadas nos casos positivos.

Desenvolver estudos de infecção com OvGHV2 para entender a situação epidemiológica, sua participação nos casos positivos e conhecer a(s) espécie(s) que o(s) mantém infectando os bovinos.

Ampliar estudos utilizando técnicas *in vitro,* com o agente BoGHV6 par a compreender e determinar a sua participação na infecção de fetos. E reconhecer o(s) hospedeiro(s) que mantem o vírus circulando em animais susceptíveis

Produzir material técnico para amplificar a divulgação dos resultados gerados nesta tese, como resumos em congresso da área, artigos para revistas técnicas, palestras para extensionistas, sanitaristas e produtores rurais.

10 ANEXOS

10.1 ARTIGO – IMMUNOHISTOCHEMICAL DETECTION OF INTRALESIONAL ANTIGENS OF OVINE GAMMAHERPESVIRUS-2 (OVHV-2) IN CATTLE WITH SHEEP-ASSOCIATED MALIGNANT CATARRHAL FEVER

Selwyn Arlington Headley, Thalita Evani Silva de Oliveira, Hong Li, Júlio Augusto Naylor Lisbôa, Gustavo Rodrigues Queiroz, Juliana Torres Tomazi Fritzen, Eduardo Furtado Flores, Amauri Alcindo Alfieri, Cristina Wetzel Cunha. **Journal of Comparative Pathology**. 2020; 174, p. 86-98. https://doi.org/10.1016/j.jcpa.2019.11.002.

SUMMARY

Sheep-associated malignant catarrhal fever (SA-MCF) is a severe lymphoproliferative disease of ruminants caused by ovine gammaherpesvirus-2 (OvHV-2). Since the initial identification of the SA-MCF there has been extensive research related to the pathogenesis of OvHV-2 based primarily on serological and molecular assays associated with typical histopathologic findings. The monoclonal antibody (MAb-15A) binds to a common epitope in MCF viruses and is frequently used in serological investigations. However, the utilization of this antibody to detect antigens of OvHV-2 in tissues was never demonstrated. Accordingly, this study developed an immunohistochemical (IHC) assay using the MAb-15A to identify antigens of OvHV-2 in tissues of cattle (*n*=5) with SA-MCF. All animals developed acute neurological signs, without ocular and nasal manifestations, and contained nucleic acids of OvHV-2 in brain tissue by polymerase chain reaction. The principal histopathologic findings were lymphocytic nephritis (*n*=5), widespread arterial proliferation and vasculitis (*n*=5), lymphocytic portal hepatitis (*n*=3), nonsuppurative meningoencephalitis (*n*=2), and atrophic enteritis with cryptal necrosis and dilation (*n*=2). Intralesional intracytoplasmic antigens of OvHV-2 were identified within multiple epithelial cells of the kidneys of all animals, the intestines of animals with and without atrophic enteritis, and within epithelial cells of bile ducts in animals with lymphocytic hepatitis. Additionally, there was positive intracytoplasmic immunoreactivity within histocytes and histiocytes in several tissues. These findings suggest that the MAb-15A is epitheliotropic and detects antigens of OvHV-2 within leucocytes. Moreover, this IHC assay would contribute significantly towards the

understanding of the pathogenesis of SA-MCF and may be an excellent tool for retrospective studies. Additionally, angiopathy in SA-MCF may be a progressive lesion, that may terminate in luminal occlusion and probably occurs irrespectively of the eye and head form of MCF.

Keywords: angiopathy; immunohistochemistry; neurological disease; ovine gammaherpesvirus 2.

INTRODUCTION

Malignant catarrhal fever (MCF) is a severe, frequently fatal, infectious viral disease with clinicopathological lesions observed in cattle and other susceptible ungulates (LI *et al.*, 2014). MCF is caused by members of the genus *Macavirus*, subfamily Gammaherpesvirinae (DAVISON *et al.*, 2009). The principal pathogenic MCF viruses include Alcelaphine herpesvirus 1 (AlHV-1) and AlHV-2, caprine herpesvirus-2, ovine gammaherpesvirus-2 (OvHV-2) and MCFV in white-tailed deer (LI *et al.*, 2014; O'TOOLE & LI, 2014). However, two important endemic and epidemiological forms of MCF are recognized within restricted geographical locations: wildebeest-associated MCF (WA-MCF) caused by AlHV-1, which occurs in ruminants from Africa and in wildlife, and sheep-associated-MCF (SA-MCF) caused by OvHV-2, which occurs predominantly in cattle, bison and deer worldwide (RUSSELL *et al.*, 2009; LI *et al.*, 2014).

SA-MCF is endemic in Brazil, with sporadic cases occurring in most geographical regions of the country, affecting predominantly cattle (BARROS *et al.*, 1983; MENDONÇA *et al.*, 2008; FURLAN *et al.*, 2012; HEADLEY *et al.*, 2012; HEADLEY *et al.*, 2015), with few descriptions in swine (COSTA *et al.*, 2010) and buffalos (COSTA *et al.*, 2009). The characteristic feature for the histopathologic diagnosis of MCF has not changed since the initial descriptions (METTAM, 1923; DANSKIN, 1955; PLOWRIGHT *et al.*, 1960), and continues to be widespread vasculitis with fibrinoid change in multiple organs (RUSSELL *et al.*, 2009; LI *et al.*, 2014). This lesion was fundamental for the initial identification of SA-MCF in cases from Brazil (BARROS *et al.*, 1983; MENDONÇA *et al.*, 2008; HEADLEY *et al.*, 2012; HEADLEY *et al.*, 2013; HEADLEY *et al.*, 2015), with OvHV-2-associated infection determined by the amplification of viral DNA from the affected animals. The

amplification of viral DNA in tissues of ruminants by polymerase chain reaction (PCR) was an historical landmark for the identification of MCF in infected animals (HSU *et al.*, 1990; WIYONO *et al.*, 1994). Moreover, the PCR method used to amplify OvHV-2 DNA in SA-MCF (BAXTER *et al.*, 1993), is used worldwide, has a significant impact on the molecular identification of SA-MCF (LI *et al.*, 2014), and is frequently used as an ancillary test to associate the viral pathogen with SA-MCF in Brazil (GARMATZ *et al.*, 2004; MENDONÇA *et al.*, 2008; HEADLEY *et al.*, 2012; HEADLEY *et al.*, 2013; HEADLEY *et al.*, 2015) and worldwide (RUSSELL *et al.*, 2009; LI *et al.*, 2014; O'TOOLE & LI, 2014).

However, PCR testing confirms the presence of the virus in the affected animal with histopathologic evidence of MCF but does not necessarily corelate with the histopathologic findings, resulting in the non-confirmation of an active infection, since the identification of viral DNA is not necessarily confirmation of disease (O'TOOLE & LI, 2014). Alternatively, *in situ* hybridization (ISH) confirms an active infection of SA-MCF due to the identification of intralesional nucleic acids within affected tissues (PESAVENTO *et al.*, 2019a). The monoclonal antibody 15A (MAb-15A) detects a epitope present in all recognized MCF viruses (LI *et al.*, 1994), and has been used effectively in several serological investigations associated with molecular testing and histopathology (LI *et al.*, 1995; MULLER-DOBLIES *et al.*, 1998; LI *et al.*, 2001). However, the efficiency of the MAb-15A to identify tissue antigens of OvHV-2 was not previously attempted. Accordingly, we have developed an immunohistochemical assay to identify intralesional antigens of OvHV-2 within tissues of cattle with SA-MCF using the MAb-15A. This paper presents the pathological and immunohistochemical findings observed in cattle from Paraná, Southern Brazil naturally infected by OvHV-2.

MATERIAL AND METHODS

Animals, study area, and sampling

The cattle in this study (*n*=5) were the source of tissue containing OvHV-2 DNA as confirmed by molecular identification (BAXTER *et al.*, 1993); detailed investigation of one of these cases is reported elsewhere (HEADLEY *et al.*, 2013). These animals were part of a previous epidemiological survey of neurological

diseases in cattle from the state of Paraná, Southern Brazil (QUEIROZ *et al.*, 2018a). The utilization of animals for these studies was approved by the Commission of Ethics Committee in Animal Usage, Universidade Estadual de Londrina (CEUA-UEL 04/2009 and 32.340.2012.04). All necropsy examinations performed during the epidemiological survey were at the location of the animals; tissues were then collected and submitted for histopathological diagnosis. As part of this study, specific brain fragments(QUEIROZ *et al.*, 2018b) from all cattle were submitted for the laboratory diagnosis of bovine rabies.

All histological slides relative to these cases were retrieved from the archives of the Laboratory of Animal Pathology, Universidade Estadual de Londrina, reviewed, and the principal histopathological pattern(s) of disease identified and recorded; these results were then tabulated. When necessary, formalin-fixed and paraffin wax-embedded (FFPE) tissue blocks of these cases were used to produce new sections that were stained by haematoxylin and eosin (HE). The FFPE tissue sections from the brain were prepared from predetermined neuroanatomical locations representing regions of the telencephalon, diencephalon (thalamus), brainstem (mesencephalon, pons and medulla oblongata), spinal cord, cerebellum and the complex of trigeminal nerve ganglia and the carotid rete mirabile, (TNG-CRM) (QUEIROZ *et al.*, 2018b). Moreover, archival laboratory files were reviewed to obtain data relative to the biological data of each animal, the clinical manifestations recorded, and gross lesions observed at autopsy. All tissues used were not maintained immersed in formalin solution for longer than one week.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on tissue sections to determine the presence of intralesional antigens of OvHV-2. FFPE tissue sections from each animal were prepared on slides treated with poly-lysine 0.1% (Sigma Aldrich, St. Louis, Missouri, USA), dewaxed, hydrated in alcohol baths and subjected to antigen retrieval by incubation in citrate buffer (pH 6.0) in a pressure cooker (Electrolux Pressure Cooker PCC10, São Paulo, Brazil) for 9 min. Endogenous peroxidase was blocked with distilled water and H2O2 (6%) for 45 min in a dark chamber. The primary incubation (24 h at 4°C) was with MAb-15A (dilution 1 in 400), designed to bind to a conserved epitope present on MCF viruses (LI *et al.*, 1994).

The utilization of the secondary antibody, counterstaining, and slide assembly were done as described (OLIVEIRA *et al.*, 2019).

Positive controls consisted of selected FFPE tissues from two pigs (Table 1), that were experimentally infected with OvHV-2 by intranasal nebulization, developed SA-MCF, and had high viral load in the tissues as identified by quantitative PCR, qPCR (LI *et al.*, 2012). Furthermore, most of these tissues were confirmed with active infections due to the detection of intralesional nucleic acids of OvHV-2 by ISH (PESAVENTO *et al.*, 2019a). Two negative controls were used to assess the quality of the IHC assay. The first consisted of the application of the MAb-15A on FFPE intestinal tissues fragments from a cow known not to contain OvHV-2 by PCR (QUEIROZ *et al.*, 2018a) using the SA-MCF primers (BAXTER *et al.*, 1993). In the second, the primary antibody was substituted by the diluent of the secondary antibody on FFPE tissues fragments of pigs that contained OvHV-2 as confirmed by PCR and ISH (Li et al., 2012) (PESAVENTO *et al.*, 2019a), and on FFPE tissues fragments of the cow known not to contain nucleic acids of OvHV-2 as described above.

Table 1 – Tissues used as positive and negative controls during for immunohistochemistry

Animals	Tissue	OvHV-2 copy number	ISH	IHC*
Positive controls				
Pig 1	Lung	225,000[a]	Positive[b]	Positive
	Mesenteric lymph node	3,090,000[a]	Positive[b]	Positive
	Spleen	649,000[a]	Positive[b]	Positive
	Stomach	330,000[a]	Not Done	Positive
Pig 2	Brain	1,000,000 [a]	Positive[b]	Negative
	Lung	3,630,000[a]	Positive[b]	Positive
	Stomach	1,060,000	Not Done	Positive
	Urinary bladder	924,000[a]	Positive[b]	Positive
Negative controls				
Cow[c]	Large and small intestine	Not applicable	Not Applicable	Negative
Pig1	Lung	225,000	Positive	Negative

*Results from this study

[a] Viral genome copy numbers per 50 mg of tissue DNA as tested by OvHV-2 qPCR in pigs with experimentally induced SA-MCF (LI *et al.*, 2012).

[b] Tissues containing OvHV-2 nucleic acids by ISH (PESAVENTO *et al.*, 2019a).

[c] OvHV-2-uninfected cow. Tissue that did not contain OvHV-2 DNA using the SA-MCF primers (BAXTER *et al.*, 1993).

Additionally, an IHC assay designed (OLIVEIRA *et al.*, 2019) to detect antigens of bovine viral diarrhoea virus (BVDV) was used to identify intralesional antigens of this virus in the intestine of animals with atrophic enteritis (# 1 and 4) and interstitial pneumonia (# 2 and 5). Positive control for the BVDV IHC assay consisted of FFPE pulmonary fragments known to contain antigens of BVDV (OLIVEIRA *et al.*, 2019); the negative control consisted of substitution of the primary antibody on pulmonary tissues known not to contain antigens of BVDV (OLIVEIRA *et al.*, 2019). Positive and negative controls were included in each IHC assay.

Molecular detection of nucleic acids of viral infectious disease pathogens

Nucleic acids of infectious disease pathogens were extracted from tissue fragments (cerebellum, mesencephalon, thalamus, TGN-RCM, as well as a pool derived from all regions from the telencephalon) maintained at -80℃, using Proteinase K (Qiagen Sample & Assay Technologies, Hilden, Germany) and a combination of the phenol/chloroform/isoamyl alcohol and silica/guanidine isothiocyanate methods as previously described (BOOM *et al.*, 1990; ALFIERI *et al.*, 2006).

The consensus PCR primers designed to amplify DNA from a wide range of herpesvirus (VANDEVANTER *et al.*, 1996), followed by sequencing of the amplicon, were used to exclude the participation of other herpesvirus in the development of the lesions herein described. Furthermore, differential molecular testing was done to determine the presence of common neurological disease pathogens of cattle by using PCR assays that targeting the glycoprotein C gene of Bovine alphaherpesvirus -1 (BoAHV1) and -5 (CLAUS *et al.*, 2005), the 16s rRNA gene of *Histophilus somni* (ANGEN *et al.*, 1998), and the 5'-UTR region of pestivirus (VILČEK *et al.*, 1994).

Viral DNA and/or RNA from previous studies (HEADLEY *et al.*, 2012; HEADLEY *et al.*, 2015), served as positive controls; nuclease-free water (Invitrogen Corporation, Carlsbad, CA, USA) was used as the negative control. Positive and negative controls were included in all PCR and/or RT-PCR assays. The generated PCR and/or RT-PCR products were separated by electrophoresis in 2% agarose gels, stained with ethidium bromide and examined under ultra-violet light. The PCR and/or RT-PCR products obtained were submitted for direct sequencing using the

forward and reverse primers of each assay. The derived nucleotide sequences were then compared by the BLAST (http://www.ncbi.nlm.nih.gov/BLAST) program with similar selected sequences deposited in GenBank.

RESULTS

Biological data and clinical manifestations

All animals were relatively young, being between 10 to 18-month-old; most (4/5; 80%) were predominantly young bulls (Table 2). The most frequent neurological manifestations observed were bilateral central blindness (*n*=3), reduced hindlimb function (*n*=3), and depression (*n*=2). Animal # 1 was found dead without any notable clinical observation; all other animals were euthanized *in extremis*. The clinical progression of neurological disease was less than 2 days in most animals, except for #4, in which 15 days of neurological manifestations were reported. Ocular and nasal secretions were not observed during the clinical evaluations of these animals.

Gross and histopathologic findings

The salient gross findings were bilateral blindness; other significant gross alterations were not observed. The principal histopathologic findings are summarized in Table 2. However, there was no uniformity in the FFPE tissue blocks and/or histologic slides from these cases, since FFPE blocks of all organs collected during autopsy were not located for each animal.

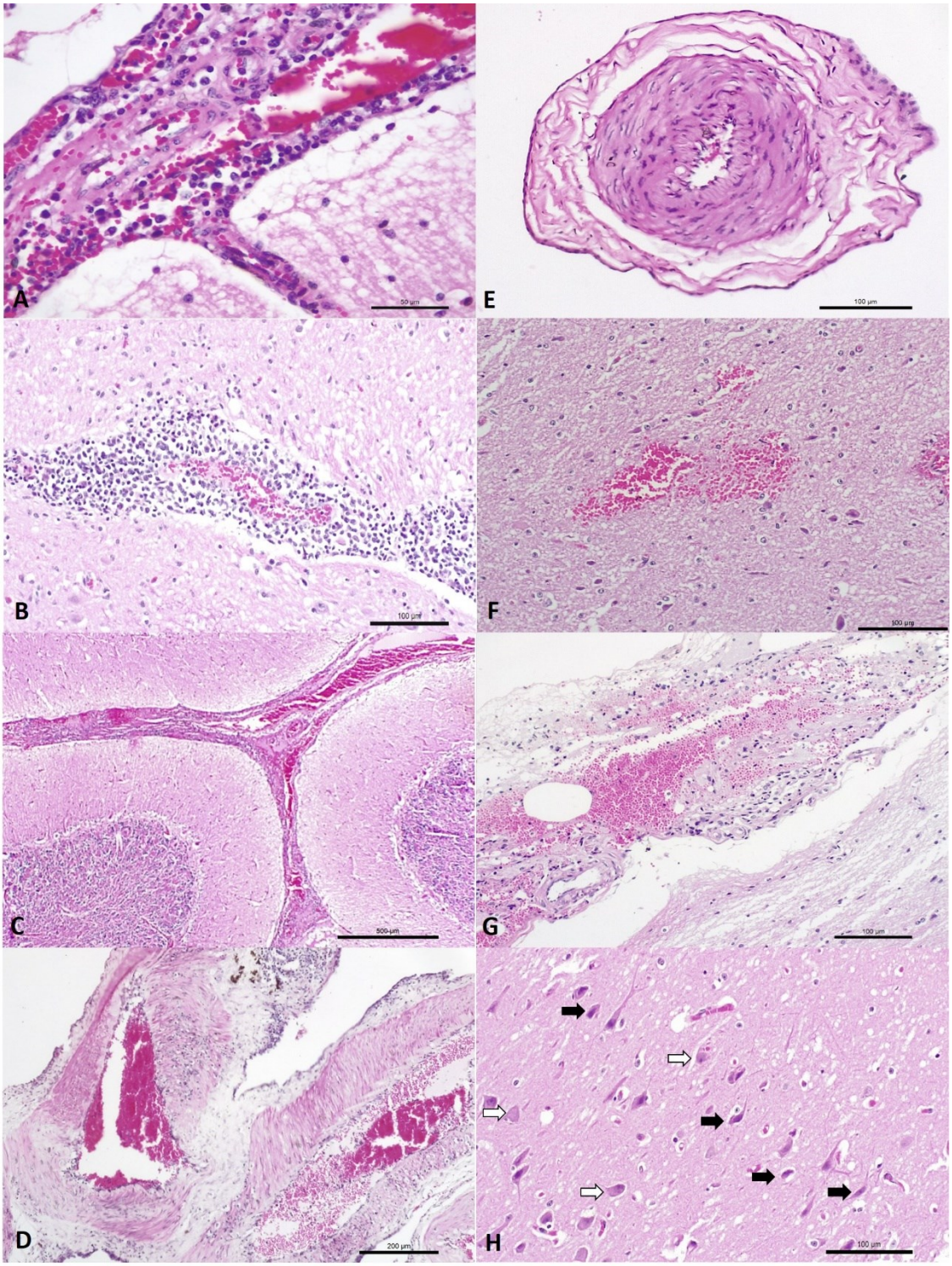
3711 **Table 2** – Biological data, clinical manifestations, histopathologic findings and outcome of cattle naturally infected by OvHV-2.

Case N°	Biological data	Principal clinic-neurological manifestations	Principal histopathologic findings	Clinical progression and outcome
1	12-month-old, Cross-bred, heifer	Sudden death	Carotid rete mirabile: vasculitis with fibrinoid change and proliferative vascular lesions Cerebrum, cerebellum, and brainstem: multifocal non-suppurative encephalitis with extensive perivascular cuffings, vasculitis, gliosis and neuronal necrosis Kidney: moderate, multifocal, lymphocytic interstitial nephritis with vasculitis Liver: mild, lymphocytic portal hepatitis and vasculitis Small intestine: atrophic enteritis Spleen: multifocal, moderate, vasculitis with proliferative vascular lesions	2 days Spontaneous death
2	18-month-old, Nelore bull	Absence of facial sensitivity Bilateral central blindness Depression Hindlimbs flaccid and static paresis Mydriasis	Cerebrum: moderate, multifocal, non-suppurative, meningoencephalitis with vasculitis and gliosis Kidney: multifocal, moderate lymphocytic interstitial nephritis Liver: mild, lymphocytic portal hepatitis Lung: moderate, multifocal, interstitial pneumonia	2 days Euthanasia *in extremis*
3	10-month-old, Nelore, bull	Depression Hindlimbs flaccid paresis	Brainstem: moderate, multifocal, haemorrhagic rhombencephalitis Cerebrum: multifocal neuronal necrosis with gliosis and neuronophagia Kidney: multifocal, moderate, lymphocytic interstitial nephritis Spleen: moderate white pulp hyperplasia with proliferative vascular lesions	1 day Euthanasia *in extremis*
4	10-month-old, Cross-bred, bull	Bilateral central blindness Incoordination Muscle tremors Mydriasis	Brainstem: moderate to severe, multifocal, haemorrhagic rhombencephalitis with neuronal necrosis Carotid rete mirabile: vasculitis with fibrinoid change and proliferative vascular lesions Kidney: multifocal, moderate lymphocytic interstitial nephritis Mesenteric lymph node: moderate lymphoid depletion and thrombosis Small intestine: atrophic enteritis	15 days Euthanasia *in extremis*

5	13-month-old, Nelore, bull	Bilateral blindness Head tremor Hindlimbs paralysis	central flaccid	Brainstem: moderate to severe, multifocal, haemorrhagic and thrombotic rhombencephalitis with neuronal necrosis Carotid rete mirabile: vasculitis with fibrinoid change and proliferative vascular lesions Cerebrum: severe, multifocal, neuronal necrosis with gliosis, satellitosis, and neuronophagia Cerebellum: non-suppurative meningitis Liver: lymphocytic portal hepatitis and thrombosis Lung: mild interstitial pneumonia with proliferative vascular lesions Kidney: multifocal, moderate, lymphocytic nephritis with ballooning degeneration of the uroepithelium and proliferative vascular lesions Spleen: moderate white pulp hyperplasia, fibrinoid change with proliferative vascular lesions Mesenteric lymph node: proliferative vascular lesion	2 days Euthanasia *in extremis*	

All cattle with clinical manifestations of brain impairment demonstrated histopathologic evidence of neurological disease. In two animals (# 1 and 2), brain lesions were predominantly inflammatory in nature, being characterized as chronic, multifocal, nonsuppurative meningoencephalitis with perivascular cuffings and vasculitis (Figure 1A-B). However, perivascular cuffings were more predominant and severe in animal #1 relative to #2, with lesions being identified throughout the cerebrum, cerebellum (Figure 1C), and brainstem of animal # 1; lesions were restricted to the cerebrum in animal #2. Histologic slides and FFPE blocks of the trigeminal nerve ganglion with the carotid rete mirabile were only available for animals #1, #3, and #5; in these animals, there was severe necrotizing vasculitis with fibrinoid change and early proliferative vascular lesions at the carotid rete mirabile (Figure 1D-E). These vascular lesions were characterized as severe proliferation at the tunica media with endothelial hypertrophy, resulting in partial occlusion of the vascular lumen. Moderate to severe, multifocal, haemorrhagic rhombencephalitis (Figure 1F) was identified in animals # 3, 4, and 5, with thrombotic rhombencephalitis occurring in animal #5 (Figure 1G). Furthermore, there was severe, multifocal neuronal necrosis at the cerebral cortex (Figure 1H) and brainstem of animals # 1, 3, and 5.

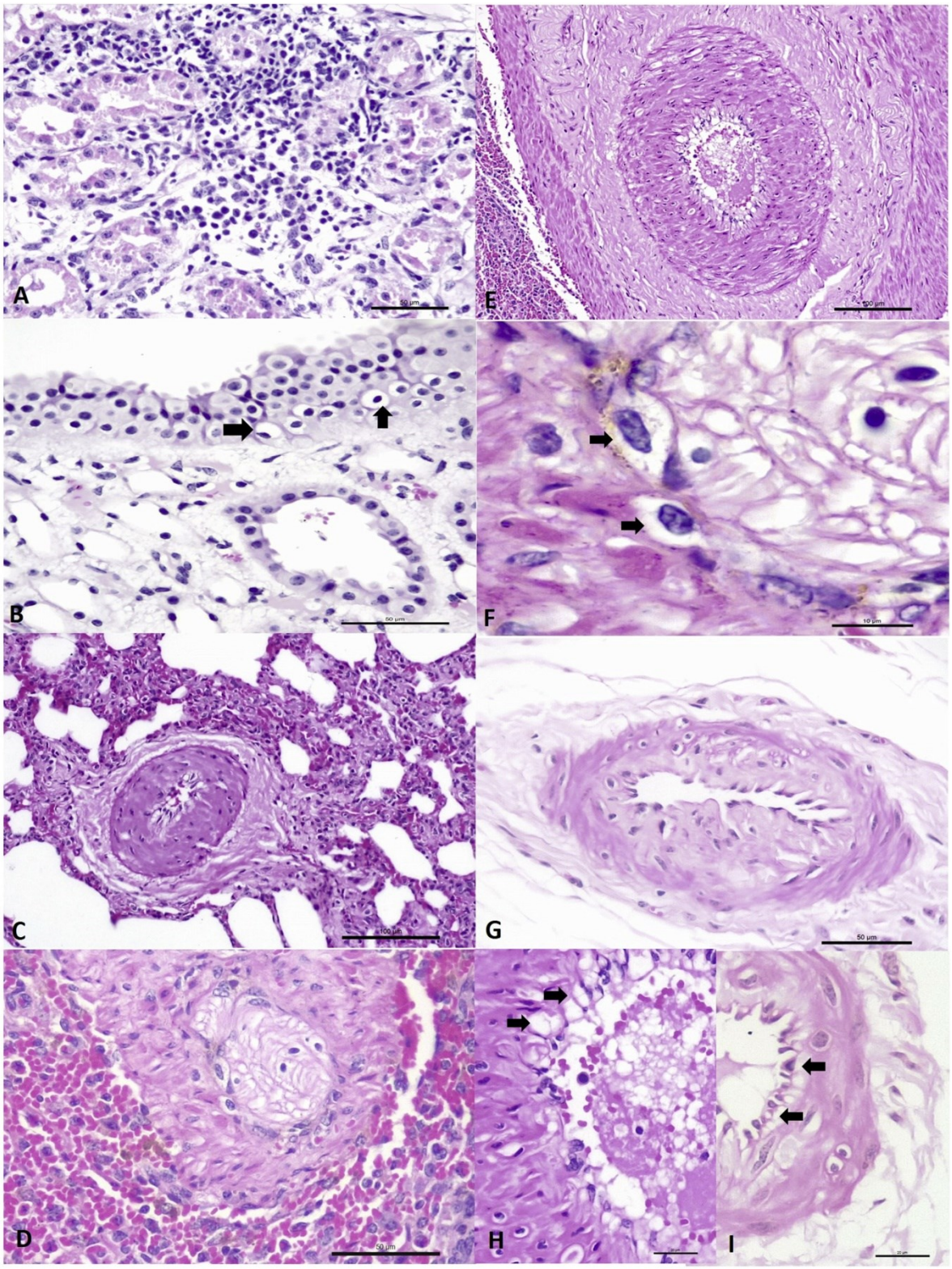
Figure 1 - Histopathological findings in the central nervous system of cattle infected naturally by OvHV-2. Non-suppurative meningoencephalitis with extensive perivascular cuffing in the cerebrum (A, B) and cerebellum (C) with lymphocytic necrotizing vasculitis in the carotid rete mirabile of case 1 (D). Early proliferative arterial lesions in the carotid rete mirabile of case 5 (E). Haemorrhagic (F) and thrombotic (G) rhombencephalitis in the brainstem of case 5. Neuronal necrosis in the cerebral cortex of case 5 (H); compare the necrotic (black arrows) with normal (open arrows) neurons. Bar, A, 50 μm; B, E-H, 100 μm; C, 500 μm; D, 200 μm.



Significant non-neurological histopathologic lesions were observed at the kidney (*n*=5), liver (*n*=3), lung (*n*=2), and small intestine (*n*=2) of the affected animals; all affected tissues demonstrated vasculitis. Histopathologic alterations at the kidneys were characterized predominantly as multifocal, lymphocytic interstitial nephritis (*n*=5; Figure 2A) with vasculitis; mild ballooning degeneration of the

uroepithelium was observed in one cow (#5; Figure 2B). Widespread proliferating vascular lesions, as described at the carotid rete mirabile, were observed in several tissues of animals #1, 3 and 5 (Figure 2C-H); the vascular lesions observed at the spleen of animal #3 was highly suggestive of atherosclerosis (Figure 2E). Additional, significant histopathologic findings included portal lymphocytic hepatitis (animals # 1, 3, and 5) and atrophic enteritis with cryptal necrosis (animals #1 and 4).

Figure 2 – Histopathologic demonstration of renal and arterial lesions in cattle naturally infected by OvHV-2. Lymphocytic interstitial nephritis (A; case 4) and ballooning degeneration (arrows) of the uroepithelium (B; case 5). Arterial proliferation in the lung with interstitial pneumonia (C; case 5). Marked proliferation of the tunica media of an artery within the spleen (D, case 3; E, case 5); higher magnification (F; case 3) demonstrating ballooning degeneration (arrows) of the endothelial cells. Proliferation of the tunica media of an artery within the capsule of the mesenteric lymph node (G; case 3); higher magnification of degenerate endothelial cells (arrows) in the spleen (H; case 5) and mesenteric lymph node (I; case 3). Bar, A, B, D, G: 50 µm; C, E: 100 µm; F, 10 µm; H, I: 20 µm.



Molecular detection of viral nucleic acids

Frozen brain samples from all animals with neurological disease (QUEIROZ *et al.*, 2018a) contained OvHV-2 using the SA-MCF PCR assay (BAXTER

et al., 1993). Nucleic acids of BoAHV-5 were amplified from frozen samples of the cerebellum, brainstem, and the TNG-CRM of case 2; tissues from all other animals did not contain BoAHV-5 DNA. Nucleic acids of BoAHV1 were not amplified from any of the samples evaluated. DNA from *H. somni* was only amplified from the cerebrum and brainstem of case 4. BVDV-1 RNA was amplified from the abomasum, mesenteric lymph node and small intestine of case 4. Bovine rabies was not identified in any of the animals.

Consequently, the molecular investigations revealed that in most (60%; 3/5) of these animals only DNA of OvHV-2 was amplified from several areas of the brain using the SA-MCF primers (BAXTER *et al.*, 1993), suggesting infections associated only with OvHV-2. Alternatively, dual (OvHV-2 and BoAHV-5) infection was identified in case 2, while triple infection (OvHV-2, *H. somni* and BVDV-1) occurred in case 4.

Immunohistochemistry

Immunohistochemical findings are summarized in Table 3. Positive immunoreactivity to the MAb-15A was cytoplasmic within epithelial cells and leucocytes. Epithelial cells from all tissues (i.e. stomach, lung, kidney and urinary bladder) derived from the control pigs, known to contain OvHV-2 DNA by qPCR (LI *et al.*, 2012) and ISH (PESAVENTO *et al.*, 2019a), demonstrated positive intracytoplasmic immunoreactivity with MAb-15A (Figs. 3A-D). Additionally, positive intracytoplasmic immunoreactivity was observed within macrophages and lymphocytes in the medullary region of the mesenteric lymph node with elevated viral load (Figure 3E). The lung from pig 1 used as negative control had no immunoreactivity with MAb-15 (Figure 3F).

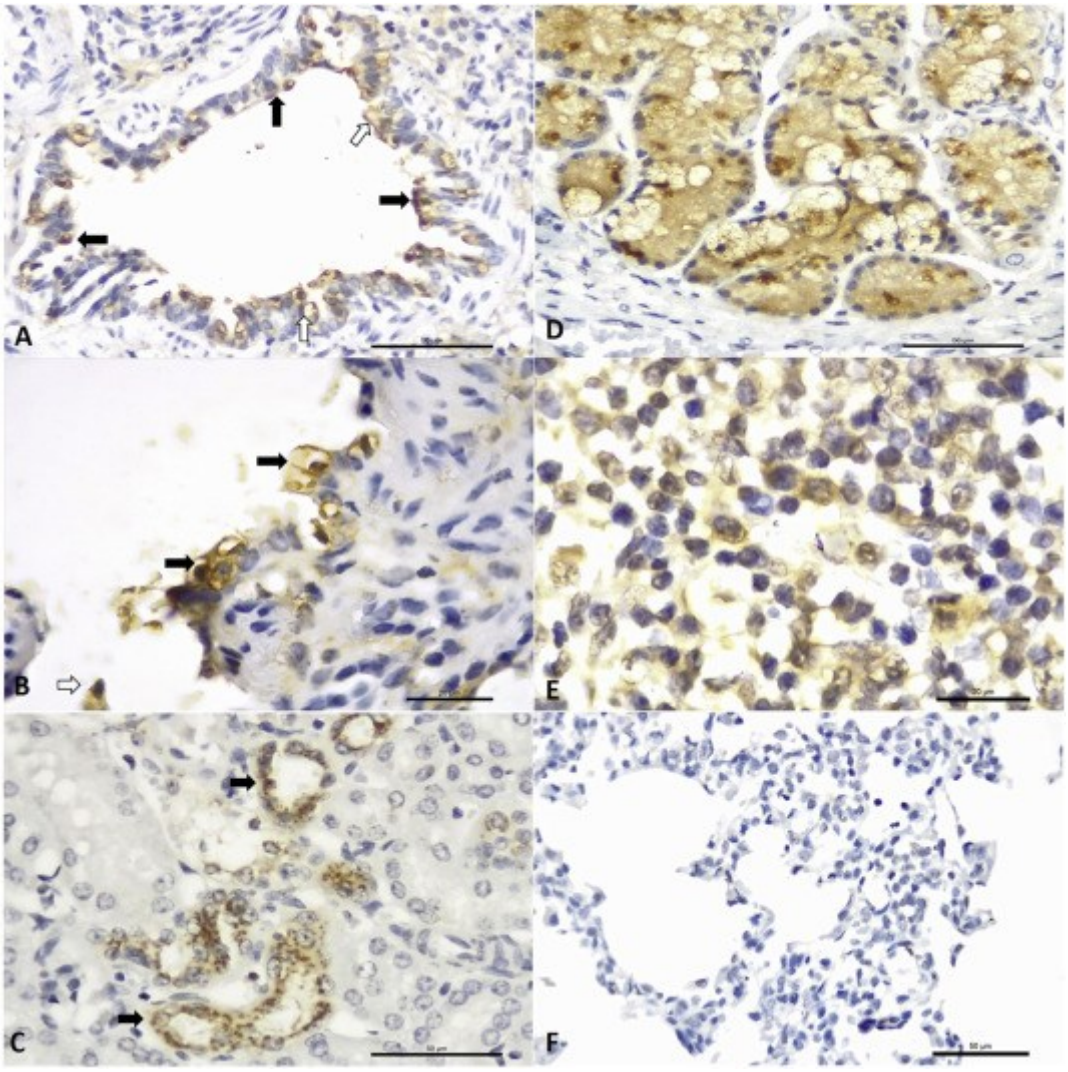
Table 3 - Immunohistochemical distribution of antigens of OvHV-2

Tissues	Cells/anatomic location	Controls[1]			Naturally infected animals				
		Pig 1[a]	Pig 2	Cow	#1	#2[a]	#3	#4[b, c]	#5
Brain	Brainstem				-	-	-	-	-
	Carotid rete mirabile				-	NL	-	NL	-
	Cerebellum				-	-	-	-	-
	Cerebrum		-		-	-	-	-	-
Kidney	Capillary vessels				+	+	+	+	+
	Collecting ducts				+	+	+	+	+
	Distal and proximal tubules				+	+	+	+	+
	Medullary rays				+	+	+	+	+
	Uroepithelium				NA	NA	NA	NA	+
Mesenteric lymph node	Histiocytes		+		+	NL	+	NL	+
	Lymphocytes		+		+	NL	+	NL	+
Large intestine	Cryptal epithelium			-	NL	NL	NL	+	+
	Enterocytes			-	NL	NL	NL	+	+
	Goblet cells			-	NL	NL	NL	+	+
	Histiocytes				NL	NL	NL	+	+
Lung	Mixed peribronchial glands	+	+		NL	-	NL	NL	-
	Pneumocytes	+	+		NL	-	NL	NL	-
	Respiratory epithelium	+	+		NL	-	NL	NL	-
Small intestine	Cryptal epithelium			-	+	+	+	+	+
	Enterocytes			-	+	+	+	+	+
	Goblet cells			-	+	+	+	+	+
	Histiocytes			-	+	+	+	+	+
Spleen	Lymphocytes				NL	+	NL	NL	+
Stomach - gastric glands	Base	+	+	NA	NA	NA	NA	NA	NA
	Isthmus	+	+	NA	NA	NA	NA	NA	NA
	Pit	+	+	NA	NA	NA	NA	NA	NA
Urinary bladder	Transitional epithelium		+	NL	NL	NL	NL	NL	NL

[1], Only tissues used as controls are shown; +, positive; -, negative; NL, tissue block not located; NA, not applicable.
[a] DNA of BoAHV-5 was amplified from the cerebrum, thalamus, pons, and TGN.
[b] RNA of BVDV was amplified from the abomasum, small intestine, and mesenteric lymph node.
[c] DNA of *Histophilus somni* was amplified at the cerebral cortex and brainstem

Figure 3 – Immunohistochemical demonstration of reactivity with MAb-15 in OvHV-2-positive, OvHV-2-negative and control tissues of pigs. Reactivity to antigens of OvHV-2 within the cytoplasm of intact (black arrows) and degenerate epithelial cells (white arrows) of the pulmonary bronchiole (A; pig 1), within type II pneumocytes (black arrows) and an alveolar macrophage (open arrow) of the lungs of pig 1 with interstitial pneumonia (B). Intracytoplasmic immunoreactivity to antigens of OvHV-2 within epithelial cells of the renal tubules (C; pig 2), gastric gland of the stomach (D, pig 2) lymphocytes and macrophages of the mesenteric lymph node (E, pig 2). Absence of

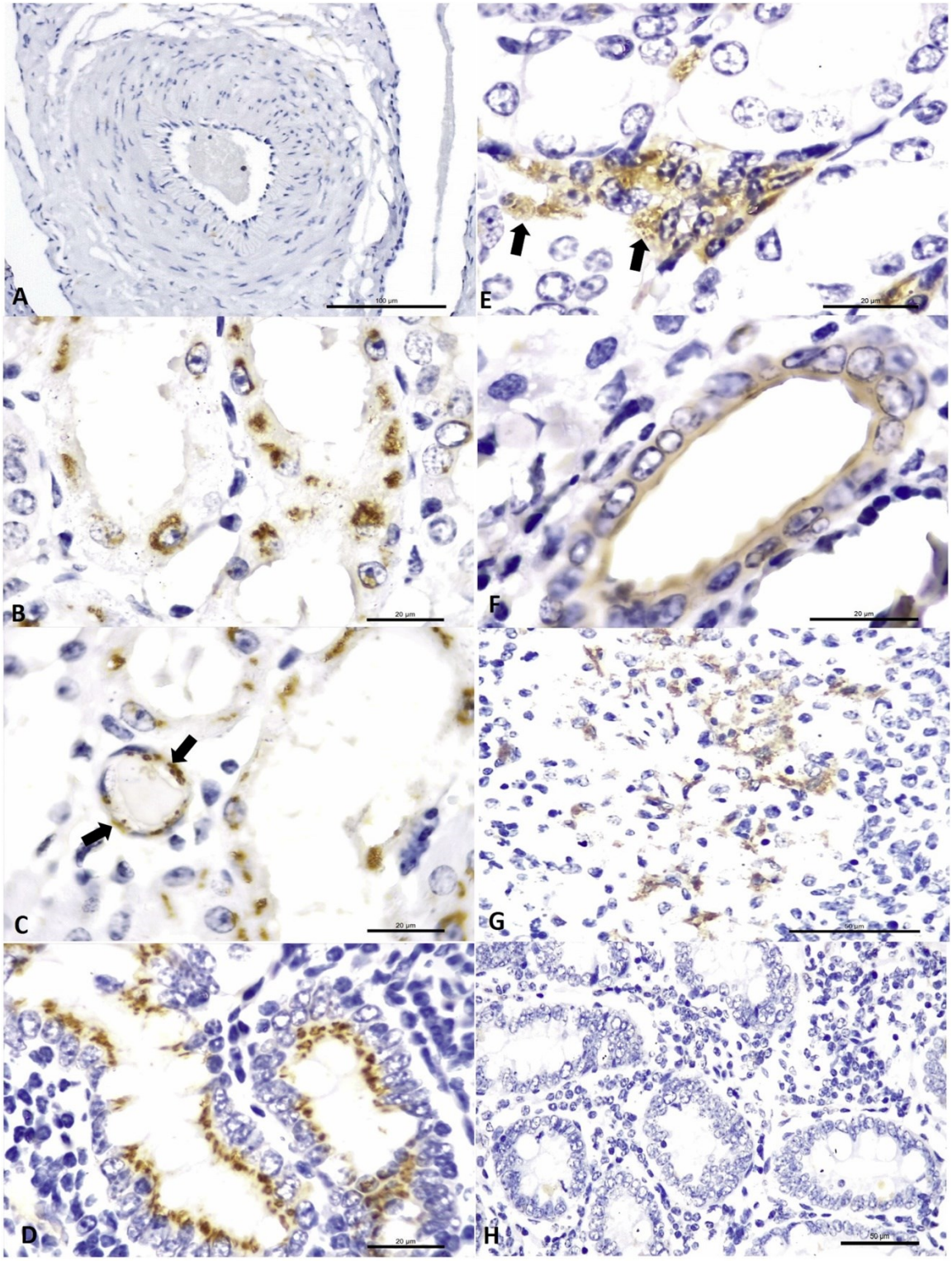
immunoreactivity in the lungs (pig 1; F). IHC. Bars, A, C, D and F, 50 µm; B, E, 20 µm



Positive immunoreactivity with MAb-15A was not observed in any brain section of animals with DNA of OvHV-2 demonstrated by PCR; this was also observed for the proliferating vascular lesions at the TNG-CRM (Figure 4A). However, there was positive immunoreactivity to antigens of OvHV-2 in non-neurological tissues, predominantly the kidneys (100%; 5/5), intestines (100%; 5/5) and mesenteric lymph nodes (60%; 3/5). Comparatively, immunohistochemical labelling for antigens of OvHV-2 was more widespread and intense within the intestine, followed by the kidneys and then the lymphoid organs. Positive intracytoplasmic immunoreactivity with MAb-15A was identified within the epithelial cells of the convoluted tubules, collecting ducts and medullary capillary vessels of the kidneys from all cows and in the degenerate uroepithelium of case 5 (Figs. 4B and C). Positive immunoreactivity occurred within the cytoplasm of epithelial cells (Figure 4D) and histiocytes of the lamina propria (Figure

4E) of the small and large intestine of all animals; immunoreactivity was more marked in case 4, which demonstrated atrophic enteritis with cryptal dilation and necrosis by histopathology. In cases 1, 2 and 3, all having lymphocytic hepatitis by histopathology, there was intralesional labelling of antigens of OvHV-2 within the cytoplasm of the epithelial cells of bile ducts (Figure 4F). Furthermore, there was positive immunoreactivity for antigens of OvHV-2 within histiocytes (Figure 4G) and lymphocytes of the mesenteric lymph nodes (cases 1, 3 and 5) and in the spleen of case 5. There was no immunoreactivity with antigens of OvHV-2 within the intestinal samples of the cow used as negative control (Figure 4H).

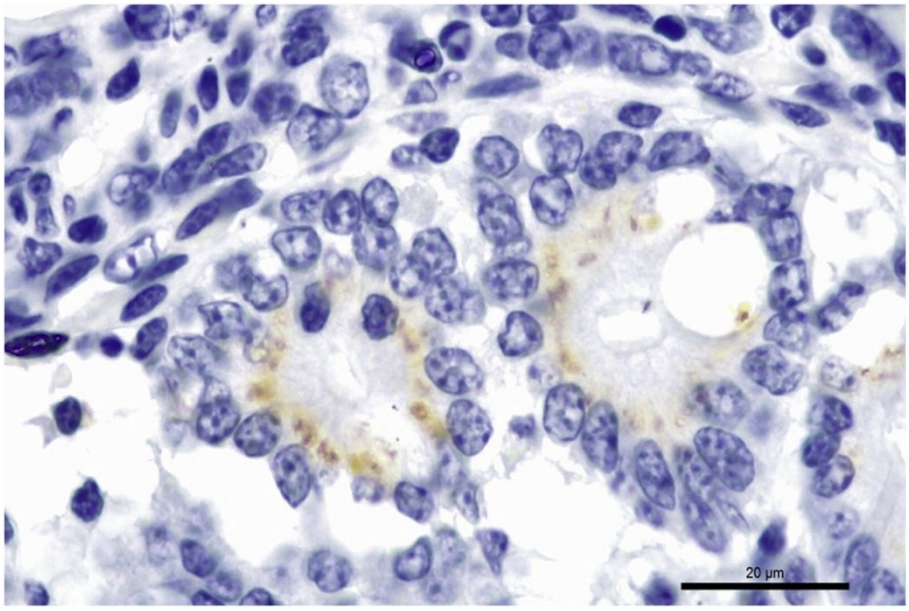
Figure 4 – Immunohistochemical identification of antigens of OvHV-2 in tissues of infected and control cattle with SA-MCF. Absence of immunoreactivity to OvHV-2 in the carotid rete mirabile (A; case 3). Intracytoplasmic immunoreactivity to MAb-15A within epithelial cells of the renal tubule (B) and endothelial cells of a medullary capillary (arrows) (C) of the kidney (case 4). Immunoreactivity within epithelial cells of the dilated intestinal crypts (D; case 4). Intracytoplasmic immunoreactivity within histiocytes (arrows) of the lamina propria of the small intestine (E), in bile duct epithelial cells (F) within the liver of case 1 and predominantly within histiocytes of the mesenteric lymph node (G; case 3). Absence of immunoreactivity to antigens of OvHV-2 within the intestine of the OvHV-2 uninfected cow used as negative control (H). IHC. Bars, A, 100 µm; B-F, 20 µm; G, H, 50 µm.



Moreover, there was positive immunoreactivity to antigens of BVDV within the epithelial cells of the dilated intestinal crypts of the small intestine (Supplementary Figure 1) of case 4, which contained nucleic acids of BVDV-1 by RT-PCR and had positive immunoreactivity for antigens of OvHV-2, and in the lungs of case

1. These findings confirmed concomitant active intestinal infections due to BVDV-1 and OvHV-2.

Supplementary data - Immunohistochemical identification of antigens of BVDV in tissue of cow with sheep-associated malignant catarrhal fever. There is positive immunoreactivity to antigens of BVDV within cryptal epithelial cells of the small intestinal (H; #4). Immunoperoxidase counterstained with haematoxylin. Bar, 20 µm



DISCUSSION

The results of this study have shown that MAb-15A can identify intralesional cytoplasmic antigens of OvHV-2 in multiple tissues of cattle with SA-MCF. Moreover, the amplification of OvHV-2 DNA with the SA-MCF PCR assay (BAXTER *et al.*, 1993) from brain tissues of all animals confirms that these animals were infected by OvHV-2. This is of fundamental importance to demonstrate the sensitivity of this IHC assay, since MAb-15A targets an epitope present in all MCF agents (LI *et al.*, 1994), and is used primarily in serological studies to identify MCF virus antibodies in infected animals (LI *et al.*, 1995; MULLER-DOBLIES *et al.*, 1998; LI *et al.*, 2001). Nevertheless, additional studies using FFPE tissues derived from animals known to contain other MCFVs are necessary to determine whether MAb-15A can identify additional viruses associated with MCF.

There was positive intracytoplasmic immunolabelling for antigens of OvHV-2 within epithelial tissues and leucocytes of the control pigs with elevated viral

loads as demonstrated by qPCR (LI *et al.*, 2012). Similarly, there was positive intracytoplasmic immunoreactivity to antigens of OvHV-2 within epithelial cells of multiple tissues from all OvHV-2-infected animals, as well as within histiocytes and lymphocytes from those cattle in which the mesenteric lymph nodes were collected, preserved, and located during the retrospective analysis. These findings are in accordance with a recent study that amplified nucleic acids of OvHV-2 by ISH within lymphocytes and circulating leucocytes from naturally- and experimentally infected animals (PESAVENTO *et al.*, 2019a), and suggest that MAb-15A detects antigens of OvHV-2 within epithelial cells and leucocytes. Epithelial immunolabelling for OvHV-2 was also demonstrated by IHC in sheep infected experimentally with OvHV-2 using the ORF25-encoded major capsid protein (ORF) of OvHV-2 (TAUS *et al.*, 2010), and in rabbits infected experimentally with OvHV-2 via the detection of ORF43 and ORF63 by ISH (MEIER-TRUMMER *et al.*, 2009). Moreover, MAb-15A demonstrated intracytoplasmic and not intranuclear reactivity for OvHV-2 within all tissues; similar intracytoplasmic labelling by IHC, to detect the ORF25 protein, was observed within perivascular fibroblasts in bison infected experimentally with OvHV-2 (NELSON *et al.*, 2013). Antigens of OvHV-2 within the brain of naturally infected animals and in a control pig with elevated viral load were not identified by IHC using Mab-15-A. Similar non-detection of nucleic acids of OvHV-2 by ISH in the brain of a naturally infected dairy cow that was PCR positive for OvHV-2 was described by PESAVENTO *et al.* (2019a). Furthermore, there were several tissues with elevated viral load, but without the corresponding detection of nucleic acids by ISH (PESAVENTO *et al.*, 2019a), as occurred during our study. The lack of detection of antigens of OvHV-2 in the brains of cattle with acute neurological manifestations by IHC, with nucleic acids of OvHV-2 detected by PCR, may reflect differences in the sensitivity of these assays. Additionally, this inconsistency observed in our study may be associated with the characteristic multifocal distribution of viral DNA in different tissues (PESAVENTO *et al.*, 2019a).

Nevertheless, there is one marked difference in the results obtained in the present study that used IHC to identify antigens of OvHV-2 when compared with the identification of nucleic acids by ISH (PESAVENTO *et al.*, 2019a). Our study demonstrated that MAb-15A detects antigens of OvHV-2 within endothelial cells, but not in the deeper layers of the walls of affected arteries. Alternatively, the ISH detected nucleic acids of OvHV-2 predominantly within the deeper layers of vascular walls, but not within endothelial cells even in animals with known elevated viral loads

(PESAVENTO *et al.*, 2019a); similar ISH detection was observed in sheep with disseminated vasculitis associated with OvHV-2 (PESAVENTO *et al.*, 2019b). These findings may be attributed to possible differences in the virus status (latent versus lytic) within tissue compartments. Consequently, IHC and ISH in combination offer a valuable way to explore this aspect of OvHV-2 infection, its relevance for pathogenesis and virus tropism in SA-MCF and may be fundamental for the differentiation between lytic and latent infections associated with OvHV-2. Additionally, these diagnostic assays would be efficient tools for retrospective studies using archival FFPE tissue blocks.

An intriguing histopathological finding in all animals with acute manifestations of neurological disease, and the absence of the head and eye form of SA-MCF (O'TOOLE & LI, 2014), were proliferating vascular lesions affecting multiple tissues. Similar vascular lesions, but with more severe luminal occlusion of affected arteries at the carotid rete mirabile and in other tissues, were described in cattle (O'TOOLE *et al.*, 1995; O'TOOLE *et al.*, 1997) and bison (SCHULTHEISS *et al.*, 1998) that survived acute manifestations of SA-MCF and had the head and eye manifestations of MCF. However, the histopathological demonstration of angiopathy in acute neurological manifestations of SA-MCF, as described herein, seems directly related to disease progression, since the intensity and severity of these vascular lesions are progressive over time (LIGGITT & DEMARTINI, 1980). Consequently, angiopathy in SA-MCF may be observed in acute presentations of this disease, with or without the eye and head form, and with vascular obliteration being more pronounced in animals that have recovered from MCF.

Case 4 was concomitantly infected by three infectious disease agents, with two of these (OvHV-2 and BVDV-1) demonstrating active intestinal infections (atrophic enteritis) due to the simultaneous amplification of viral nucleic acids by molecular testing and the identification of viral antigens by IHC. Additionally, the large and small intestine of all animals contained antigens of OvHV-2, irrespective of the histopathological evidence of disease. Therefore, one wonders as to the participation of the intestine in the pathogenesis of this viral infection, considering the elevated viral load identified in the intestinal tract of sheep infected with OvHV-2 (PESAVENTO *et al.*, 2019b) and the positive immunoreactivity within leucocytes of the mesenteric lymph nodes shown in the present study. Interestingly, OvHV-2 proteins were detected only within the intestinal epithelia, and more specifically within the intestinal M cells, of rabbits infected experimentally with OvHV-2 (MEIER-TRUMMER *et al.*, 2009). Additionally,

elevated viral loads of OvHV-2 were detected in the mesenteric lymph nodes of several mammals with MCF (PESAVENTO *et al.*, 2019a) as well as in the mesenteric lymph nodes and intestine of sheep with disseminated vasculitis induced by OvHV-2 (PESAVENTO *et al.*, 2019b). Consequently, additional combined studies using IHC, and ISH are necessary to effectively understand the role of the intestine in the development of SA-MCF.

In conclusion, the results from this study demonstrate that MAb-15A detects antigens of OvHV-2 within the cytoplasm of epithelial cells and leucocytes of cattle with SA-MCF and may be an adequate tool to help further understand the pathogenesis of OvHV-2 infection. Moreover, angiopathy in SA-MCF may be a progressive histological manifestation of OvHV-2 infection and may occur irrespective of the eye and head form of MCF.

REFERENCES

ALFIERI, A. A.; PARAZZI, M. E.; TAKIUCHI, E.; MEDICI, K. C.; ALFIERI, A. F. Frequency of group A rotavirus in diarrhoeic calves in Brazilian cattle herds, 1998-2002. **Tropical Animal Health and Production**, v. 38, n. 7-8, p. 521-526, 2006.

ANGEN, O.; AHRENS, P.; TEGTMEIER, C. Development of a PCR test for identification of *Haemophilus somnus* in pure and mixed cultures. **Veterinary Microbiology**, v. 63, n. 1, p. 39-48, 1998.

BARROS, S. S.; SANTOS, M. N.; BARROS, C. S. L. Surto de febre catarral maligna em bovinos no Rio Grande do Sul. **Pesquisa Veterinária Brasileira**, v. 3, n. 3, p. 81-86, 1983.

BAXTER, S. I.; POW, I.; BRIDGEN, A.; REID, H. W. PCR detection of the sheep-associated agent of malignant catarrhal fever. **Archives of Virology**, v. 132, n. 1-2, p. 145-159, 1993.

BOOM, R.; SOL, C. J.; SALIMANS, M. M.; JANSEN, C. L.; WERTHEIM-VAN DILLEN, P. M.; VAN DER NOORDAA, J. Rapid and simple method for purification of nucleic acids. **Journal of Clinical Microbiology**, v. 28, n. 3, p. 495-503, 1990.

CLAUS, M. P.; ALFIERI, A. F.; FOLGUERAS-FLATSCHART, A. V.; WOSIACKI, S. R.; MEDICI, K. C.; ALFIERI, A. A. Rapid detection and differentiation of bovine herpesvirus 1 and 5 glycoprotein C gene in clinical specimens by multiplex-PCR. **Journal of Virological Methods**, v. 128, n. 1-2, p. 183-188, 2005.

COSTA, E. A.; BOMFIM, M. R.; FONSECA, F. G.; DRUMOND, B. P.; COELHO, F. M.; VASCONCELOS, A. C.; FURTINI, R.; PAIXAO, T. A.; TSOLIS, R. M.; SANTOS, R. L.; RESENDE, M. Ovine herpesvirus 2 infection in Foal, Brazil. **Emerging Infectious Diseases**, v. 15, n. 5, p. 844-845, 2009.

COSTA, É. A.; VIOTT, A. D. M.; MACHADO, G. D. S.; BOMFIM, M. R. Q.; COELHO, F. M.; LOBATO, Z. I. P.; RESENDE, M.; GUEDES, R. M. C. Transmission of ovine herpesvirus 2 from asymptomatic boars to sows. **Emerging Infectious Diseases**, v. 16, n. 12, p. 2011-2012, 2010.

DANSKIN, D. Elementary bodies in bovine malignant catarrh. **Nature**, v. 176, n. 4480, p. 518, 1955.

DAVISON, A. J.; EBERLE, R.; EHLERS, B.; HAYWARD, G. S.; MCGEOCH, D. J.; MINSON, A. C.; PELLETT, P. E.; ROIZMAN, B.; STUDDERT, M. J.; THIRY, E. The order Herpesvirales. **Archives of Virology**, v. 154, n. 1, p. 171-177, 2009.

FURLAN, F. H.; AMORIM, T. M.; JUSTO, R. V.; MENDES, E. R. S.; ZILIO, M. G.; COSTA, F. L.; NAKAZATO, L.; COLODE, E. M. Febre catarral maligna em bovinos no norte de Mato Grosso - Brasil. **Acta Scientiae Veterinariae**, v. 40, n. 2, p. 1043, 2012.

GARMATZ, S. L.; IRIGOYEN, L. F.; RECH, R. R.; BROWN, C. C.; ZHANG, J.; BARROS, C. S. L. Malignant catarrhal fever in cattle in Rio Grande do Sul, Brazil: Experimental transmission to cattle and characterization of the etiological agent (in Portuguese). **Pesquisa Veterinária Brasileira**, v. 24, n. 2, p. 93-106, 2004.

HEADLEY, S. A.; LISBÔA, J. A. N.; FRITZEN, J. T. T.; QUEIROZ, G. R.; ALFIERI, A. F.; OLIVEIRA, R. A. M.; BRACARENSE, A. P. F. R. L.; FLAIBAN, K. K. M. C.; ALFIERI, A. A. Ovine herpesvirus type 2-induced malignant catarrhal fever in a heifer. **Semina: Ciências Agrárias**, v. 34, n. 6,S2, p. 3903-3908, 2013.

HEADLEY, S. A.; PIMENTEL, L. A.; OLIVEIRA, V. H.; TOMA, H. S.; ALFIERI, A. F.; CARVALHO, A. M.; DOS SANTOS, M. D.; ALFIERI, A. A. Transplacental transmission of Ovine Herpesvirus 2 in cattle with sheep-associated malignant catarrhal fever. **Journal of Comparative Pathology**, v. 153, n. 4, p. 206-211, 2015.

HEADLEY, S. A.; SOUSA, I. K. F.; MINERVINO, A. H. H.; BARROS, I. O.; BARRÊTO JÚNIOR, R. A.; ALFIERI, A. F.; ORTOLANI, E. L.; ALFIERI, A. A. Molecular confirmation of ovine herpesvirus 2-induced malignant catarrhal fever lesions in cattle from Rio Grande do Norte, Brazil. **Pesquisa Veterinária Brasileira**, v. 32, n. 12, p. 1213-1218, 2012.

HSU, D.; SHIH, L. M.; CASTRO, A. E.; ZEE, Y. C. A diagnostic method to detect alcelaphine herpesvirus-1 of malignant catarrhal fever using the polymerase chain reaction. **Archives of Virology**, v. 114, n. 3-4, p. 259-263, 1990.

LI, H.; BROOKING, A.; CUNHA, C. W.; HIGHLAND, M. A.; O'TOOLE, D.; KNOWLES, D. P.; TAUS, N. S. Experimental induction of malignant catarrhal fever in pigs with ovine herpesvirus 2 by intranasal nebulization. **Veterinary Microbiology**, v. 159, n. 3, p. 485-489, 2012.

LI, H.; CUNHA, C. W.; TAUS, N. S.; KNOWLES, D. P. Malignant catarrhal fever: inching toward understanding. **Annual Review of Animal Biosciences**, v. 2, n. 1, p. 209-233, 2014.

LI, H.; MCGUIRE, T. C.; MULLER-DOBLIES, U. U.; CRAWFORD, T. B. A simpler, more sensitive competitive inhibition enzyme-linked immunosorbent assay for detection of

4023 antibody to malignant catarrhal fever viruses. **Journal of Veterinary Diagnostic**
4024 **Investigation**, v. 13, n. 4, p. 361-364, 2001.

4025 LI, H.; SHEN, D. T.; KNOWLES, D. P.; GORHAM, J. R.; CRAWFORD, T. B. Competitive
4026 inhibition enzyme-linked immunosorbent assay for antibody in sheep and other
4027 ruminants to a conserved epitope of malignant catarrhal fever virus. **Journal of Clinical**
4028 **Microbiology**, v. 32, n. 7, p. 1674-1679, 1994.

4029 LI, H.; SHEN, D. T.; O'TOOLE, D.; KNOWLES, D. P.; GORHAM, J. R.; CRAWFORD,
4030 T. B. Investigation of sheep-associated malignant catarrhal fever virus infection in
4031 ruminants by PCR and competitive inhibition enzyme-linked immunosorbent assay.
4032 **Journal of Clinical Microbiology**, v. 33, n. 8, p. 2048-2053, 1995.

4033 LIGGITT, H. D.; DEMARTINI, J. C. The pathomorphology of malignant catarrhal fever:I.
4034 Generalized lymphoid vasculitis. **Veterinary Pathology**, v. 17, n. 1, p. 58-72, 1980.

4035 MEIER-TRUMMER, C. S.; TOBLER, K.; HILBE, M.; STEWART, J. P.; HART, J.;
4036 CAMPBELL, I.; HAIG, D. M.; GLAUSER, D. L.; EHRENSPERGER, F.; ACKERMANN,
4037 M. Ovine herpesvirus 2 structural proteins in epithelial cells and M-cells of the appendix
4038 in rabbits with malignant catarrhal fever. **Veterinary Microbiology**, v. 137, n. 3, p. 235-
4039 242, 2009.

4040 MENDONÇA, F. S.; DÓRIA, R. G. S.; SCHEIN, F. B.; FREITAS, S. H.; NAKAZATO, L.;
4041 BOABAID, F. M.; PAULA, D. A. J.; DUTRA, V.; COLODEL, E. M. Febre catarral maligna
4042 em bovinos no Estado de Mato Grosso. **Pesquisa Veterinária Brasileira**, v. 28, n. 3,
4043 p. 155-160, 2008.

4044 METTAM, R. W. M. **Snotsiekte in cattle**. Union of South Africa, p.395-432 pp. 1923

4045 MULLER-DOBLIES, U. U.; LI, H.; HAUSER, B.; ADLER, H.; ACKERMANN, M. Field
4046 validation of laboratory tests for clinical diagnosis of sheep-associated malignant
4047 catarrhal fever. **Journal of Clinical Microbiology**, v. 36, n. 10, p. 2970-2972, 1998.

4048 NELSON, D. D.; TAUS, N. S.; SCHNEIDER, D. A.; CUNHA, C. W.; DAVIS, W. C.;
4049 BROWN, W. C.; LI, H.; O'TOOLE, D.; OAKS, J. L. Fibroblasts express OvHV-2 capsid
4050 protein in vasculitis lesions of American bison (*Bison bison*) with experimental sheep-
4051 associated malignant catarrhal fever. **Veterinary Microbiology**, v. 166, n. 3, p. 486-
4052 492, 2013.

4053 O'TOOLE, D.; LI, H. The pathology of malignant catarrhal fever, with an emphasis on
4054 ovine herpesvirus 2. **Veterinary Pathology**, v. 51, n. 2, p. 437-452, 2014.

4055 O'TOOLE, D.; LI, H.; MILLER, D.; WILLIAMS, W. R.; CRAWFORD, T. B. Chronic and
4056 recovered cases of sheep-associated malignant catarrhal fever in cattle. **Veterinary**
4057 **Record**, v. 140, n. 20, p. 519-524, 1997.

4058 O'TOOLE, D.; LI, H.; ROBERTS, S.; ROVNAK, J.; DEMARTINI, J.; CAVENDER, J.;
4059 WILLIAMS, B.; CRAWFORD, T. Chronic generalized obliterative arteriopathy in cattle:
4060 a sequel to sheep-associated malignant catarrhal fever. **Journal of Veterinary**
4061 **Diagnostic Investigation**, v. 7, n. 1, p. 108-121, 1995.

OLIVEIRA, T. E. S.; PELAQUIM, I. F.; FLORES, E. F.; MASSI, R. P.; VALDIVIEZO, M. J. J.; PRETTO-GIORDANO, L. G.; ALFIERI, A. A.; SAUT, J. P. E.; HEADLEY, S. A. *Mycoplasma bovis* and viral agents associated with the development of bovine respiratory disease in adult dairy cows. **Transboundary and Emerging Diseases**, v. doi: 10.1111/tbed.13223., 2019.

PESAVENTO, P. A.; CUNHA, C. W.; LI, H.; JACKSON, K.; O'TOOLE, D. *In situ* hybridization for localization of Ovine herpesvirus 2, the agent of sheep-associated malignant catarrhal fever, in formalin-fixed tissues. **Veterinary Pathology**, v. 56, n. 1, p. 78-86, 2019a.

PESAVENTO, P. A.; DANGE, R. B.; FERRERAS, M. C.; DASJERDI, A.; PEREZ, V.; LAROCA, A.; SILVAN, J. B.; DIAB, S.; JACKSON, K.; PHILLIPS, I. L.; LI, H.; CUNHA, C. W.; WESSELS, M. Systemic necrotizing vasculitis in sheep is associated with Ovine Herpesvirus 2. **Veterinary Pathology**, v. 56, p. 87-92, 2019b.

PLOWRIGHT, W.; FERRIS, R. D.; SCOTT, G. R. Blue Wildebeest and the ætiological agent of bovine malignant catarrhal fever. **Nature**, v. 188, n. 4757, p. 1167-1169, 1960.

QUEIROZ, G. R.; OLIVEIRA, R. A. M. D.; FLAIBAN, K. K. M. C.; SANTIS, G. W. D.; BRACARENSE, A. P. F. R. L.; HEADLEY, S. A.; ALFIERI, A. A.; LISBÔA, J. A. N. Differential diagnosis of neurologic diseases of cattle in the state of Paraná. **Pesquisa Veterinária Brasileira**, v. 38, p. 1264-1277, 2018a.

QUEIROZ, G. R.; PEREIRA, P. F. V.; FLAIBAN, K.; DI SANTIS, G. W.; ALFIERI, A. A.; LISBOA, J. A. N. Cerebrospinal fluid changes in cattle with rabies or with BoHV-5 meningoencephalitis and its correlation with the severity of CNS inflammatory process. **Research in Veterinary Science**, v. 118, p. 389-394, 2018b.

RUSSELL, G. C.; STEWART, J. P.; HAIG, D. M. Malignant catarrhal fever: A review. **Veterinary Journal**, v. 179, n. 3, p. 324-335, 2009.

SCHULTHEISS, P. C.; COLLINS, J. K.; AUSTGEN, L. E.; DEMARTINI, J. C. Malignant catarrhal fever in bison, acute and chronic cases. **Journal of Veterinary Diagnostic Investigation**, v. 10, n. 3, p. 255-262, 1998.

TAUS, N. S.; SCHNEIDER, D. A.; OAKS, J. L.; YAN, H.; GAILBREATH, K. L.; KNOWLES, D. P.; LI, H. Sheep (*Ovis aries*) airway epithelial cells support ovine herpesvirus 2 lytic replication in vivo. **Veterinary Microbiology**, v. 145, n. 1, p. 47-53, 2010.

VANDEVANTER, D. R.; WARRENER, P.; BENNETT, L.; SCHULTZ, E. R.; COULTER, S.; GARBER, R. L.; ROSE, T. M. Detection and analysis of diverse herpesviral species by consensus primer PCR. **Journal of Clinical Microbiology**, v. 34, n. 7, p. 1666-1671, 1996.

VILČEK, Š.; HERRING, A. J.; HERRING, J. A.; NETTLETON, P. F.; LOWINGS, J. P.; PATON, D. J. Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis. **Archives of Virology**, v. 136, n. 3, p. 309-323, 1994.

4102 WIYONO, A.; BAXTER, S. I.; SAEPULLOH, M.; DAMAYANTI, R.; DANIELS, P.; REID,
4103 H. W. PCR detection of ovine herpesvirus-2 DNA in Indonesian ruminants-normal sheep
4104 and clinical cases of malignant catarrhal fever. **Veterinary Microbiology**, v. 42, n. 1, p.
4105 45-52, 1994.

10.2 PROTOCOLO IMUNO-HISTOQUÍMICO

REAGENTE/SOLUÇÃO	EQUIPAMENTO	T°C	TEMPO
1. Desparafinização			
	Estufa	60°C	12 h
Xilol I	Berço da IHQ		20 min
Xilol II			20 min
2. Reidratação			
Álcool 100% I			10 min
Álcool 100% II			10 min
Álcool 90%			5 min
Álcool 80%			5 min
Ligar a panela de pressão			
Álcool 70%			5 min
Lavar em H_20 corrente	Fraca		10 min
Água destilada	Mergulho, transporte		
3. Recuperação Antigênica			
Citrato pH 6,0, cuba azul	Panela de pressão	86-90°C	3 min
Perda da pressão da panela			20 min
Retirar da panela com auxílio de luva de cozinha			
Estabilização da °T do material em °T ambiente			20 min
Lavar em H_20 corrente	Fraca		10 min
Água destilada	Mergulho, transporte, cuba de vidro		
4. Bloqueio da Peroxidase Endógena			
Metanol (110ml) + H_20 oxigenada (40 ml)		°T amb	30 min
Trabalhar em cuba e berço de vidro. Proteger da luz (utilizar pano do micrótomo			
Descartar solução de metanol em descarte de álcool			
Lavar em H_20 corrente	Fraca		10 min
Água destilada	Mergulho, transporte		
Quando necessário, bloqueio com Leite Mollico®			
10g de leite + 200ml de H_20 dest	Cuba azul	°T amb	10 min
Enxaguar em H_20 destilada			2x
5. Incubação com Anticorpo Primário			
Secar ao redor dos cortes com papel toalha			
Ac Primário diluído em diluente	Câmara úmida	4°C	22h
PBS pH7,2			5 min
PBS pH7,2			5 min
6. Incubação com Anticorpo Secundário			
Secar ao redor dos cortes com papel toalha			
Ac Secundário diluído	Câmara úmida	°T amb	30 min
PBS pH7,2			5 min
PBS pH7,2			5 min
7. Solução Cromógena (CANCERÍGENA!) Use 2 luvas! Forre com papel			
Secar ao redor dos cortes com papel toalha			
DAB	Capela ligada	°T amb	3 min
Parar a reação em H_20 corrente	Cuba de vidro + berço do HE	°T amb	-
Descartar em local próprio – CUIDADO CANCERÍGENO, em caso de contaminação LAVAR COM H_20 SANITÁRIA. Após o uso da cuba deixar de molho 12h com 50mL de H_20 SANITÁRIA + 400 mL de H_20 torneal			
Lavar em H_20 corrente	Fraca		10 min
Água destilada	Mergulho, transporte		
8. Coloração e desidratação			
Hematoxilina de Harris	Tempo relativo		35 seg

Descartar a 1ª H_20, em descarte de corante. TROCAR O BERÇO PARA O DA IHQ.			
Álcool 70%			5 min
Álcool 80%			5 min
Álcool 90%			5 min
Álcool 100% I			10 min
Álcool 100% II			10 min
Deixar secar as lâminas na capela para não hidratar o xilol			
Xilol I			20 min
Xilol II			20 min
Montar com lamínula (tamanho adequado + resina)			

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10.3 SOLUÇÕES IMUNO-HISTOQUÍMICA

Tampão Fosfato Salino (PBS) pH7,2

$Na_2HPO_4.7H_20$ 3,96g

$NaH_2PO_4.H_20$ 0,72g

NaCl 16,34g

H_2O q.s.p. 2000mL

Ajustar o pH para 7,20 - 7,24

Calibração do pH

Lavar eletrodo com H_20 destilada e secar com cuidado (papel folha dupla)

Ligar o aparelho na tomada **110v** (on)

Introduzir eletrodo no tampão pH 4,0

Aperte a tecla **CAL**. Deixe estabilizar até aparecer uma letra **A com traço no canto inferior direito do aparelho.**

Lavar o eletrodo e secar com cuidado (papel folha dupla)

Introduzir eletrodo no tampão pH 7,0

Aperte a tecla **CAL**. Deixe estabilizar até aparecer uma letra **A com traço no canto inferior direito do aparelho.**

Para ler as amostras, deixe organizadas de menor para maior pH.

Introduza eletrodo no material desejado para calibração.

Aperte tecla **READ**.

Lavar o eletrodo e secar com cuidado (papel folha dupla) e colocar no local apropriado.

Solução bloqueio da Biotina endógena

Solução de leite em pó a 5% - usar cuba azul

Leite em pó 10g

Solução de lavagem q.s.p. 200mL

Solução de Bloqueio da Peroxidase endógena

Solução 36% de H_2O_2 em metanol, para bloqueio da peroxidase endógena.

H_2O_2 (20 vol) 40 mL

Metanol 110 mL

Utilizar cuba e berço de vidro.

Solução de Revelação

H_2O destilada	1 mL
Solução 1	1 gota
Solução 2	1 gota
Solução 3	1 gota

Essa solução é **estável por 30 min**. Solução **CANCERÍGENA**. Tomar muito cuidado para não contaminar a sala com a luva, solução em água e frascos utilizados.