UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE VETERINÁRIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

CAUSAS INFECCIOSAS DE ABORTAMENTOS EM RUMINANTES NO ESTADO DO RIO GRANDE DO SUL

LUAN CLEBER HENKER

PORTO ALEGRE

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Orientador: Prof. Dr. Saulo Petinatti Pavarini

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Membro da Comissão

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Membro da Comissão

Dedico este trabalho a minha querida mãe, Ivanelde Henker, que sempre será meu maior exemplo e minha maior incentivadora.

RESUMO

Abortamentos acarretam grandes perdas econômicas em sistemas de produção animal em todo o mundo. A investigação patológica das causas de abortamento em animais de fazenda faz-se crucial para a melhor compreensão das enfermidades reprodutivas e visa a redução de perdas econômicas e a mitigação do risco atrelado a disseminação de doenças zoonóticas. Este estudo busca caracterizar determinadas doenças infeciosas que cursam com abortamentos em ruminantes a partir de dados obtidos através da avaliação anatomopatológica de fetos e membranas fetais remetidos ao Setor de Patologia Veterinária da Universidade Federal do Rio Grande do Sul (SPV-UFRGS). A tese é composta por quatro artigos científicos e um material técnico ilustrado voltado a avaliação anatomopatológica em casos de abortamentos. O primeiro artigo tem por objetivo caracterizar os achados patológicos e etiológicos de casos de perdas fetais e perinatais associadas a infecção transplacentária por agentes do complexo Tristeza Parasitária Bovina (TPB) (*Anaplasma marginale* e *Babesia bovis*). Seis bezerros (dois fetos abortados em terço gestacional final, dois natimortos e dois neonatos) foram remetidos para a avaliação patológica. Os achados de necropsia foram consistentes com TPB e incluíram hepatoesplenomegalia, icterícia, bile grumosa, rins escuros e encéfalo cor de cereja. Através de ferramentas moleculares, detectou-se a infecção exclusivamente por *Babesia* sp. em três casos, por *Anaplasma* sp. em um caso, além de coinfecções por *Babesia* sp e *Anaplasma* sp. em dois casos. No segundo artigo realizou-se um estudo retrospectivo no arquivo do SPV-UFRGS (2004–2019) dos casos de abortamento bovino causados por agentes bacterianos e fúngicos esporádicos/oportunistas. Dezenove casos de abortamento bacteriano e cinco casos de abortamento fúngico foram avaliados. Alterações macroscópicas foram incomuns em casos de abortamento de origem bacteriana e dois padrões microscópicos foram observados: 1) broncopneumonia primária com ocasional disseminação em casos de infecção por *Staphylococcus* sp., *Streptococcus* sp., e *Mannheimia haemolytica*; 2) doença sistêmica com septicemia em casos de infecções por *Escherichia coli* e *Listeria* sp. *Aspergillus* sp. foi o principal agente fúngico identificado, e casos de aborto micótico apresentaram placentite, dermatite e pneumonia. O terceiro artigo traz os achados patológicos e microbiológicos de um caso de abortamento bovino devido a infecção por uma cepa de *Staphylococcus aureus* de alta virulência. O quarto artigo relata os achados epidemiológicos, sorológicos, moleculares e patológicos de um surto de abortamentos em um rebanho ovino no estado do Rio Grande do Sul devido a infecção por *Toxoplasma gondii* (genótipo clonal tipo III). Por fim, o material técnico ilustrado produzido busca compilar informações referentes a avaliação *post mortem* em fetos de ruminantes e equinos. Tópicos abordados nesse material incluem especificidades da técnica de necropsia, método de coleta de amostras, lesões macroscópicas, não lesões, diferenciação de morte fetal intrauterina e extrauterina, achados *post mortem*, particularidades microscópicas e artefatos. Este material tem por objetivo servir como uma ferramenta adicional de consulta para estudantes, médicos veterinários e patologistas veterinários que trabalham no diagnóstico de casos de abortamento.

Palavras-chave: Doenças reprodutivas. Abortamentos. Ruminantes. Hemoparasitas. *Toxoplasma gondii*.

ABSTRACT

Abortions lead to significant economic losses in livestock systems worldwide. Pathological investigations on causes of abortion in farm animals are crucial to improve the understanding about reproductive diseases, aiming to reduce economic losses and mitigate the risk of dissemination of zoonotic diseases. The aim of this study is to characterize certain aspects of infectious diseases that lead to abortion in ruminants with data obtained through the *postmortem* examinations of fetuses and fetal membranes referred for analysis at Setor de Patologia Veterinária, Universidade Federal do Rio Grande do Sul (SPV-UFRGS). This dissertation comprises four scientific articles and an illustrated technical material focused on the anatomopathological examination in cases of abortion. In the first article we aimed to characterize the pathological and etiological findings of cases of fetal loss and perinatal death due to transplacental infection by tick fever agents (*Anaplasma marginale* e *Babesia bovis*). Six calves (two third-trimester aborted fetuses, two stillborn fetuses, and two calves that died in the neonatal period) were submitted for pathological examination. Necropsy findings were consistent with tick fever and included hepatosplenomegaly, icterus, grumous bile, dark kidneys, and "cherry-pink" discoloration of the encephalon. Through molecular assays, it was possible to detect three cases of *Babesia* sp. infection alone, and one case of *Anaplasma* sp. infection alone. Co-infections with *Anaplasma* sp. and *Babesia* sp. were detected in two cases. For the second manuscript, we conducted a retrospective study compiling cases of bovine abortion due to sporadic/opportunistic fungal and bacterial agents diagnosed at SPV-UFRGS (2004 – 2019). Nineteen cases of bacterial etiology and five cases of fungal etiology were assessed. In cases of bacterial etiology, gross changes were uncommon and two different microscopic patterns were observed: 1) primary bronchopneumonia with occasional dissemination in cases of *Staphylococcus* sp., *Streptococcus* sp., and *Mannheimia haemolytica* infections; and 2) systemic disease with sepsis in cases of *Escherichia coli* and *Listeria* sp. infections. *Aspergillus* sp. was the main fungal agent identified, and cases of mycotic abortion were characterized by placentitis, dermatitis, and pneumonia. In the third manuscript, we report the pathological and microbiological findings of a case of bovine abortion due to the infection by a highly virulent *Staphylococcus aureus* strain. In the fourth manuscript, we report the epidemiological, serological, molecular and pathological findings of an abortion outbreak in a sheep flock in the State of Rio Grande do Sul due to infection by *Toxoplasma gondii* (clonal Type III). Finally, we produced an illustrated technical material aiming to compile information on the postmortem examination of fetuses of ruminants and horses. Topics covered in this material include necropsy technique specificities, sampling method, gross lesions, nonlesions, findings to differentiate intrauterine and extrauterine fetal death, postmortem changes, normal microscopic findings, and artifacts. This technical material aims to provide an additional tool for students, veterinarians and veterinary pathologists working with the diagnosis of abortion cases.

Keywords: Reproductive diseases. Abortion. Ruminants. Hemoparasites. *Toxoplasma gondii*.

SUMÁRIO

1 INTRODUÇÃO

Perdas reprodutivas representam importantes limitantes econômicos em sistemas de produção animal em todo o mundo (JONKER, 2004; CABELL, 2007). Eventos de reabsorção embrionária, abortamentos, natimortalidade e morte perinatal estão diretamente associados à baixos índices de produtividade e rentabilidade em sistemas de criação de bovinos de corte, bovinos de leite, pequenos ruminantes, suínos e equinos (JONKER, 2004; DE VRIES, 2006; REESE et al., 2020).

Abortamentos levam a perdas econômicas decorrentes do aumento do intervalo entre partos, diminuição do número de dias em lactação/número de lactações (bovinos de leite) (DE VRIES, 2006), redução do número de bezerros desmamados ao ano (bovinos de corte) (REESE et al., 2020), elevação das taxas anuais de descarte e reposição, além da elevação dos custos de produção decorrentes de gastos com alimentação, insumos, medicamentos, assistência técnica, entre outros (DE VRIES, 2006). O estabelecimento do valor monetário atrelado a uma morte fetal individual é de difícil estimação, e variáveis do cálculo incluem o estágio de gestação, o nível de produção individual, a composição genética e valor comercial dos animais, o tipo de sistema de produção e o nível de tecnificação da propriedade (DE VRIES, 2006). Apesar da difícil determinação desses custos, um estudo anterior indica que as perdas decorrentes de um único abortamento alcançam valores médios de U$ 555 em propriedades leiteiras com sistemas intensivos na América Do Norte (DE VRIES, 2006).

Além do relevante impacto econômico decorrente das perdas fetais, inúmeros agentes infecciosos relacionados a desordens reprodutivas em ruminantes possuem caráter zoonótico, e desta maneira, apresentam relevância a nível de saúde pública (JONKER, 2004). Entre estes agentes tem-se a infecção por *Brucella* abortus, *Leptospira* spp., *Coxiella burnetii*, entre outros (JONKER, 2004; CLOTHIER e ANDERSON, 2012; ANTONIASSI et al., 2016). Desta forma, a investigação patológica das causas de abortamento em animais de produção visa embasar a elaboração de estratégias com foco na redução de perdas econômicas bem como o monitoramento e a mitigação do risco atrelado a disseminação de doenças potencialmente zoonóticas (JONKER, 2004).

A determinação das causas de abortamento em animais domésticos é uma tarefa desafiadora e laboriosa (NJAA 2012). A avaliação anatomopatológica de fetos e membranas fetais é uma importante ferramenta empregada no diagnóstico de doenças reprodutivas, entretanto, esta apresenta inúmeras dificuldades intrínsecas o que leva a baixas taxas de

diagnóstico (SCHLAFER e FOSTER, 2016; NJAA, 2012). Por exemplo, estudos retrospectivos que avaliam causas de abortamentos em bovinos indicam taxas diagnósticas que variam de 30-55% (ANTONIASSI, 2012; CLOTHIER e ANDERSON, 2016; MACÍAS-RIOSECO et al., 2020; WOLF-JÄCKEL et al., 2020).

Entre as dificuldades para o estabelecimento do diagnóstico tem-se a grande variedade de agentes não infecciosos e infecciosos potencialmente envolvidos em casos de abortamentos (NJAA, 2012). Entre os agentes não infecciosos, citam-se fatores individuais, doenças sistêmicas que afetam a matriz, eventos estressores, além de doenças nutricionais, tóxicas, genéticas e metabólicas, estas que majoritariamente não cursam com lesões significativas ou específicas nos tecidos fetais e placenta (SCHLAFER e FOSTER, 2016; NJAA, 2012). Dentre as causas infecciosas citam-se agentes primários e oportunistas, incluindo uma vasta gama de bactérias, fungos, protozoários e vírus (CABELL, 2007; NJAA, 2012; SCHLAFER e FOSTER, 2016).

Outros fatores limitantes ao diagnóstico das causas de abortamentos incluem o elevado grau de autólise, frequentemente observado em fetos remetidos para a avaliação laboratorial, aliado ao fato de que determinados agentes infecciosos com frequência não encontram-se associados ao desenvolvimento de alterações morfológicas apreciáveis (NJAA, 2012; SCHLAFER e FOSTER, 2016). Ainda, a investigação das causas de abortamento constitui uma tarefa laboriosa e dispendiosa, visto que o procedimento de necropsia aliado a histopatologia, embora fundamentais, com frequência não são suficientes para o estabelecimento do diagnóstico definitivo, desta forma, a realização de exames complementares faz-se essencial (NJAA, 2012; SCHLAFER e FOSTER, 2016). Portanto, estudos com o foco em expandir a compreensão a respeito das causas de abortamentos, dos achados anatomopatológicos em tecidos e membranas fetais e das técnicas diagnósticas complementares rotineiramente empregadas são imprescindíveis para a melhoria das taxas de diagnóstico laboratorial.

No Brasil, estudos realizados a partir dos anos 1990 e 2000, por laboratórios de patologia veterinária, com destaque para o Setor de Patologia Veterinária da Universidade Federal do Rio Grande do Sul (SPV-UFRGS), visaram estabelecer um panorama geral de algumas das principais causas de abortamentos em ruminantes, equinos e suínos (CORBELLINI et al., 2002; PESCADOR, 2005; CORBELLINI et al., 2006; PESCADOR et al., 2007a; PESCARDOR et al., 2007b; PESCADOR, 2008; ANTONIASSI, 2012; MARCOLONGO-PEREIRA et al., 2012; JUFFO, 2016; ANTONIASSI et al., 2016; DA

COSTA, 2020; WITHOEFT, 2021). Embora estes trabalhos representem peças fundamentais para a compreensão da ocorrência de doenças associadas a abortamentos em território nacional, principalmente na região sul do Brasil, o conhecimento científico sobre inúmeras doenças/causas de abortamento ainda é limitado a nível nacional.

A observação da rotina diagnóstica do SPV-UFRGS permite determinar lacunas de conhecimento no campo de estudo de causas de abortamento em animais de fazenda, favorecendo a realização de estudos adicionais, o que leva a expansão do corpo de conhecimentos sobre o tópico. Esses estudos podem ser úteis para médicos veterinários que acompanham sistemas de produção animal, bem como para pós-graduandos e patologistas veterinários que trabalham na rotina anatomopatológica e avaliam casos de abortamentos.

Entre as lacunas mencionadas, citam-se os casos de morte fetal e neonatal por *Anaplasma marginale* e *Babesia bovis*. Estes agentes pertencem ao complexo Tristeza Parasitária Bovina (TPB), que constitui uma das principais causas de doença e mortalidade em bovinos da região sul do país (ALMEIDA et al., 2006; LUCENA et al., 2010; MELLO et al., 2017). Entretanto, descrições de doenças reprodutivas, como abortamentos, natimortalidade e morte neonatal associadas a estes agentes, em especial a protozoários do gênero *Babesia*, são extremamente escassas na literatura internacional (CORREA, 1978; TRUEMAN e MCLENNAN, 1987; COSTA et al., 2016).

Outra lacuna de conhecimento observada refere-se à caracterização da infecção por micro-organismos considerados oportunistas. Agentes bacterianos e fúngicos esporádicos, apesar de não representarem etiologias primárias de doença reprodutiva, são listados como causas relativamente frequentes de abortamentos em inúmeras espécies domésticas (KIRKBRID, 1993; MARCOLONGO-PEREIRA et al., 2012) Entretanto, na espécie bovina esses agentes são pouco discutidos na maior parte dos estudos, devido a sua menor importância enquanto doença reprodutiva primária ou como causadores de surtos de abortamentos (CLOTHIER e ANDERSON, 2012). A avaliação da literatura disponível sobre o tópico permite observar que estudos focados na caracterização patológica de episódios de abortamento bovino associados a estes agentes são escassos (KIRKBRID, 1993).

A infecção por *Toxoplasma gondii* é uma das principais causas de abortamentos em pequenos ruminantes em diversos países (DUBEY, 2009). No Brasil, surtos de abortamentos decorrentes da infecção por este protozoário são relatados principalmente em caprinos (PESCADOR et al., 2007b), e menos frequentemente em ovinos (WITHOEFT, 2021). Entretanto, descrições dos aspectos moleculares observados em surtos espontâneos de

abortamentos em ovinos são escassos no Brasil. Uma vez que a infecção por *T. gondii* é extremamente prevalente em humanos e animais no Brasil (DUBEY et al., 2012), e que isolados de *T. gondii* identificados na América do Sul apresentam alta variabilidade genética (DARDÉ et al., 2014), destaca-se a importância da expansão do conhecimento sobre os achados moleculares de *T. gondii* em surtos de abortamento ovino no Brasil.

Por fim, a avaliação anatomopatológica de fetos e membranas fetais é repleta de particularidades, o que pode ser desafiador para veterinários, pós-graduandos e patologistas veterinários. Ao decorrer do desenvolvimento da tese detectou-se uma pronunciada escassez de bibliografia disponível a respeito de particularidades da avaliação anatomopatológica e do processo diagnóstico em casos de abortamentos em animais de fazenda. Embora algumas iniciativas importantes estejam disponíveis, como por exemplo, um artigo com foco no processo diagnóstico em casos de abortamento suíno (PESCADOR et al., 2010), a literatura científica sobre o tema em língua portuguesa é bastante escassa e fragmentada, especialmente tratando-se de ruminantes e equinos.

Desta maneira, esta tese tem por objetivo principal compilar e documentar aspectos patológicos e etiológicos pouco descritos de causas infecciosas de abortamentos em bovinos e ovinos diagnosticadas no estado do Rio Grande do Sul. Além disso, um objetivo adicional deste trabalho consiste na elaboração de um material técnico ilustrado focado em particularidades da avaliação anatomopatológica e do processo diagnóstico em casos de abortamentos em ruminantes e equinos.

2 ARTIGO 1

Neste item é apresentado o artigo intitulado "**Bovine abortion, stillbirth and neonatal death associated with *Babesia bovis* and *Anaplasma* sp. infections in southern Brazil**". Este artigo encontra-se publicado no periódico científico "*Ticks and Tick-borne Diseases*" com endereço de DOI: https://doi.org/10.1016/j.ttbdis.2020.101443.

Bovine abortion, stillbirth and neonatal death associated with *Babesia bovis* and *Anaplasma* sp. infections in southern Brazil

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Abstract

Anaplasmosis and Babesiosis are tick-borne diseases widely disseminated in cattle herds in many parts of the world. These diseases represent important causes of death and economic losses in several countries, including Brazil, and are characterized by hemolytic disease and anemia. Animals of all ages may be affected. Although transplacental infections are known to occur, abortion, stillbirth and neonatal death directly associated with *Anaplasma* marginale and especially *Babesia* spp. infections have rarely been documented in cattle. The objective of the present study is to describe the pathological and molecular findings of two cases of bovine abortion, two cases of stillbirth and two cases of neonatal death associated with intrauterine anaplasmosis and/or babesiosis in Southern Brazil. All cases occurred in beef farms in the state of Rio Grande do Sul, between 2017 and 2019. Angus and crossbred calves were affected. At the necropsy, the main gross lesions observed included different degrees of splenomegaly, enlarged and yellow liver, thick and grumous bile, pallor or jaundice of mucous membranes and carcass, and dark kidneys. Four calves also presented cherry-pink discoloration of the central nervous system. Cytological slides enabled the observation of intraerythrocytic organisms consistent with *Babesia bovis* (3/6) and *A. marginale* (2/6). Through PCR assays, it was possible to detect three cases of *Babesia* sp. infection alone, and

one case of *Anaplasma* sp. infection alone. Co-infections with *Anaplasma* sp. and *Babesia* sp. were detected in two cases. These findings reaffirm that anaplasmosis and babesiosis should be considered as an important differential diagnosis of fetal loss, stillbirth and neonatal death in cattle in areas where these diseases occur.

Key-words: cattle; hemoparasites; fetal loss; Anaplasmosis; Babesiosis.

Introduction

Anaplasmosis and babesiosis, which may be referred to conjunctly as "tick fever", represent important causes of disease, death and major economic losses in cattle in many countries in different parts of the world (Mcleod and Kristjanson, 1999). Anaplasmosis in cattle is associated with infection by the intraerythrocytic bacterium of the family Anaplasmataceae, *Anaplasma marginale* (Aubry and Geale, 2011). Bovine babesiosis is generally caused by *Babesia bigemina* or *Babesia bovis*, which are apicomplexan protozoans (Bock et al., 2014). These conditions are associated with mild to severe febrile hemolytic disease in cattle, and many times present overlapping clinical signs and lesions (Valli et al., 2016). Both anaplasmosis and babesiosis are tick-borne diseases (Aubry and Geale, 2011; Bock et al., 2014). *B. bovis* and *B. bigemina* have *Rhipicephalus (Boophilus) microplus* as the main biological vector (Bock et al., 2014), while *A. marginale* may be transmitted by *R. (B.) microplus* as well as several other tick species, which act as biological vectors, and mechanically by biting flies and blood-contaminated fomites (Kocan et al., 2010).

The occurrence and severity of the referred diseases are related to many factors, including seasonal or artificially induced fluctuations in the tick population and consequently, the immune status of the affected cattle (Mahoney and Ross, 1972; Bock et al., 2014). In Brazil, bovine anaplasmosis and babesiosis represent leading causes of death, especially in areas of enzootic instability, including the state of Rio Grande do Sul (Almeida et al., 2006; Lucena et al., 2010; Mello et al., 2017). These diseases are associated with anemia, and may be directly related to neurological disease, in the case of *B. bovis* infection (Aubry and Geale, 2011; Bock et al., 2014). Even though transplacental transmission of *A. marginale* and *Babesia* spp. have been described (Grau et al., 2013; Costa et al., 2016), cases of fetal or neonatal death associated with direct damage induced by such agents have rarely been reported (Correa et al., 1978; Trueman and Mclennan, 1987). Therefore, the objective of this

work is to describe the pathological and molecular findings of six cases of fetal and neonatal death associated with transplacental babesiosis and anaplasmosis in southern Brazil.

Material and methods

From November 2017 to July 2019, two aborted fetuses, two stillborn, and two newborn calves, which died within 24 hours after birth, were referred for necropsy at the Veterinary Pathology Department of the Universidade Federal do Rio Grande do Sul (UFRGS), presenting gross lesions consistent with anaplasmosis, babesiosis or the association of both. These cases occurred in farms from different cities in the state of Rio Grande do Sul, southern Brazil.

Epidemiological information regarding affected animals, and farm abortion status were obtained from the referring veterinarians and farm owners. Necropsy procedures were routinely conducted, gross lesions were recorded, and fetal age was estimated through the measurement of crown-rump length (Barr et al., 1990) along with the information provided by the farm owner. Tissue scraping slides of brain, spleen, and smears of blood when available, were prepared and routinely stained with PanóticoRápido®, (Laborclin, Brazil), to search hemoparasites. Tissue samples of brain, spinal cord, skeletal muscle, tongue, bone marrow, skin, eyelid, placenta when available, and the main organs of the thoracic and abdominal cavities were collected, fixed in 10% buffered formalin, and routinely processed. Tissue fragments were trimmed, dehydrated in a sequence of alcohol containers of increasing concentration, and cleared with xylene solution. Cleared tissue fragments were embedded in paraffin wax. Sections (3 µm thick) were prepared, placed in glass slides and stained by hematoxylin and eosin. Histological slides were evaluated under light microscopy.

In all cases, tissue samples of lung and heart were submitted for immunohistochemistry (IHC) anti-*Brucella abortus*, using polyclonal antibody as previously described (Antoniassi et al., 2016). In addition, IHC anti-*Neospora caninum* using polyclonal antibody (VMRD, Pullman, WA), was performed in tissue sections of brain, skeletal muscle, and heart according to previous description (Pescador et al., 2007). Slides from known cases of *B. abortus* and *N. caninum* infections were used as positive controls. Primary antibodies were replaced with PBS in the negative control sections.

Fresh samples of several organs were collected aiming to conduct further molecular and microbiological testing. To rule out the involvement of a bacterial etiology, lung

fragments and abomasal fluid were inoculated on 5% sheep blood agar and MacConkey agar, and incubated at 37°C for 72 h in aerobic atmosphere. Total DNA was extracted from fresh frozen samples using QIAamp®DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. PCR assays were conducted to search hemoparasites, pestivirus (BVDV), and *Leptospira* spp., as shown in Table 1. Tissues tested for hemoparasites included spleen and CNS, except for calves 3 and 6, in which CNS and liver were tested (Table 2).

The PCR products were submitted to electrophoresis through a 1.5% agarose gel and examined by a UV transilluminator. Amplicons of the expected size were purified with the PureLink kit (Invitrogen®), following manufacturer's instructions, and sequenced in an automatic sequencer (Sanger) according to the manufacturer's protocol. Generated sequences were submitted to BLAST® analysis (Altschul et al., 1990) to determine the closest similarities in GenBank®. Partial sequences of the 18S rRNA gene of *Babesia* were aligned with corresponding 18S rRNA sequences of six *Babesia* species retrieved from GenBank®, using Clustal/W v.1.8.1 (Thompson et al., 1994). An identity matrix was calculated with the BioEdit software using sequences of *Babesia* spp. and *B. bovis* deposited in GenBank® and the ones found in this study to evaluate the similarity.

Results

Epidemiological findings

All cases compiled were referred from cities in the State of Rio Grande do Sul, southern Brazil. One case (calf 1) was referred in November 2017, calves 2-5 were submitted from August to October 2018, and calf 6 was referred in July 2019. All animals were referred from extensive beef farms of different sizes, ranging from 145 to 1500 animals in total. In all referring farms, vaccinations against *B. abortus*, using B19 vaccine strain, were routinely conducted in female calves with age ranging from three to eight months. This vaccination practice used to be performed according to the guidelines and requirements established by the National Program for the Control and Eradication of Animal Brucellosis and Tuberculosis conducted by the Ministry of Agriculture, Livestock and Food Supply (MAPA, 2006).

Farms of calves 1, 2, 4, and 6 informed conducting annual vaccinations against the main reproductive pathogens; farms of calves 3 and 5 reported no vaccination against reproductive agents. Regarding reproductive management practices, farm of calf 1 used only

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fixed-time artificial insemination (FTAI); farms of calves 2, 4, and 6 performed FTAI, and bulls were used to breed empty cows; farms from calves 3 and 5 used bulls only.

The corresponding farms of calves 1, 3, 4, 5, and 6 reported increased abortion rates in the comprehended calving season, which reached up to 7.8% of fetal loss (farm of calf 1). Farm owners reported that pregnant heifers were more affected than multiparous cows. Calves were purebred Aberdeen Angus (calves 1, 2, and 6) or crossbred (calves 3, 4, and 5) (Table 2). All dams were Angus, and even though anaplasmosis and babesiosis were reported to occur in these farms, none of the aborting dams was reported to present clinical signs of these conditions at the time of parturition or abortion. Four fetuses were male, and two were female.

Two calves submitted for necropsy were third trimester mildly autolyzed aborted fetuses (calf 2 and 6). Calf 2 crown-rump length was consistent with 8 months of gestation, while calf 6 measurement was compatible with 7 months of gestation. Calves 1 and 4 were stillborn full-term calves. Both of which resulted from difficult deliveries, which required veterinary assistance, including fetotomy procedure in calf 4. Calves 3 and 5 were born alive; however, both animals were weak, prostrated, unable to stand and nurse, and died within 24 hours after birth. Calf 5 was premature (compatible with 7 months of gestation) and significantly small, measuring 60 cm of crown-rump length, while calf three was a full-term calf. Information regarding the studied calves is shown in Table 2.

Gross lesions

The gross lesions observed during necropsy are depicted in Table 3, along with the level of severity. The main macroscopic findings included different degrees of liver enlargement in all cases (Figure 1.A-B). The liver of all calves presented yellow to orange discoloration, and in all cases the gallbladder was moderately to severely distended by dark orange, grumous, and thick bile (Figure 1.C). Dark discoloration of the kidneys was present in three cases (calves 1, 5, and 6) (Figure 1.D). Splenomegaly was consistently observed in all animals (Figure 1.E). Multiple hemorrhages were noted in several organs in calves 1 and 5. Changes in the coloration of mucous membranes and carcass were consistently observed in all calves, and included pallor (calves 4 and 5), mild to moderate jaundice (calves 2 and 3), and congestion (calves 1 and 6). In addition, calves 1, 2, 5, and 6 presented moderate to marked cherry-pink discoloration of the central nervous system (CNS), affecting mainly the gray

matter of the encephalon (Figure 1.F) and spinal cord. No significant lesions were observed in the remaining organs inspected.

Microscopic findings

At the cytological evaluation of slides prepared during necropsy, intraerythrocytic structures compatible with *B. bovis* were detected in three cases (calves 1, 5 and 6), and compatible with *A. marginale* in two cases (calves 3 and 4) (Table 2). Calf 2 was shipped to the laboratory frozen; therefore, freezing artifacts prevented adequate search for hemoparasites. In calves 1, 5, and 6, tissue smears of brain enabled the observation of capillaries filled with erythrocytes, which frequently were parasitized by *Babesia*-like organisms, consistent with merozoites, a typical pathological finding in cases of cerebral babesiosis (Figure 2.A). Smears of brain, spleen (calf 3), and blood (calf 4) enabled the observation of numerous round basophilic intraerythrocytic peripheral pinpoint structures, which measured around 1 µm in diameter, and were consistent with *A. marginale* (Figure 2.B).

Histologically, all calves presented moderate to severe spleen congestion, associated with mild to moderate splenic erythrophagocytosis and hemosiderosis (Figure 2.C). In the liver, all cases showed mild to marked accumulation of gold yellow pigment in the bile ducts, in the biliary canaliculi, and in the cytoplasm of hepatocytes (cholestasis) (Figure 2.D). In the kidneys of calves 3, 4, and 6, moderate to marked accumulation of intracytoplasmic granular yellow to brown pigment was noted in the tubular epithelial cells (Figure 2.E). Calves 1, 3, and 6 also showed mild multifocal renal interstitial inflammatory infiltrate of lymphocytes.

In the CNS, severe diffuse congestion was noted in calves 1, 2, 5, and 6, and this finding was more evident in the grey matter (Figure 2.F). Calves 5 and 6 presented mild multifocal areas of pulmonary hemorrhage associated with fibrin deposition. Calves 1, 2, and 5 also showed mild to moderate meconium aspiration in the lumen of airways and alveolar spaces. In only one case (calf 1), histological observation of intraerythrocytic organisms was possible, in the blood vessels of the CNS and small intestine, and these were consistent with *B. bovis*. The other organs inspected did not show histological changes. All tissue samples subjected to IHC anti-*B. abortus* and anti-*N. caninum* yielded negative results.

Molecular findings

Samples of all cases yielded negative results for pestivirus (BVDV), and *Leptospira* spp. Additionally, none of the samples presented significant bacterial growth. Hemoparasite search revealed positive PCR results for *Anaplasma* sp. alone in one case (calf 3) and *Babesia* alone in three cases (*Babesia* sp. in calf 1, and *B. bovis* in calves 2 and 6). Co-infections with *Anaplasma* sp. and *Babesia* were detected in two cases (*Babesia* sp. in calf 4, and *B. bovis* in calf 5). Positive cases are depicted in Table 2, along with the positive tissues tested. At the same time, obtained sequences of *Anaplasma* sp. and *Babesia* sp., with their respective accession numbers and sequences with highest similarities are shown in Table 4. The divergence matrix indicates high identity among obtained sequences and *B. bovis* and *Babesia* sequences deposited in GenBank (supplementary file 1). None of the samples showed positive results for *B. bigemina* and '*Candidatus* M. haemobos'.

Discussion

In this study, the diagnosis of babesiosis and anaplasmosis were made through the association of the epidemiological, gross, microscopic, and molecular findings. Additionally, some other important causes of reproductive disorders in cattle were ruled out through molecular and immunohistochemical techniques. In Brazil, anaplasmosis and babesiosis are described in several states; however, these are certainly more frequent in areas of enzootic instability, such as the state of Rio Grande do Sul (Almeida et al., 2006; Lucena et al., 2010; Mello et al., 2017). The intricate regional climatic characteristics propitiate the frequent occurrence of isolated cases and outbreaks of anaplasmosis and babesiosis (Evans 1992), and these diseases have been listed as one of the major causes of death in beef and dairy cattle in the state of Rio Grande do Sul by several previous studies (Almeida et al., 2006; Lucena et al., 2010; Mello et al., 2017), which corroborate the present findings.

In the present case series, all farms reported the enzootic occurrence of cases of tick fever; however, no specific clinical signs were described in the aborting dams. This may be partly explained by the intrinsic difficulties of observation of clinical signs and events of fetal loss in cattle kept in extensive grazing systems (Campero et al., 2003). Still, pregnant cows presenting clinical signs of tick fever may abort (Kocan et al., 2010), which could possibly be a result of the systemic disease shown by these animals, and associated clinical abnormalities including fever (Bock et al., 2014). However, reports of abortion, stillbirth, and neonatal death

associated with direct fetal tissue damage induced by these agents, especially *B. bovis*, are exceedingly limited (Correa et al., 1978; Trueman and Mclennan, 1987; Costa et al., 2016).

Some previous descriptions of neonatal death associated with intrauterine *A. marginale* and *Babesia* infections, which are similar to the present findings, have reported that the dams were asymptomatic at the time of parturition (De Vos et al., 1976; Costa et al., 2016). The pathogenesis of fetal infection by these hemoparasites remains poorly understood (Costa et al., 2016), mainly when it comes to *Babesia* sp. infection, since most articles on this subject are represented by individual case reports (Trueman and Mclennan, 1987). Moreover, it is plausible to think that, in the case of ruminants, which present synepitheliochorial placentation, fetal infection may be favored by the limited transfer of molecules, mainly antibodies, from the dam to the fetus (Wooding and Buton, 2008).

High prevalence of '*Candidatus* M. haemobos' has been described in cattle in southern Brazil (Girotto et al., 2012); additionally, detection of such hemoparasite has been recorded in bovine fetuses in the same geographic region (Girotto-Soares et al., 2016). Thus, the search for hemoparasites in the present study included '*Candidatus* M. haemobos'; however, no positive results were obtained.

The gross and microscopic findings observed in all cases were typical of bovine anaplasmosis and babesiosis, and were identical to lesions commonly seen in affected cattle of all ages (Valli et al., 2016; Pupin et al., 2019). These findings are mostly induced by the severe hemolytic disease and erythrocyte recycling, including enlarged liver and spleen, pallor, jaundice, and cholestasis (Valli et al., 2016). Hemolysis leads to anemia and consequently hypoxia (Bock et al., 2014), which likely plays an important role in these episodes of fetal loss. Moreover, *B. bovis* may be involved in the production of cytokines, which may induce vascular impairment including coagulation disorders, hypotension, and blood stasis, which impacts mainly the CNS and the lungs (Wright et al., 1989; Ahmed 2002).

Another interesting gross finding detected in this study was cerebral babesiosis, which was diagnosed in four calves, characterized by marked CNS congestion, which was more evident in the grey matter, due to the broader vascularization present in this region when compared to the white matter (Valli et al., 2016). These lesions are known to be characteristic of *B. bovis* infection and are frequently associated with neurologic clinical signs (Valli et al., 2016). As previously mentioned, *B. bovis* transplacental infection is poorly documented, and to the best of the author's knowledge, this study represents the first case series describing the

occurrence of several cases of cerebral babesiosis in aborted, stillborn, and newborn calves, providing supporting pathological and molecular evidence of such infections.

Cytological examination of tissue smears is considered a practical diagnostic technique commonly used for the postmortem confirmation of these hemoparasites (Everitt et al., 1986), and herein enabled the observation of intraerythrocytic organisms in five out of six cases. In the remaining calf, search for hemoparasites was compromised because this fetus was frozen before being shipped to the laboratory, which resulted in lysed erythrocytes. PCR assays enabled the detection of *Anaplasma* sp. (calves 3, 4, and 5) and *Babesia* (calves 1, 2, 4, 5, and 6), as well as the exclusion of *B. bigemina* and '*Candidatus* M. hemobos'. While in calves 2, 5, and 6, sequencing confirmed the involvement of *B. bovis*, in calves 1 and 4 it was impossible to reach the species level, which is probably due to the use of a highly conserved gene in the search (18S rRNA). That also stands for what was found in the obtained *Anaplasma* sp. sequences, in which 16S rRNA gene was searched. Furthermore, some sequences did not yield large amplified fragments, which led to the generation of small contings, and may have ultimately interfered in the classification at the species level, since several *Babesia* spp. and *Anaplasma* spp. deposited sequences presented high similarities with the sequences obtained in this study. This is highlighted by the divergence matrix, which demonstrates high similarity among the sequences presented here and sequences of *Babesia*, including *B. bovis*, deposited in GenBank.

However, considering the lesions observed (cerebral babesiosis, for example), as well as hemoparasite morphology, it is highly likely that these agents were indeed *A. marginale* and *B. bovis*. No cases of *B. bigemina* infection were detected, corroborating previous studies that indicated that *B. bigemina* transplacental infection presents lower frequency when compared with *B. bovis* and *A. marginale* (Costa et al., 2016).

Therefore, this study indicates that *Anaplasma* sp. and *B. bovis* infections should be considered as a differential diagnosis in cases of fetal loss and neonatal death in cattle, highlighting the importance of transplacental infections associated with these hemoparasites in the bovine species. In the present case series, gross and microscopic findings were identical to lesions commonly seen in cattle of all ages affected by anaplasmosis and babesiosis; additionally, cytological, and molecular techniques were useful to reach a final diagnosis.

Conflict of interest

The authors declare no conflict of interest regarding this publication.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.ttbdis.2020.101443.

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Figure 1: Gross lesions detected during the necropsy of fetuses and newborn calves with anaplasmosis, babesiosis, or both conditions. **Fig.1.A (calf 3) and 1.B (calf 5)**: evaluation of open cavities enabled the observation of markedly enlarged and yellow liver. Fig. A also shows moderate jaundice of the carcass and collapsed lungs. **Fig. 1.C (calf 5)**: the opening of the gallbladder reveals large amounts of thick grumous bile. **Fig. 1.D (calf 1)**: the kidneys show diffuse marked dark discoloration. **Fig. 1.E (calf 3)**: The spleen presents severe enlargement in contrast with abomasum and fore stomachs. **Fig. 1.F (calf 1):** Marked cherry-pink discoloration of the encephalic grey matter is noted.

Figure 2: Microscopic findings in cases of anaplasmosis and babesiosis diagnosed in fetuses and newborn calves. **Fig 1.A (calf 1):** CNS cytological evaluation reveals capillaries filled with erythrocytes highly parasitized by *Babesia*-like organisms, consistent with merozoites (asterisks) (PanóticoRápido®, Bar 40 µm). **Fig 1.B (calf 3):** Blood smear reveals numerous round intraerythrocytic structures consistent with *A. marginale* (arrows) (PanóticoRápido®, Bar 30 µm). Histological findings. **Fig. 1.C (calf 1):** Spleen, moderate accumulation of brown pigment is observed in the cytoplasm of macrophages (hemosiderosis) (arrows) (H&E, Bar 120 µm). **Fig. 1.D (calf 3):** Liver, marked bile accumulation is noted in bile ducts (arrowhead) and biliary canaliculi (arrows) (cholestasis) (H&E, Bar 75 µm). **Fig. 1.E (calf 4):** Kidney, multifocal moderate accumulation of granular pigment is noted in the cytoplasm of epithelial tubular cells (asterisks) (H&E, Bar 120 µm). **Fig. 1.F (calf 1):** Cerebellum, the central nervous system grey matter shows diffuse marked congestion. Arrowheads indicate capillaries filled with erythrocytes (H&E, 400 µm).

Table 1: Nucleotide sequences of primers used to amplify specific fragments of the agents cited by polymerase chain reaction.

Agent	Primers	Fragment size ($\simeq$)	Gene	Tissues tested	Reference
Anaplasmataceae	F*:GE2 5' GTTAGTGGCATACGGGTGAAT 3' R*:HE3 5' CTTCTATAGGTACCGTACATTATCTTCCCTAT 3'	360 bp	16S rRNA	Spleen, CNS and liver	Breitschwerdtetet al., 1998
Piroplasm	F: BAB143-167 5' CCGTGCTAATTGTAGGGCTAATACA 3' R: BAB694-667 5' GCTTGAAACACTCTARTTTTCTCAAAG 3'	500 bp	18S rRNA	Spleen, CNS and liver	Soares et al., 2011
Babesia bigemina	F: GAU5 5' TGGCGGCGTTTATTAGTTCG 3' R: GAU8 5'GCCAGCGAAAAGACCCAAC 3'	458 bp	18S rRNA	Spleen and liver	Linhares et al., 2002
Babesia bovis	F: GAU9 5' CTGTCGTACCGTTGGTTGAC 3' R: GAU10 5' CGCACGGACGGAGACCGA 3'	541 bp	18S rRNA	Spleen, CNS and liver	Linhares et al., 2002
"*Candidatus* M. haemobos"	F: 5′ ATCTAACATGCCCCTCTGTA 3′ R: 5' GTAGTATTCGGTGCAAACAA 3′	500 bp	16S rRNA	Spleen and liver	Nishizawa et al., 2010
Pestivirus	F: 5' ATG CCCWTA GTA GGA CTA GCA 3′ R: 5' TCA ACT CCA TGT GCC ATG TAC 3′	288 bp	5'UTR	Spleen and lymph node	Vilcek et al., 1994
Leptospira spp.	F: Lep 5' GGCGGCGCGTCTTAAACATG 3′	330 bp	16S rRNA	Kidney, lung and liver	Merien et al., 1992

R: Lep 5' TCCCCCCATTGAGCAAGATT 3′

F*= Forward; R*= Reverse; Base pairs**; CNS: central nervous system.

Table 2: Epidemiological information and samples used to diagnose anaplasmosis and babesiosis in fetuses, stillborn, and newborn calves.

| | Epidemiological information | | | | | Positive tissue samples and agent detected | | | |
| | | | | | | Cytological evaluation | | Molecular diagnosis | |
Calf	Category	Sex	Breed	City	Diagnosis	*A. marginale*	*B. bovis*	*Anaplasma* sp.	*Babesia* sp.
1	Stillbirth	M	Angus	Itapuã - RS	Babesiosis	-	+ (CNS)	-	+ (spleen and CNS)
2	Abortion	F	Angus	Glorinha - RS	Babesiosis	-	-	-	+ (spleen and CNS)
3	Neonatal death	M	Angus x Devon	Eldorado do Sul–RS	Anaplasmosis	+ (CNS and spleen)	-	+ (liver)	
4	Stillbirth	M	Brangus	Viamão - RS	Anaplasmosis/ Babesiosis	+ (blood and spleen)		+ (spleen and CNS)	+ (spleen)
5	Neonatal death	M	Brangus	Jaquirana -RS	Anaplasmosis/ Babesiosis	-	+ (CNS)	+ (spleen)	+ (spleen and CNS)
6	Abortion	F	Angus	Soledade - RS	Babesiosis	-	+ (CNS)	-	+ (CNS)

M: male; F: female; CNS: central nervous system; (-) indicates negative results. The columns depicting cytological and molecular results only show the organs that presented positive results in the referred tests. RS: state of Rio Grande do Sul, Brazil.

Table 3: Gross lesions observed in cases of bovine abortion, stillbirth, and neonatal death associated with babesiosis and anaplasmosis.

Calf	Mucous membranes and carcass	Hemorrhages	Thick bile	Enlarged liver	Enlarged spleen	Kidneys	Cherry-pink brain
1	Congested	++	Present	+++	+++	Dark coloration	+++
2	Mild jaundice	-	Present	++	+++	-	++
3	Moderate jaundice	-	Present	+++	+++	-	-
4	Moderate pallor	-	Present	+	+++	-	-
5	Moderate pallor	+	Present	+++	+++	Dark coloration	+++
6	Congested	-	Present	+++	++	Dark coloration	++

Change severity is represented by (+). +: mild; ++: moderate; +++: severe. (-) indicates absent change.

Table 4: Identification of calves and the obtained sequences of *Anaplasma* sp. and *Babesia* sp. detected in the present study, along with the sequences with highest identity deposited in Genbank.

Calf	Percentage of similarity (GenBank®)	Query cover	Fragment size (bp)	Accession number
Calf 1	99.82% *Babesia* sp. (MG682492)	100 %	551 bp	[MN625742]
Calf 2	98.84% *Babesia bovis* (EF458214)	100 %	404 bp	[MN625741]
Calf 3	100% *Anaplasma* sp. (MN317257)	99 %	337 bp	[MN607601]
Calf 4	100% *Anaplasma* sp. (MN317257)	99 %	341 bp	[MN607602]
	100% *Babesia* sp. (MK580475)	100 %	267 bp	[MN625743]
Calf 5	100% *Anaplasma* sp. (MN317257)	99 %	333 bp	[MN607600]
	98.80% *Babesia bovis* (MN053043)	100 %	333 bp	[MN625744]
Calf 6	98.94% *Babesia bovis* (EF458214)	100%	376 bp	[MN625745]

bp: base pair.

Supplementary file 1: Nucleotide identity matrix between the 18S rRNA gene sequences of the five *Babesia* sp. sequences identified in the present study, and six of the most similar *Babesia* species retrieved from GenBank.

Sequence	1	2	3	4	5	6	7	8	9	10	11
1. [MK580475] *Babesia* spp. - North America	ID										
2. [MN053043] *Babesia bovis* - Cuba	0.917	ID									
3. [MG682492] *Babesia* spp. - Uruguay	1	0.917	ID								
4. [AF030058] *Babesia bovis* - Brazil	0.386	0.4	0.386	ID							
5. [FJ588013] *Babesia bovis* - southern Brazil	0.386	0.4	0.386	0.993	ID						
6. [KC894394] *Babesia bovis* - South Africa	0.386	0.4	0.386	0.993	1	ID					
7. [MN625741] *Babesia bovis* (calf 2)	0.91	0.988	0.91	0.4	0.4	0,4	ID				
8. [MN625742] *Babesia* sp. (calf 1)	1	0.917	1	0.386	0.386	0,386	0.91	ID			
9. [MN625743] *Babesia* sp. (calf 4)	1	0.917	1	0.386	0.386	0,386	0.91	1	ID		
10. [MN625744] *B. bovis* (calf 5)	0.913	0.996	0.913	0.4	0.4	0,4	0.985	0.913	0.913	ID	
11. [MN625745] *B. bovis* (calf 6)	0,91	0,988	0.91	0.4	0.4	0,4	1	0.91	0.91	0.985	ID

ID: Identical.

3 ARTIGO 2

Neste item é apresentado o artigo intitulado **"Pathological and etiological characterization of cases of bovine abortion due to sporadic bacterial and mycotic infections."** O artigo foi submetido para publicação no periódico "*Brazilian Journal of Microbiology*".

Pathological and etiological characterization of cases of bovine abortion due to sporadic bacterial and mycotic infections

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Abstract

Opportunistic bacteria and fungi are commonly reported causes of bovine abortion in a small percentage of fetal losses of infectious etiology in cattle. The objective of this study was to characterize the pathological and etiological findings in fetuses aborted due to secondary bacterial and fungal infections submitted for postmortem examination between 2004–2019 in the state of Rio Grande do Sul, Brazil. Nineteen cases of bacterial etiology and five cases of fungal etiology were assessed. In cases of bacterial etiology, gross changes were uncommon and two different microscopic patterns were observed: 1) primary bronchopneumonia with occasional dissemination in cases of *Staphylococcus* sp., *Streptococcus* sp., and *Mannheimia haemolytica* infections; and 2) systemic disease with sepsis in cases of *Escherichia coli* and *Listeria* sp. infections. *Aspergillus* sp. was the main fungal agent identified, and cases of mycotic abortion were characterized by placentitis, dermatitis, and pneumonia. Fetal membranes were available for examination in less than half of the submissions (11/24), and

placental lesions were observed in all cases. This study reaffirms the importance of postmortem examinations in the determination of causes of fetal loss in cattle and highlights pathological findings commonly observed in fetuses aborted due to sporadic bacterial and fungal agents.

Keywords: fetal loss, postmortem examination; histopathology; microorganism; opportunist; placenta.

1. Introduction

Reproductive diseases, including embryonic and fetal losses, represent major economic limitations in livestock systems worldwide [1–3]. Bovine abortion has been associated with a plethora of infectious and non-infectious agents, and the task of reaching a final diagnosis in these cases is commonly challenging [4]. Studies compiling causes of abortion in cattle indicate that diagnostic rates are highly variable. Nevertheless, a final diagnosis is commonly made in around 30–55% of postmortem submissions and most are represented by infectious etiologies [5–9].

Among infectious causes, numerous bacterial agents have been associated with cattle abortion [10] and are classified as primary reproductive pathogens and secondary or opportunistic agents [4]. The first group is represented by bacteria known to lead to primary reproductive disorders such as abortion, and include *Brucella abortus*, *Leptospira* sp., *Campylobacter* sp., and *Chlamydophila* sp. [4]. The second group is comprised mainly of bacterial agents commonly found in the environment or colonizing the skin, mucous membranes, and gastrointestinal tract of cattle, which under certain circumstances, e.g. hematogenous spread in the dam, may cause placentitis or fetal dissemination, leading to abortion [4]. Common opportunistic bacterial agents include *Staphylococcus aureus*, *Trueperella pyogenes*, and *Bacillus* sp. [7, 10–12]. Likewise, fungal agents, including *Aspergillus fumigatus*, are occasionally described in association with cases of sporadic abortion in cattle [13].

Even though secondary or opportunist bacterial and fungal agents are not major causes of fetal loss in cattle, they are often documented in retrospective studies as relatively common causes of bovine abortion [6–8] and may account for around 25% of fetal losses of infectious etiology in the species [7]. This represents a significant number of submissions that are handled by veterinary pathologists and diagnosticians and need to be differentiated from other

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causes of abortion in cattle, including primary reproductive pathogens. However, systematic studies thoroughly compiling and describing the pathological findings associated with abortions linked to these agents are scant.

Even though several approaches have been used to diagnose and study reproductive diseases in livestock, the postmortem examination of aborted fetuses remains an important tool for this purpose, allowing the observation of pathological changes and collection of samples for confirmatory ancillary testing. Therefore, the objective of this study is to retrospectively characterize the pathological and etiological findings of cases of bovine abortion due to sporadic/opportunistic bacterial and fungal agents, diagnosed in the State of Rio Grande do Sul, Brazil.

2. Material and methods

2.1. Case selection

A retrospective search was performed at the database of the Veterinary Pathology Laboratory of the Universidade Federal do Rio Grande do Sul (UFRGS), Brazil. Cases of bovine abortion associated with sporadic bacterial and fungal agents diagnosed from 2004 to 2019 were compiled.

Fetuses met the criteria to be included if: i) opportunist bacterial or fungal agents were isolated in pure or nearly pure culture from fetal tissues or the placenta; ii) fetal tissues or the placenta presented microscopic findings consistent with a bacterial or fungal infection, including suppurative lesions associated or not with intralesional agents; and iii) no other abortifacient agent could be retrieved from the case at the time of the postmortem examination. Although *Listeria monocytogenes* is commonly classified as a primary reproductive agent, we included abortion cases due to this agent because infections leading to fetal loss are rarely seen in our diagnostic routine. Exceptions to the first criterion were made in cases of mycotic abortion in which culture was not available, but etiology identification was possible with immunohistochemistry (IHC) against *Aspergillus* sp.

Fetuses that met the following criteria were excluded from the study: (i) cases of abortion associated with primary reproductive pathogens, such as *Brucella abortus*, *Campylobacter* sp., *Coxiella burnetii*, and *Leptospira* sp.; (ii) fetuses positive for Bovine Viral Diarrhea Virus infection; (iii) cases presenting histopathological findings indicative of a

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bacterial etiology, in which no significant growth occurred or culture results were not available; (iv) cases with gross or microscopic lesions consistent with a fungal etiology, in which culture results were not available and IHC against *Aspergillus* sp. was negative; (v) severely autolyzed aborted fetuses and calves that died in the neonatal period but were referred as aborted fetuses; (vi) and cases lacking sufficient tissue to perform re-evaluations.

2.2. Case information and pathological evaluation

Postmortem examination reports and corresponding photographic files were reviewed in the selected cases. Compiled case information included breed, sex, and gestational age estimated through the measurement of crown-rump length [14] or through reproductive records, when available. Compiled information also included the presence of placenta with the referred submission, gross lesions and complementary diagnostic results documented in the postmortem examination reports. Since only formalin-fixed paraffin-embedded (FFPE) tissue blocks were available in most cases, all investigations were conducted using histopathology, histochemistry and/or IHC. FFPE tissue blocks were searched in the archives of the Veterinary Pathology Laboratory of UFRGS. Histological sections (3–4 μm thick) were prepared, routinely stained by hematoxylin and eosin (HE), and reassessed.

Microscopic findings were classified according to their distribution in the different organs, and inflammatory lesions were characterized and subjectively graded as mild, moderate, and severe. In addition to that, the presence and morphology of bacterial and fungal agents were recorded. Available sections of the gastrointestinal tract (forestomachs, abomasum, small and large intestine) were searched for evidence of bacterial or fungal colonization. When necessary, Grocott Methenamine Silver (GMS) and Brown-hopps stains were employed in tissue sections to evidence fungi and Gram-positive bacteria, respectively.

2.3. Microbiology

Bacteriology and mycology results available in the postmortem examination reports were used to determine the etiological agent involved in each case. Standard bacterial culture was routinely conducted in all fetuses with fresh samples of lung, abomasum content, placenta (when available), and any additional organ showing gross changes at postmortem examination. Tissues were inoculated on 5% sheep blood agar plates (Mueller Hinton,

Kasvi®, Brazil) and MacConkey agar (Kasvi®, Brazil) and incubated at 37 °C for 72 hours. Identification of bacterial isolates was based on culture, morphotinctorial, and biochemical features. When gross and microscopic changes were consistent with mycotic abortion, fresh samples of skin, placenta, abomasum content, and lung were inoculated on Sabouraud dextrose agar and incubated at 25–30 °C [15].

2.4. Immunohistochemistry

IHC was conducted using rabbit polyclonal antibodies to characterize pathogen tissue distribution in cases of abortions due to *E. coli* (1:200 dilution; ViroStat®, Westbrook, Maine, USA) and *L. monocytogenes* (1:200 dilution; BD®, Franklin Lakes, New Jersey, USA), previously diagnosed by bacterial culture. The occurrence of *Aspergillus* sp. infection in cases of mycotic abortion was confirmed with IHC against *Aspergillus* sp. (1:80 dilution; antibody mouse anti-*Aspergillus* spp., Bio-rad®, Hercules, California, USA) when the infection was inferred by fungal culture or presumed by fungi morphology at histopathology (cases with no fungal culture available). In these cases, IHC positive results were considered diagnostic of *Aspergillus* sp. infection, even when fungal culture was absent. Additionally, sections from all selected cases were submitted for IHC anti-*B. abortus* (1:20 dilution; polyclonal antibody, rabbit anti-*B. abortus*, non-commercial) [16].

For each case, one slide was selected from organs presenting visible bacteria/fungi and associated inflammatory changes on histopathology. Sections of cases of *E. coli* mastitis, *L. monocytogenes* meningoencephalitis, *Aspergillus fumigatus* pneumonia, and *B. abortus* bronchopneumonia were used as positive control sections. Primary antibodies were replaced by Universal Negative Control Serum (Biocare medical, Concord, California, USA) in the negative control sections. Antigen retrieval was carried out in microwave oven for 5 min. for *E. coli*, with Citrate buffer (pH 6.0) in a heating chamber (40 min./96 °C) with Tris EDTA buffer (pH 9.0) for *Aspergillus* spp., with proteinase K for 10 min. for *B. abortus*, and was not performed for *Listeria monocytogenes*. In all cases, amplification was performed with MACH 4: Universal HRP-Polymer (Biocare medical, Concord, California, USA). The reaction was detected with 3'-Diaminobenzidine chromogen (Dako, Glostrup, Denmark) (IHC anti-*E. coli*, anti-*Aspergillus* spp., and anti-*L. monocytogenes*), and with 3-amino-9-ethylcarbazole chromogen (AEC; Sigma, St. Louis, MO, USA) (IHC anti-*B. abortus*). Slides were counterstained with Harris' hematoxylin.

2.5. Additional tests

Results of additional tests aiming to rule out the involvement of important abortigenic agents were retrieved from the postmortem examination reports. Tissue sections of thymus and brain from all fetuses submitted between 2004 and 2013 were tested for BVDV through IHC as previously described [17], and fresh samples of thymus and spleen of fetuses submitted between 2014 and 2019 were tested for BVDV through PCR [18]. Direct fluorescent antibody test (FAT) for *Leptospira* sp. was performed in imprint preparations of kidney from fetuses submitted between 2004 and 2014 [19], and fresh samples of liver and kidney from fetuses submitted between 2015 and 2019 were tested for *Leptospira* sp. through PCR [20]. IHC anti-*Neospora caninum* was performed in tissue sections of fetuses presenting microscopic lesions suggestive of protozoal infection, including non-suppurative encephalitis, myocarditis, and myositis, as previously described [21].

3. Results

The screening process resulted in a total of 24 cases which met the inclusion criteria, consisting of 19 cases of sporadic/opportunistic bacterial infections (cases 1–19) and 5 cases of fungal infections (cases 20–24). Diagnosed agents were *Staphylococcus* sp. (8/24), *Escherichia coli* (5/24), *Listeria* sp. (3/24), *Streptococcus* sp. (2/24), *Mannheimia haemolytica* (1/24), *Aspergillus* sp. (4/24), and *Geotrichum candidum* (1/24). Information of individual cases of bacterial abortion and *Aspergillus* sp. abortion are shown in Table 1 and 2, respectively. The placenta was referred for examination in 11/24 cases, and placental microscopic lesions were observed in all cases. All included cases were negative for *B. abortus* (IHC), BVDV (IHC or PCR) and *Leptospira* sp. (IFA or PCR), and none of the cases showed microscopic lesions that warranted IHC anti-*N. caninum*.

3.1. Bacterial infections

3.1.1. Staphylococcus sp.

Gross lesions were observed in only one case of *Staphylococcus* infection (fetus 7). These changes were characterized by moderate fibrin deposition covering the visceral pleura

in the lung cranioventral region (Fig 1A), and this case has been published elsewhere [22]. Microscopic lesions were observed primarily in the lungs in all cases of *Staphylococcus* sp. and *Staphylococcus aureus*-associated abortions. These changes were multifocal and ranged from mild to severe. Histological patterns were classified as suppurative bronchopneumonia (3/8), necrosuppurative and fibrinous bronchopneumonia (4/8), and alveolar exudation and thrombosis with minimal inflammation (1/8). Other pulmonary changes included fibrinous pleuritis (3/8), thickening of the interlobular septa by fibrin exudation and edema (3/8), and thrombosis (3/8). Coccoid bacterial aggregates were seen amidst lung lesions in seven cases, and these aggregates were abundant (5/8) (Fig 1B and Fig 1C) or rare (2/8).

The placenta was available for examination in only two cases, both of which presented multifocal fibrinosuppurative placentitis associated with necrosis and bacterial cocci (Fig 1D). Brown-hopps stain revealed that cocci observed in the lungs (Fig 1C) and placenta (Fig 1E) were Gram-positive. Sections of the gastrointestinal tract were available for reassessment in 7/8 cases, and bacteria were seen in the lumen or adhered to the mucosa without associated inflammatory changes in four fetuses (Fig 1F). Microscopic lesions seen in other organs included thrombosis in the thymus, thrombosis in the epicardium, and multifocal necrosuppurative hepatitis (one case each).

3.1.2. Streptococcus *sp. and* Mannheimia haemolytica

The lung was the primarily affected organ in these cases. Gross changes were observed in only one case of *Streptococcus* sp. abortion (fetus 10), and lesions consisted of multifocal to coalescing, firm, and white areas (0.2–4cm in diameter) in the cranioventral region of the lung (Fig 2A). Microscopically, these foci were represented by large numbers of Gram-positive coccoid bacterial aggregates surrounded by moderate inflammatory infiltrate of neutrophils, fibrin deposition, and necrosis (Fig 2B-C). Marked fibrin deposition was also observed in the visceral pleura. The second case of *Streptococcus* sp. associated abortion presented mild, multifocal fibrinosuppurative bronchopneumonia with rare Gram-positive cocci and marked expansion of interlobular septa by edema, fibrin deposition, and congestion.

The abortion associated with *Mannheimia haemolytica* was characterized by marked, multifocal suppurative bronchopneumonia associated with large numbers of rod-shaped bacteria inside bronchioles, bronchi, and alveolar spaces (Fig 2D). The placenta was not

available for evaluation in any of these cases. Bacteria were not observed in the lumen or adhered to the mucosa of the gastrointestinal tract in any of the cases.

3.1.3. *Escherichia coli*

No gross changes were recorded in cases of *E. coli*-associated abortions. Histologic lesions were observed in two or more organs in 4/5 fetuses. Microscopic findings were characterized by large amounts of free bacterial rods occasionally associated with mild necrosis, mild inflammatory infiltrate of neutrophils, and mild fibrin deposition. Most commonly, numerous bacterial aggregates were seen freely in the interstitial space and inside blood vessels with minimal inflammatory or necrotic changes (Fig 3A). Affected organs included the lung (3/5), liver (2/5), kidney (2/5), heart (2/5), skeletal muscle (2/5), brain (2/5), eyelid (1/5), spleen (1/5), and thymus (1/5).

Bacterial aggregates were found in the lumen and adhered to the mucosa of the gastrointestinal tract in 2/3 cases (sections unavailable in two cases). The placenta was available for evaluation in 3/5 calves. These three cases showed multifocal, mild to severe necrosuppurative placentitis associated with fibrin deposition, deposition of necrotic debris (Fig 3B), and rare (2/3) to numerous (1/3) rod-shaped bacterial aggregates. IHC was positive in 5/5 cases. Immunolabeling evidenced numerous *E. coli* in the interstitial space and inside blood vessels in the lungs and kidney (fetus 15), in the heart (fetus 13), and adhered to the chorionic epithelium and amidst necrotic areas in the placenta (fetus 12, 14, and 16) (Fig 3B, inset).

3.1.4. *Listeria sp.*

Gross changes were observed solely in the placenta of one fetus, and these were represented by diffuse, severe thickening of cotyledons and intercotiledonary regions due to marked deposition of friable, dull, and yellow material (fetus 19). The placenta was available for analysis in 2/3 fetuses, both of which showed similar histologic lesions characterized as moderate (fetus 17) to marked (fetus 19), multifocal necrosuppurative placentitis (Fig 3C) associated with large numbers of small, Gram-positive bacterial rods amidst necrotic areas, adhered to the remaining chorionic epithelium, and inside chorionic blood vessels (Fig 3D). In one case, lesions were restricted to the placenta (fetus 17). In the second case, additional

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findings included multifocal, moderate fibrinosuppurative bronchopneumonia (Fig 3E) associated with numerous small, Gram-positive bacterial rods and few bacteria in the intestinal lumen (fetus 19). The third case, in which placenta was unavailable for examination (fetus 18), presented similar, small Gram-positive bacterial rod aggregates nearly occluding blood vessels in the brain, liver, spleen, thymus, lung, skeletal muscle, eyelid, forestomaches, abomasum, and small and large intestine. Additionally, mild, multifocal suppurative bronchopneumonia associated with numerous bacterial rods filling alveolar spaces, bronchioles, and bronchi were seen. In this case, bacteria were also observed invading the mucosa of the small and large intestine, in association with vasculitis and thrombosis in the submucosa. IHC yielded positive results in the three cases. Immunolabeling confirmed *Listeria* sp. aggregates inside blood vessels in the brain (fetus 18) and chorionic blood vessels of the placenta (fetuses 17 and 19) (Fig 3F). In cases 17 and 19, IHC also labeled large numbers of *Listeria* sp. amidst areas of inflammation and necrosis in the placenta and adhered to the remaining chorionic epithelium (Fig 3F).

3.2. Fungal infections

Four cases of *Aspergillus* sp. or *Aspergillus fumigatus* (cases 20-23) and one case of *Geotrichum candidum* (case 24) infections were assessed. This latter fetus presented dermatitis and pneumonia, and detailed pathological and etiological features of this case have been published elsewhere [23] and will not be addressed here. The etiological diagnosis of *Aspergillus sp.* associated abortion was made through fungal culture and IHC (cases 20 and 23) or only through positive IHC immunolabeling (cases 21 and 22) (Table 2).

Gross lesions were restricted to the skin and placenta in cases of *Aspergillus* sp. abortion. The placenta was available for examination in all four cases, and gross changes were observed in all of them. The cotyledons and, often, the intercotiledonary regions were moderately to severely thickened, reddened, and showed deposition of friable, dull, and yellow material. In one case (case 23), small cotyledon-like structures were observed in the intercotiledonary regions in the chorioallantois (adventitial placentation) (Fig 4A). This particular fetus was reported to be nine months of gestation according to the farmer's record; however, the fetus seemed smaller, had very sparse haircoat, and crown-rump measurement was consistent with seven months of gestation. Cutaneous lesions (fetuses 20, 22, and 23) were represented by irregular, multifocal to coalescing, raised, round to oval, white to grey

areas (1–4 cm) located predominantly in the head, neck, trunk, and fore and hind limbs (Fig 4B).

Microscopic placental changes were observed in all cases of *Aspergillus* sp. associated abortion (4/4). Changes were characterized by severe, multifocal to coalescing necrosuppurative and fibrinous placentitis, which effaced the superficial chorionic epithelium and frequently extended to the deep layers of the chorioallantois. Fungal hyphae were seen in all cases, sometimes amidst inflammatory changes and frequently presenting angioinvasion (Fig 4C). Moderate to severe fibrinoid necrosis of blood vessel walls and thrombosis (Fig 4D) were seen in all cases. Additional changes included marked edema in the intercotiledonary regions and multifocal areas of hemorrhage.

In the skin, microscopic lesions in cases of *Aspergillus* sp. abortion were represented by marked suppurative or necrosuppurative dermatitis, folliculitis, and vasculitis, as well as hyperkeratosis and intraepidermal pustules. Lesion severity and predominant histological pattern varied among cases, and among skin sections of the same fetus. In some cases, histological findings included marked dilatation of hair follicles, which were filled with large numbers of neutrophils, fewer macrophages, lymphocytes, and plasma cells, as well as necrotic debris (Fig 4E). Sometimes, hair follicles were ruptured, and inflammation extended to the adjacent dermis (Fig 4F). In other cases, skin lesions were more superficial and characterized by intracorneal pustules, moderate to severe hyperkeratosis, and superficial dermatitis and perifolliculitis.

Aspergillus sp. hyphae were seen in all cases amidst lesions. Fungal hyphae presented amphophilic to basophilic staining on HE slides (Fig 4C), and these structures were strongly positive on GMS (Fig 4F, inset). Hyphae were 3–6 μm wide, commonly showed dichotomous branching at acute angles (45°) and septation.

In the lung, microscopic changes were observed in 3/4 cases of *Aspergillus* sp. infection. Lesions consisted of multifocal areas of moderate neutrophilic and histiocytic bronchopneumonia, often forming small nodules. Fungal hyphae were observed in the lungs of two fetuses. Additional findings included moderate, multifocal necrotic hepatitis in one case (fetus 23). Sections of the gastrointestinal tract were available for examination in 2/4 cases, none of which showed microscopic changes. IHC anti-*Aspergillus* sp. was positive in all cases, showing moderate, multifocal labeling of fungal hyphae in the skin (fetus 23) and placenta (fetuses 20, 21, 22, and 23) (Fig 4C, inset), amidst areas of inflammation and necrosis.

4. Discussion

The pregnant uterus and fetal membranes show increased susceptibility to colonization by numerous infectious agents. Several factors are believed to be associated with this susceptibility, including fetus isolation from the maternal immune system, elevated temperatures, slow local inflammatory response, among others [24]. Hematogenous spread seems to be the most common route of infection of the fetomaternal interface in cattle, while ascending infections are uncommon in this species [24]. The colonization of fetal membranes may occur as a result of an episode of bacteremia or fungal systemic infections, leading to placentitis, impairment of blood and nutrient supply to the fetus, and dissemination to the amniotic fluid [25]. After colonizing the placenta, microorganisms may gain entry to the fetus by two routes [4]. The first occurs after microorganisms spread to the amniotic fluid, where fungal or bacterial agents may be inhaled, ingested, or colonize the skin of the fetus [25]. A second route occurs in specific infections, for instance *Listeria monocytogenes* and *Salmonella* spp., in which after colonizing the placenta, microorganisms enter the fetus through the umbilical cord circulation, leading to systemic lesions [4].

Fungi and certain bacteria observed in this study, such as *Staphylococcus* sp., *Streptococcus* sp., and *Mannheimia haemolytica*, may have gained access to the fetus predominantly through the first route [4, 25]. This is corroborated by the fact that bronchopneumonia was a major and a consistent finding in most of these fetuses, especially in cases involving the mentioned bacteria above, and dermatitis was a common finding in cases of mycotic abortion. Nevertheless, some fetuses presented mild changes in other organs, which could indicate that systemic dissemination may occur with disease progression, as previously reported [7]. As previously mentioned, *Listeria monocytogenes* is believed to enter the fetus through the bloodstream after placental colonization, leading to a systemic and septicemic disease in the fetus [4], as observed in the reported cases. In Brazil, the main clinical presentation of bovine listeriosis is the neurologic form [26], and cases of fetal loss attributed to this disease have rarely been documented [27]. Therefore, this study brings supporting evidence to corroborate the occurrence of *L. monocytogenes* as a cause of fetal loss in Brazilian cattle.

Cases of *E. coli* infection herein observed seem to differ from cases of *Staphylococcus* sp, *Streptococcus* sp., and *Manhemia hemolytica* infections. For *E. coli* infection, even though pneumonia and gastrointestinal tract colonization were present in some cases, the main

finding consisted of large numbers of bacteria in several organs, frequently inside blood vessels and without a significant inflammatory response. Even though previous studies have documented the occurrence of cattle abortions due to *E. coli* infection [5, 9], the role of this bacterium in bovine fetal loss seems controversial [5]. Nevertheless, herein we were able to determine *E. coli* tissue colonization, vascular dissemination, and sometimes associated inflammatory changes by HE and IHC, which corroborates the participation of *E. coli* in certain cases of sporadic bovine abortion.

Placental lesions were consistently observed in our cases, reinforcing the importance of submitting and evaluating fetal membranes in order to increase diagnostic accuracy, mainly in cases where a bacterial or fungal etiology is suspected, since abortions may occur in the absence of significant lesions in fetal tissues [28]. Placental lesions were especially severe in cases of mycotic abortion, with marked vascular lesions, which agrees with the literature [13]. One particular case of fungal abortion had adventitial placentation, and the corresponding fetus seemed smaller than the expected according to the gestational age reported by the owner. Adventitial placentation is a compensatory mechanism that occurs in cases of placental insufficiency in cattle [29] that may occur as a result of inadequate number of caruncles (congenital) or more commonly due to placental inflammation [29]. In the case reported, fungal placentitis may have led to placental insufficiency, resulting in inadequate fetal supply and underdevelopment, leading ultimately to adventitial placentation as a compensatory mechanism.

Gross changes were not common in cases of opportunist bacterial infections, highlighting the importance of standard sampling for microbiological culture and histopathology during postmortem examinations in fetuses. In contrast, fungal infections led to typical macroscopic lesions in most cases, predominantly in the placenta and in the skin, which agrees with the scientific literature [13, 24]. Even though microbiological culture is crucial for the diagnosis of bacterial/fungal abortion, the simple identification of opportunistic agents from the placenta or fetal tissues is not considered of diagnostic value, since several of them may represent environmental contamination after fetal expulsion or cervix opening [5]. Therefore, the association of microbiological results with the observation of pathological findings and exclusion of other important abortigenic agents, as conducted in this study, appears to be an appropriate approach when dealing with this type of submission [5].

Another interesting finding of this study was the observation of bacterial agents in the lumen and adhered to or invading the mucosa of the gastrointestinal tract of some examined

fetuses. Although this event is known to occur in aborted fetuses [24], these pathological findings seem to be commonly overlooked in the postmortem diagnostic routine. Colonization of the gastrointestinal tract is believed to be associated with ingestion of amniotic fluid containing bacteria or fungi after placental dissemination [24] and explains why abomasum contents are commonly sampled to perform microbiological cultures. Therefore, the search for bacteria in histological sections of the gastrointestinal tract may represent a useful complementary tool in the diagnostic routine of aborted fetuses.

Main limitations of this study include the small number of cases assessed and inherent shortcomings associated with retrospective studies (e.g., variability and change in procedures conducted in different years by different professionals and dependence on the quality of previous postmortem records). Additional limitations include shortcomings that are intrinsic to studies on causes of abortion through postmortem examinations (e.g., autolysis, insufficient or incomplete submissions, multiplicity of possible etiologies, and constant need for complementary tests).

In conclusion, the postmortem examination of bovine fetuses is a fundamental tool in the diagnostic approach of fetal losses. Abortions due to opportunist bacterial and fungal agents commonly show typical gross and/or histological features that, when taken in conjunction with complementary ancillary testing (e.g., bacterial and fungal cultures, and IHC), allow the determination of such agents as causes of abortion. This work highlights the importance of complete submissions, especially the placenta, to increase the likelihood of reaching a final diagnosis. Also, we point out the importance of evaluating the gastrointestinal tract in aborted fetuses, organs that are commonly overlooked in this type of submission and may yield important complementary information to the pathological diagnosis.

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Declarations

Conflict of interest: The authors declare no conflict of interest regarding this publication.

Ethics approval: All cases described herein occurred spontaneously, with no experimentation, inoculation, or treatment of live animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Consent to participate: not applicable.

Consent for publication: not applicable.

Availability of data and material: All data generated or analyzed during this study are included in this published article.

Code availability: not applicable.

Authors' contributions: Luan C. Henker was the main responsible for data collection and interpretation, as well as drafting the manuscript. Luan C. Henker and Marcele B. Bandinelli performed immunohistochemical exams. Bruna C. Lopes assisted with microbiological cultures of samples submitted from 2017 to 2019. Luan C. Henker and Igor R. dos Santos performed the histochemical preparations. Luan C. Henker and Saulo P. Pavarini re-evaluated H&E slides and delineated the study. Luan C. Henker, Marina P. Lorenzett, Daniele M. Bassuino, Gregory D. Juffo, Nadia A. B. Antoniassi, Caroline A. Pescador, Luciana Sonne, David Driemeier, and Saulo P. Pavarini performed the postmortem examinations and pathological examinations throughout the years. All authors critically revised and made intellectual contributions to the manuscript.

Fig. 1 Bovine fetuses, *Staphylococcus aureus*-associated abortions. (A) At the opening of the thoracic cavity, moderate amount of fibrin is seen covering the visceral pleura of the cranioventral area of the lung. (B) Lung, suppurative pneumonia associated with moderate fibrin deposition and numerous coccoid bacterial aggregates, HE, Bar, 180 μm. (C) Lung, numerous Gram-positive cocci aggregates are observed, Brown-hopps stain, Bar, 180 μm. (D) Placenta, chorionic epithelium showing necrosis, fibrin deposition and numerous cocci, HE, Bar, 90 μm. (E) Placenta, numerous Gram-positive cocci are observed colonizing the surface of the chorionic epithelium. Brown-hopps stain, Bar, 180 μm. (F) Abomasum, numerous cocci are seen in the abomasal lumen and adhered to the mucosa, HE, 90 μm.

Fig. 2 Bovine fetuses, abortions due to opportunistic bacterial agents. Abortion due to *Streptococcus* sp. infection (A–C). (A) At the opening of the thoracic cavity, firm, multifocal to coalescing, white areas (0.2–4cm in diameter) are seen in the cranioventral region of the lung. (B) Lung, severe necrosuppurative and fibrinous bronchopneumonia associated with numerous coccoid bacterial aggregates, HE, 360 μm. (C), which are Gram-positive cocci, Brown-hopps stain, Bar, 240 μm. (D) Abortion due to *Mannheimia haemolytica* infection. Lung, numerous bacterial rods are observed filling a bronchiole, and some adjacent alveolar spaces, HE, Bar 120 μm.

Fig. 3 Bovine fetuses, abortions due to opportunistic/sporadic bacterial agents. Abortions due to *Escherichia coli* infection (A–B). (A) Heart, numerous bacterial rods are observed filling the lumen of a blood vessel HE, Bar 120 μm. (B) Placenta, marked necrosuppurative placentitis, HE, Bar 240. Inset: placenta, marked immunolabeling for *E. coli* adhered to the surface of the remaining chorionic epithelium, IHC. Bar, 240 μm. Abortions due to *Listeria* sp. infection (C–F). (C) Placenta, marked necrosis, inflammatory infiltrate of neutrophils, and deposition of debris are observed affecting chorionic villi, HE, Bar 360 μm. (D) Placenta, chorioallantois blood vessel, numerous bacterial rods are seen in the lumen and invading the endothelium and vessel wall, HE, Bar 240 μm. Inset: Placenta, numerous Gram-positive rods are observed inside blood vessels of the chorioallantois, Brown-hopps stain, Bar, 240 μm. (E) Lung, mild, diffuse inflammatory infiltrate of neutrophils and multifocal moderate fibrin deposition are observed, HE, Bar 240 μm. (F) Placenta, marked immunolabeling for *Listeria monocytogenes* adhered to the surface of the remaining chorionic epithelium and inside blood vessels, IHC. Bar, 240 μm.

Fig. 4 Bovine fetuses, abortions due to *Aspergillus* sp. Infection. (A) Placenta, the cotyledons are markedly thickened and irregular, and show deposition of yellow and dull material. Numerous, small, cotyledon-like structures are observed in the intercotiledonary regions (adventitial placentation). (B) multifocal, irregular, raised, round to oval, white to grey areas (1–3 cm in diameter), are observed in the skin of the head and neck. (C) Placenta, large numbers of basophilic fungal hyphae are observed in the stroma of the chorioallantois, frequently invading the walls of blood vessels, HE, Bar, 240 μm. Inset: Multifocal immunolabeling for *Aspegillus* sp. hyphae is observed in the placenta, IHC. Bar, 240 μm. (D) Placenta, blood vessels of the chorioallantois show marked fibrinoid necrosis and thrombosis. Also, severe inflammatory infiltrate of neutrophils is observed in the adjacent placental tissue, HE, Bar, 240 μm. (E) Skin, hair follicles are markedly dilated and filled predominantly with neutrophils and necrotic debris. Also, multifocal intraepidermal pustules and diffuse hyperkeratosis are observed, HE, Bar, 360 μm. (F) Skin, hair follicles are ruptured, and marked inflammatory infiltrate composed predominantly by neutrophils, associated with deposition of fibrin and necrotic debris are seen in the dermis, HE, Bar, 240 μm. Inset: Numerous fungal hyphae are observed inside hair follicles and in the adjacent dermis, GMS, Bar, 240 μm.

Table 1: Bacterial isolates, case information, and main pathological findings in cases of sporadic bacterial abortion in cattle

Fetus no.	Bacterial isolate	Gestational age (mo)	Sex	Breed	Gross lesions	Main microscopic findings
1	*Staphylococcus* sp.	NI	NI	NI	-	Necrosuppurative and fibrinous bronchopneumonia
2	*Staphylococcus aureus*	8	F	Holstein	-	Suppurative bronchopneumonia. Necrosuppurative and fibrinous placentitis
3	*Staphylococcus* sp.	6	M	NI	-	Necrosuppurative and fibrinous bronchopneumonia
4	*Staphylococcus* sp.	3	M	NI	-	Necrosuppurative and fibrinous bronchopneumonia. Necrosuppurative placentitis
5	*Staphylococcus aureus*	6	M	Holstein	-	Suppurative bronchopneumonia
6	*Staphylococcus aureus*	6	M	Crossbred	-	Suppurative bronchopneumonia
7*	*Staphylococcus aureus*	6	M	Crossbred	Fibrin deposition in the lung cranioventral area	Necrosuppurative and fibrinous bronchopneumonia and pleuritis
8	*Staphylococcus aureus*	5	F	Angus	-	Alveolar exudation and thrombosis with minimal inflammation
9	*Streptococcus* sp.	NI	NI	NI	-	Fibrinosuppurative bronchopneumonia
10	*Streptococcus* sp.	7	F	Holstein	Multifocal, firm, white areas in the lung cranioventral region	Necrosuppurative and fibrinous bronchopneumonia and pleuritis
11	*Mannhemia haemolytica*	6	F	Angus	-	Suppurative bronchopneumonia
12	*Escherichia coli*	5	F	Angus	-	Multifocal hepatic necrosis. Multifocal necrosuppurative placentitis
13	*Escherichia coli*	5	M	Holstein	-	Systemic disease with septicemia
14	*Escherichia coli*	3	M	Angus	-	Necrosuppurative placentitis. Multifocal hepatic necrosis. Bacteria inside airways and blood vessels with minimal inflammation
15	*Escherichia coli*	6	F	NI	-	Systemic disease with septicemia
16	*Escherichia coli*	6	F	Holstein	-	Necrosuppurative placentitis
17	*Listeria monocytogenes*	8	M	Braford	-	Necrosuppurative placentitis with large amounts of intravascular bacteria
18	*Listeria monocytogenes*	NI	M	Braford	-	Systemic disease with septicemia. Suppurative bronchopneumonia
19	*Listeria* sp.	7	M	Hereford	Marked placental thickening and deposition of white, friable, and dull material	Necrosuppurative placentitis with large amounts of intravascular bacteria. Fibrinosuppurative bronchopneumonia

M: male; F: female; mo: months (gestational age estimated through the crown-rump length or through farmers' reproductive records); NI: not informed; -: no gross lesion observed; * Case published elsewhere (Henker et al., 2020).

Table 2: Case information and diagnostic techniques used in cases of bovine abortion due to *Aspergillus* infection.

Fetus no*	Fungal isolate or IHC	Gestational age (mo)	Sex	Breed	Gross lesions	Main microscopic findings
20	*Aspergillus fumigatus*	NI	M	Holstein	Raised areas in the skin. Placental thickening and reddening with deposition of yellow material	Hyperkeratosis and intraepidermal pustules. Necrosuppurative and fibrinous placentitis
21	*Aspergillus* sp.	4	NI	NI	Placental thickening and reddening with deposition of yellow material	Necrosuppurative and fibrinous placentitis
22	*Aspergillus* sp.	7	M	Holstein	Raised areas in the skin. Placental thickening and reddening with deposition of yellow material	Necrosuppurative dermatitis and folliculitis, and intradermal pustules. Necrosuppurative and fibrinous placentitis
23	*Aspergillus fumigatus*	9	M	Jersey	Raised areas in the skin. Placental thickening and reddening with deposition of yellow material. Adventitial placentation.	Suppurative dermatitis and folliculitis. Necrosuppurative and fibrinous placentitis

NI: not informed; M: male; IHC: immunohistochemistry; mo: months; Gestational age estimated through the crown-rump length. *All cases were positive for *Aspergillus* spp. IHC

4 ARTIGO 3

Neste item é apresentado o artigo intitulado: "**Bovine abortion associated with** *Staphylococcus aureus* **infection – Case report and characterization of** *S. aureus* **strain isolated from fetal tissues.**" Este artigo foi publicado no periódico científico "*Ciência Rural*" com endereço de DOI: https://doi.org/10.1590/0103-8478cr20190901.

Bovine abortion associated with *Staphylococcus aureus* infection – Case report and characterization of *S. aureus* strain isolated from fetal tissues

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Abstract

Staphylococcus aureus is a gram-positive bacterium, commonly found colonizing the skin and mucous membranes of humans and animals. The present report describes a case of fetal loss associated with *S. aureus* infection in a cow. A six-month old, crossbred male bovine fetus from a beef farm was submitted for necropsy. At gross examination fibrinous pleuropneumonia was observed. Histologically, lesions were restricted to the lungs and consisted of marked multifocal to coalescing areas of inflammatory infiltrate of neutrophils, abundant fibrin exudation, necrosis of bronchiolar epithelium and numerous aggregates of coccoid bacteria. Lung and abomasal fluid bacterial culture yielded pure culture of *S. aureus*, which was characterized as a multidrug resistant strain. Molecular analysis indicated that the studied strain presented several genes of virulence factors including toxic shock syndrome toxin-1 (*tst*), staphylococcal enterotoxin type A (*sea*), Panton–Valentine leukocidin (*pvl*), alpha-hemolysin (*hla*), and delta-hemolysin (*hld*). The present report documents an infrequent case of fetal loss in cattle due to infection with a highly virulent *S. aureus* strain.

Keywords: fetal loss; reproduction; cattle; bacteria, virulence genes.

Resumo

Staphylococcus aureus é uma bactéria gram-positiva, comumente encontrada colonizando a pele e as membranas mucosas de humanos e animais. O presente relato descreve um caso de aborto bovino associado à infecção por *S. aureus*. Um feto bovino, macho, cruzado, com seis meses de idade gestacional proveniente de uma fazenda de gado de corte foi submetido para a necropsia. Pleuropneumonia fibrinosa foi observada na avaliação macroscópica. Histologicamente as lesões encontravam-se restritas aos pulmões e eram representadas por infiltrado inflamatório acentuado, multifocal a coalescente de neutrófilos, acentuada exsudação de fibrina, necrose do epitélio bronquiolar e numerosos agregados bacterianos cocoides. A cultura bacteriana de fragmento de pulmão e líquido do abomaso revelou o crescimento puro de *S. aureus*, que foi caracterizado como uma cepa multirresistente a drogas. Análises moleculares indicaram que a cepa estudada apresentava vários fatores de virulência, incluindo toxina 1 da síndrome do choque tóxico (TSST-1), enterotoxina estafilocócica tipo A (*sea*), leucocidina Panton-Valentine (*pvl*), hemolisina alfa (*hla*) e hemolisina delta (*hld*). O presente relato documenta um caso infrequente de aborto bovino devido à infecção por uma cepa altamente virulenta de *S. aureus*.

Palavras-chave: perda fetal, reprodução, bovinos, bactérias, genes de virulência.

Staphylococcus aureus is a gram-positive, catalase-positive bacterium which is commonly found colonizing the skin, mucous membranes and other sites of healthy carriers, and may be associated with a wide range of clinical conditions in animals and humans (WERTHEIM et al., 2005; PETON & LOIR, 2013). *S. aureus* may carry numerous genes encoding virulence factors, which have been associated to its capacity to evade host immune response and ultimately cause disease (FOSTER, 2005). In cattle, such bacterium is commonly associated with cases of chronic mastitis, leading to significant economic losses in the dairy industry (RAINARD et al., 2017).

Abortion in cattle due to *S. aureus* has been infrequently described, and such event is classified as a sporadic cause of fetal loss in the bovine species (CORBELLINI et al., 2006). Thorough pathological descriptions of *S. aureus*-induced abortions are scarce in the bovine species. Additionally, data characterizing the virulence factors of *S. aureus* involved in these cases are exceedingly limited. Therefore, the objective of the present work is to describe the

gross, histopathological, microbiological and molecular findings of a case of bovine abortion associated with a highly virulent *S. aureus* strain.

In August 2018, a crossbred male bovine fetus was referred for postmortem examination at the Department of Veterinary Pathology, Universidade Federal do Rio Grande do Sul (UFRGS). The fetus was referred from a cow-calf operation beef farm, in the state of Paraná, Southern Brazil. Herd was composed of 2,500 cows raised in grassland, and only sporadic abortions had been previously documented. The aborting animal was a 6-year-old, multiparous Zebu cow (forth calving), with no history of health problems or treatments, which did not show any clinical signs before and after abortion. In the referred farm, vaccination against the main reproductive pathogens used to be routinely conducted, including Bovine herpesvirus I, bovine viral diarrhea and *Leptospira* spp., and the herd overall health status was good. Reproductive management used to be performed with artificial insemination, and natural service was only used in empty cows.

Fetus crown-rump length measured 57cm, compatible with six months of gestation. External examination revealed no abnormalities. Fetal membranes were not available for evaluation. At the necropsy, gross lesions were restricted to the lungs and were characterized by moderate fibrin deposition covering the visceral pleura surface in the pulmonary cranioventral area (Figure 1A). Samples of various organs were collected and fixed in 10% formalin. Fixed tissues were routinely processed, embedded in paraffin wax, and sections (3 - 4 µm) were stained by hematoxylin and eosin. In addition, lung sections were stained with modified Brown-Hopps method.

Histologically, marked multifocal to coalescing areas characterized by inflammatory infiltrate of neutrophils, fewer lymphocytes and macrophages, as well as abundant fibrin exudation, necrosis, accumulation of cell debris and numerous aggregates of 0.5 - 1µm coccoid bacteria were observed affecting alveolar spaces, bronchioles and bronchi (Figure 1B-C). Also, interlobular septa were markedly expanded by fibrin exudation and edema, and abundant fibrin deposition associated with inflammatory infiltrate of neutrophils was seen covering the visceral pleura. No microscopic lesions were detected in other organs. Brown-Hopps staining showed that bacterial aggregates observed in the lungs were gram-positive cocci (Figure D).

Fresh samples were collected aiming to perform microbiological diagnostic. Lung fragments and abomasal fluid were inoculated on 5% sheep blood agar and MacConkey agar

and incubated at 37 °C for 72 h in aerobic and microaerophilic atmosphere. The isolated bacterium was identified by MALDI-TOF mass spectrometry (MS), using Microflex LT instrument and MALDI Biotyper 3.1 software (Bruker Daltonik, Bremen, Germany), and antimicrobial susceptibility test was performed using Kirby-Bauer method accordingly to the Clinical and Laboratory Standard Institute guidelines (CLSI, 2018). Methicillin resistance was verified by conventional PCR for *mecA* gene and by the Kirby-Bauer method using cefoxitin and oxacilin.

Pure white, bright, hemolytic, medium size colonies grew in the blood agar in aerobic and microaerophilic conditions, in both inoculated samples, while no growth was observed from MacConkey agar. The isolated bacterium was classified as a catalase and coagulase positive gram-positive coccus, which was identified by MALDI-TOF as *S. aureus*. This bacterium showed resistant phenotype to tetracycline, gentamicin, ceftazidime, ciprofloxacin, erythromycin, and chloramphenicol, characterizing the isolate as a multidrug resistant (MDR) bacteria. Otherwise, the strain was susceptible to sulfamethoxazole+trimethoprim, penicillin, imipenem and amoxicillin. Besides that, the *S. aureus* showed intermediary susceptibility to oxacillin and cefoxitin. However, the strain was negative to *mecA* gene.

Genomic DNA from the isolated bacterium was extracted and a molecular characterization was conducted by PCR assays to search the following virulence marker genes: toxic shock syndrome toxin-1 (*tst*), staphylococcal enterotoxin type A (*sea*), Panton–Valentine leukocidin (*pvl*), alpha-hemolysin (*hla*) and delta-hemolysin (*hld*), as previously described (Rossato et al., 2018). All the referred virulence marker PCR assays yielded positive results, providing information to assume the highly virulent character of the *S. aureus* studied. In addition, fresh kidney and liver samples were tested for *Leptospira* spp. through PCR as previously described (Ahmed et al., 2012), and thymus and spleen samples were tested for pestivirus (BVDV) (Vilcek et al., 1994), both of which yielded negative results.

Abortion is considered a significant cause of economic losses in livestock systems worldwide and frequently represents a diagnostic challenge (CABELL, 2007). The main differential diagnosis in the present case should include other causes of pleuropneumonia and bronchopneumonia in bovine aborted fetuses, mainly *B. abortus* infection (POESTER et al., 2013), as well as sporadic bacterial agents (ANDERSON et al., 1990). However, based on histological and microbiological examinations, the abovementioned agents may be easily differentiated. Species in the genus *Staphylococcus* that have been implicated with abortion in

cattle include *Staphylococcus lugdunensis (*ARDIGÒ et al., 2014) and *S. aureus* (CLOTHIER & ANDERSON, 2016). *S. aureus* infection has been associated mainly with bronchopneumonia, and rarely with skin lesions in bovine fetuses (CORBELLINI et al., 2006).

In the present case, the lungs were the only affected organs, and large numbers of coccoid aggregates were seen inside bronchioles and bronchi, which may be associated with inhalation of bacteria present in the amniotic fluid (MILLER, 1977). Bacterial infections leading to abortion may be a result of systemic bacterial spread, as well as reproductive tract ascending infections (PARTHIBAN et al., 2015). In the present case, however, it was not possible to infer the route of fetal infection, since no previous disease that could justify fetal spread was detected in the aborting dam.

S. aureus isolated was a methicillin sensitive strain (MSSA), but resistant to several antibiotics. *S. aureus* may express several virulence factors which have been associated with adhesion to host cells, tissue invasion and damage, host immune system escape, disease promotion, cytokine production, and systemic inflammation (FOSTER, 2005). Although well characterized and described in human isolates, to the best of our knowledge, no information regarding virulence factors present in animal abortion associated-*S. aureus* strains is currently available. The identified genes from the *S. aureus* here described encoding toxins, *tst*, *sea*, *pvl*, *hla* and *hld,* have been described as some of the most frequent virulence genes of *S. aureus* (ROSSATO et al., 2018). The expression of these virulence factors is coordinated by cell-communication system (quorum-sensing) in response to population density (KONG et al., 2016).

In conclusion, *S. aureus* should be considered as a sporadic cause of bacterial abortion in cattle, mainly in fetuses presenting fibrinosuppurative pleuropneumonia and bronchopneumonia. In the present case, the isolate was a multidrug resistant strain which presented several genes of virulence factors, indicating that these may play a role in *S. aureus*-induced abortion in cattle.

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Declaration of conflict of interest:

The authors declare that they have no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Authors' contributions

The authors contributed equally to the manuscript.

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Figure 1 – Bovine abortion associated with *Staphylococcus aureus* infection. Gross appearance. (A) The lungs present moderate fibrin deposition covering the visceral pleura surface in the pulmonary cranioventral area. Bar: 4cm. *S. aureus*-induced bovine abortion histopathological evaluation (B) In the lungs, the alveolar spaces show marked inflammatory infiltrate of neutrophils, as well as abundant fibrin exudation, necrotic debris accumulation and numerous aggregates of coccoid bacteria. HE. Bar, 120µm. (C) Large numbers of coccoid bacteria are noted inside a bronchiole intermixed with fibrin exudation. HE. Bar, 350 µm. (D) Numerous gram-positive coccoid bacterial aggregates are seen in the pulmonary parenchyma. Brown-Hopps staining. Bar, 120µm.

5 ARTIGO 4

Neste item é apresentado o artigo intitulado: **"Abortion outbreak in a sheep flock caused by *Toxoplasma gondii* clonal Type III"**. Este artigo foi submetido para publicação no periódico "*Parasitology Research*".

Abortion outbreak in a sheep flock caused by *Toxoplasma gondii* clonal Type III

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Abstract

Toxoplasma gondii is a major cause of reproductive losses in small ruminants in several countries. We describe here an outbreak of *T. gondii*-associated abortion in sheep in Southern Brazil. The flock was comprised of 55 adult sheep, and late-term abortions and stillbirths were detected in 15/36 (41.66%) gestating ewes. Serum samples collected from 45 sheep were tested for *T. gondii* through indirect immunofluorescence assay; IgM and IgG positive results were detected in 44.44% (20/45) and 86.67% (39/45) of the cases, respectively. Four fetuses and two placentas were pathologically evaluated. Gross changes were restricted to fetal membranes and were characterized by multifocal white areas in the cotyledons. Microscopically, these areas corresponded to necrotic foci affecting the chorionic epithelium accompanied by rare cysts of *T. gondii*. The main histological change in fetal tissues consisted of well-demarked and sparsely distributed necrotic foci in the central nervous system. Tissue samples from all four fetuses and one placenta had positive PCR results for *T. gondii*. Restriction fragment length polymorphism (RFLP) genotyping using ten markers (SAG1, 5'-3'SAG2, alt.SAG2, SGA3, BTUB, GRA6, c22-8, c29-2, L358 and PK1) was carried out on one sample, and results were consistent with *T. gondii* clonal Type III (ToxoDB-PCR-RFLP genotype #2, TgCpBr4).

Introduction

Toxoplasma gondii is a cosmopolitan apicomplexan protozoan of global importance that has been associated with a plethora of clinical manifestations in a broad range of hosts, including humans, representing a major public health concern (Tenter et al. 2000; Dubey et al. 2008). *T. gondii* definitive hosts (ie, domestic and wild cats) usually become infected through predation and may excrete oocysts in the feces, contaminating the environment (Tenter et al. 2000). Mammals, including humans and birds, become infected primarily through consumption of food or water contaminated with sporulated oocysts (fecal-oral route) or by ingesting cysts containing bradyzoites through carnivorism (Dubey et al. 2008). The fecal-oral route is the main route of transmission in herbivores (Dubey et al. 2008). Congenital transmission is also a known form of infection in several species (Dubey et al. 2008), and some studies have indicated that this form of transmission may be more common than previously believed in sheep (Williams et al. 2005).

T. gondii has been first documented as a cause of abortion in sheep in 1957 in New Zealand (Hartley and Marshall 1957). Since then, this parasite has been reported as a major cause of reproductive losses in the sheep industry in several countries (Dubey 2009; Moeller 2012; Dorsch et al. 2021; Dorsch et al. 2022), including Brazil (De Moraes et al. 2011; Withoeft 2021). The infection is asymptomatic in non-gestating ewes (Dubey 2009), however, exposure of seronegative sheep flocks to *T. gondii* while ewes are gestating may lead to significant abortion storms and various reproductive losses (Dubey 2009; Edwards and Dubey 2013).

Toxoplasma gondii is classically classified as having three clonal lineages: type I, II and III; all other lineages are grouped as atypical (Su et al. 2010). Molecular techniques, such as restriction fragment length polymorphism (RFLP) genotyping, have been extensively used in the last decades to study *T. gondii* genetic diversity (Dubey and Su 2009; Shwab et al. 2014). Current knowledge indicates relatively limited genetic diversity with predominance of few genotypes of *T. gondii* in Europe and North America, suggesting a clonal population structure (Dardé et al. 2014; Shwab et al. 2014). Nevertheless, *T. gondii* strains identified in Central and South America show high genetic diversity, with detection of numerous atypical or exotic genotypes, without a clear predominance of any particular genotype (Dubey and Su

2009; Dardé et al. 2014; Shwab et al. 2014). Human and animal toxoplasmosis are highly prevalent in Brazil (Dubey et al. 2012); however; information on the molecular findings and strain genotype involved in naturally occurring cases of *T. gondii* abortion in sheep are scarce in the country.

We describe here the epidemiological, serological, pathological, and molecular findings of a spontaneous abortion storm due to infection by *T. gondii* type III strain in sheep in Southern Brazil.

Material and methods

Case report and pathological examination

A sheep flock owner contacted the Setor de Patologia Veterinária, Universidade Federal do Rio Grande do Sul (UFRGS), after observing numerous late-term abortions and stillbirths in the lambing season of 2021 (May – June). The farm was located in the city of Encruzilhada do Sul (30°42'50.4"S 52°34'07.5"W), state of Rio Grande do Sul (RS), Southern Brazil (Fig. 1). This flock was initiated in 2019 when Australian Merino ewes (n = 25) and ram (n = 1) were purchased from a farm located in Dom Feliciano (30°42'00.4"S 52°06'32.7"W), RS. No significant reproductive losses were detected in 2020. Sheep did not have any type of identification, such as ear tags, and reproductive records were limited.

Sheep were kept in areas of native pastures and ewes with nursing lambs had access to cultivated pastures of *Avena sativa* and *Lolium multiflorum*, and were supplemented with corn silage. Apart from the sheep flock, this farm had a beef cattle cow-calf operation, several dogs, and 15 cats; the latter had free access to pastures, sources of water, and feed storage facilities.

In 2021, the flock consisted of 51 ewes, 36 of which shared the facilities with the ram, and were believed to be gestating in the mentioned lambing season (no ultrasonography confirmation). Late-term abortions (around 4 months of gestation) and stillbirths (5 months of gestation, term fetuses) were detected in 15/36 gestating ewes, leading to an estimated abortion rate of 41.66%. The flock owner reported that several lambs were born weak, with difficulty to stand and a few lambs needed assistance to nurse in the first few hours after parturition. Additionally, all aborting ewes and several ewes that delivered weak lambs had retained placentas (Fig. 2a), many of which developed myiasis in the vulva and adjacent skin.

Products of three abortions (four aborted fetuses; fetuses 1 and 2 were twins, and fetuses 3 and 4 were singletons) and two placentas (chorioallantois) were referred for pathological examination at Setor de Patologia Veterinária, UFRGS. Postmortem examinations were routinely conducted, fetal age was estimated through crown-rump measurement (Njaa 2012), and gross changes were recorded. Representative tissue samples of skeletal muscle, eyelids, organs of the abdominal and thoracic cavities, both eyes, and the entire encephalon were collected in all cases. The spinal cord was collected in two cases (fetuses 3 and 4), and the placenta was collected when available (fetus 2 and 3). All samples were fixed in 10% formalin, routinely processed for histopathology, and stained with hematoxylin and eosin. Additionally, fresh samples of several tissues were collected aiming to perform molecular search for *T. gondii* and *Neospora* sp. An on-site visit was performed approximately 2 weeks after the onset of the outbreak aiming to collect flock serum samples.

Serological analysis

During the on-site visit, blood samples were collected by jugular venipuncture from 45 sheep (one ram and 44 ewes, including aborting, gestating, and empty ewes). Due to the lack of sheep identification in the flock, samples were numbered 1-45, and no accurate annotation of which ewe had aborted could be definitely drawn for future correlations.

Detection of immunoglobulin G (IgG) and immunoglobulin M (IgM) for *T. gondii* was performed by indirect immunofluorescence assay (IFA) at Laboratório de Doenças Parasitárias, Universidade Federal de Santa Maria. For detection of these antibodies, antigens were obtained from tachyzoites of *T. gondii* (RH strain) grown in Vero cells (epithelial cells from African green monkeys). All cells were maintained at 37ºC in RPMI 1640 medium supplemented with 10% fetal bovine serum in a 5% CO_2 incubator. Species-specific antibodies conjugated to fluorescein, Anti-sheep IgG (Rabbit Anti-Sheep IgG FITC®, F5137, Sigma-Aldrich, San Luis, Missouri, USA) and Anti-sheep IgM (Rabbit Anti-Sheep IgG FITC®, A130-109F, BETHYL Laboratories INC, Montgomery, Alabama, USA), were used as secondary antibodies. Serum samples known to be positive or negative for the presence of antibodies against *T. gondii* were used as controls. Sheep samples were considered positive when fluorescence was found on the entire surface of the parasite and negative when fluorescence was absent or present only on the apical portion of the parasite. Samples for IgG and IgM were tested at 1:64 dilution; all positive IgG serum samples at this dilution were

submitted to serial dilutions to determine the maximum titer of the reaction, as previously described (Garcia, et al. 1999).

Molecular analysis

Fresh samples of central nervous system (CNS), lung, heart, thymus, spleen, and liver of all four aborted fetuses, and samples of placenta from fetuses 2 and 3 were referred for *T. gondii* and *Neospora* sp. molecular detection at Laboratório de Doenças Parasitárias, Universidade Federal de Santa Maria. DNA was extracted using a Genomic DNA Purification Kit (Promega®, Madison, USA), following the manufacturer instructions. Extracted DNA samples were stored at -20°C prior to PCR.

To detect the presence of *T. gondii* DNA in the samples, PCR was performed using the primers TOX4 CGCTGCAGGGAGGAAGACGAAAGTTG 5'-3') and TOX5 (CGCTGCAGACACAGTGCATCTGGATT 5'-3'), with an expected amplification product of 529bp, following the methodology described by Homan et al. (2000). Negative and positive controls for the reactions consisted of ultrapure water (MilliQ) and tachyzoite DNA from a cell culture of RH reference strain, respectively. PCR amplification conditions consisted of: initial denaturation at 94 °C for 5 min followed by 35 cycles at 94 °C for 30 s, annealing at 63 °C for 45s, extension at 72°C for 45s, and a final extension at 72 °C for 5 min. Agarose gel electrophoresis (2%) was used to separate PCR products, and amplified DNA was viewed under ultraviolet light.

One sample with positive PCR results for *T. gondii* (placenta of fetus 3) was submitted to Multilocus nested PCR-RFLP genotyping using 10 markers: SAG1, 5'-3'SAG2, alt.SAG2, SGA3, BTUB, GRA6, c22-8, c29-2, L358 and PK1, as previously described (Su et al. 2006; Su et al. 2010). Genotype was determined through comparison of the amplified products with positive control strains: GTI, PTG e CTG (Darde et al. 1992). The suggestive genotype was compared with genotypes previously deposited in ToxoDB (https://toxodb.org/toxo/app).

To detect the presence of *Neospora* sp. in the samples, PCR was performed using primers Np21 (3′ AAC ACT ACG ACT TGC AAT CC 5′) and Np6 (3′ GGT TCC TTA GGA CTC CGT CG 5′), with an expected amplification product of 328pb, following the described methodology by Yamage et al. (1996). The negative control for the detection of *Neospora* sp. was ultrapure water (MilliQ) and the positive control used consisted of DNA products extracted from a culture of Vero cells infected with *N. caninum* tachyzoites. The conditions

used for amplification were as follows: initial denaturation 95ºC for 2min, followed by 40 cycles at 95ºC for 1min, 60ºC for 1min and 72ºC for 1 min, with final extension at 72ºC for 2 min. Agarose gel electrophoresis (2%) was used to separate PCR products, and amplified DNA was viewed under ultraviolet light.

Results

Pathological findings

Gross lesions were observed in both placentas (fetus 2 and 3). In the chorioallantois, there were multifocal 0.5 - 4mm white pinpoints on the surface and cut section of the cotyledons (Fig 2b). The intercotiledonary space was not affected. Lambs submitted for examination were term fetuses, with crown-rump length measurement consistent with 5 months of gestation. Partial lung inflation was observed in fetuses 2 and 3, indicating death during or immediately after birth, and fetuses 1 and 4 had collapsed lungs, indicating intra-uterine death. No significant gross lesions were observed in fetal tissues.

Histologically, the white areas in the cotyledons consisted of multifocal, well-demarked areas of marked necrosis, which partially effaced the chorionic epithelium of villi (Fig 2c). These areas had abundant deposition of cell debris, mild fibrin deposition and hemorrhage, mild inflammatory infiltrate of lymphocytes, macrophages, and fewer neutrophils, and multifocal areas of mineralization. Mild diffuse similar inflammatory component was observed in the chorionic epithelium in the remaining areas. Rare parasitic structures measuring from 15 to 20µm in diameter, consistent with *T. gondii* cysts, were observed amidst areas of necrosis in both placentas, and occasionally in the epithelium adjacent to necrotic foci (Fig. 3). In both cases, multifocal areas with large bacterial rods were observed in the cotyledonary and intercotiledonary areas (presumed ascending infection secondary to retained placentas).

All four fetuses had microscopic lesions in the encephalon. These consisted of sparse well-demarcated foci of neuropil necrosis, containing central areas with deposition of hypereosinophilic cell debris, surrounded by a thin rim of glial cells (Fig. 2d). Multifocal areas of gliosis were also observed. Foci of necrosis and gliosis were distributed with paucity, predominantly in the white matter and periventricular areas of the prosencephalon, mesencephalon, and rhombencephalon. Similar changes were observed in the thoracic and lumbar spinal cord segments of fetus 4. Multifocal, mild inflammatory infiltrate of

lymphocytes and plasma cells was observed in perivascular spaces within the neuropil and in the leptomeninges in all cases.

Other histologic changes included multifocal, mild to moderate inflammatory infiltrate of lymphocytes, plasma cells, and macrophages, occasionally accompanied by mild necrosis and rare neutrophils, in the lung (fetus 2 and 3), heart (fetus 2), liver (fetus 3 and 4), skeletal muscle (fetus 4), and adrenal gland (fetus 2). Multifocal areas of mild necrosis were observed in the mucosa of the small intestine of fetus 1, affecting the epithelium lining the tip of villi, accompanied by mild fibrin deposition and inflammatory infiltrate of neutrophils.

Serological and Molecular findings

Out of 45 serum samples tested for *T. gondii* antibodies, 20/45 (44.44%) and 39/45 (86.67%) tested positive por IgM and IgG, respectively. As for the IgG anti-*T. gondii* titer, in 32 samples the titer was 1064 (82.05%; 32/39), in one sample was 512 (2.56%; 01/39), and in six samples was 64 (13.39%; 6/39). All four fetuses had at least one tissue sample with positive PCR result for *T. gondii* (Table 1). The results of Multilocus nested PCR-RFLP carried out in one positive sample (placenta of fetus 3) are shown in Table 2. The genotype was identified as clonal Type III (ToxoDB-PCR genotype #2, TgCpBr4). All tested samples were negative for *Neospora* sp.

Table 1 PCR results of samples from sheep fetuses tested for *Toxoplasma gondii*.

Organ tested	Fetus 1	Fetus 2	Fetus 3	Fetus 4
Liver	-	-	-	-
CNS	-	+	-	-
Heart	+	-	-	-
Pool of spleen and thymus	-	-	-	-
Lung	+	-	+	+
Placenta	NA	-	+*	NA

(+): PCR positive; (-): PCR negative; (NA): not available for testing; CNS: central nervous system; * sample used for restriction fragment length polymorphism (RFLP) genotyping.

Table 2 Multilocus PCR- RFLP (Restriction fragment length polymorphism) typing of the *Toxoplasma gondii* isolate obtained from the placenta of fetus 3.

	Genetic markers									
	SAG1	(5'+3') SAG2	Alt. SAG2	SAG3	BTUB	GR6	c22-8	c29-2	L358	PK1
Isolate 1	II or III	I or III	III	III	III	III	II or III	III	III	III

      The farm owner was contacted on multiple occasions following the outbreaks, and no significant reproductive losses were detected on the lambing season of 2022.

Discussion

      Epidemiological, pathological, serological, and molecular findings corroborated the diagnosis of *T. gondii*-associated abortion storm in a sheep flock in this study. Numerous serological studies, employing different detection techniques and study designs, have demonstrated high exposure to *T. gondii* in Brazilian sheep flocks (Garcia et al. 1999; Dubey 2009; Romanelli et al. 2007). Nevertheless, reports on spontaneous abortion storms or studies with pathological and confirmatory evidence of fetal loss due to this protozoan are somewhat limited in Brazilian sheep (De Moraes et al. 2011; Withoeft 2021) and goat flocks (Pescador et al. 2007; Caldeira et al. 2011).

      Natural exposure to *T. gondii* by nonpregnant sheep is generally believed to result in protective immunity (Dubey 2009). Alternatively, sheep with no previous exposure to *T. gondii* are more susceptible to reproductive losses when infected with the parasite during gestation (Dubey 2009); which may lead to major abortion storms (Edwards and Dubey 2013). In the reported case, although not proven, we hypothesize that purchased ewes may have come from a flock with no significant environmental exposure to *T. gondii*, consequently with no protective immunity against the agent at a flock level. This is corroborated by our

serological results for IgM, which indicated recent exposure for *T. gondii* in almost half of the tested ewes. Similar epidemiological findings have been previously described in *T. gondii* abortion outbreaks in sheep (Edwards and Dubey 2013). Since numerous cats had direct access to the premises where sheep were kept, water sources, and feed storage facilities, environmental contamination by *T. gondii* oocysts could have coincided with the period where a high percentage of ewes were gestating, leading to numerous abortions.

Pathological findings observed were similar to previous descriptions of *T. gondii*-associated abortion in sheep, corresponding to the clinical presentation known as 'classical' abortion from mid-pregnancy (Benavides et al. 2017). In this presentation, abortion is likely to occur 28 days post-infection, with development of white necrotic foci in the cotyledonary placenta and absence of gross lesions in fetal tissues (Benavides et al. 2017). This reiterates the importance of complete submissions in cases of abortion investigations, since the examination of the placenta may yield crucial information to direct and expedite the diagnostic process (Taylor and Njaa 2012). In this outbreak, several ewes likely had retained placentas following the abortion as a consequence of placentitis, which may lead to subsequent metritis and to vulvar and perivulvar myiasis, leading to further economic losses.

Aside from placental findings, all analyzed fetuses consistently presented multifocal necrotic foci and gliosis in the encephalon and one fetus in the spinal cord, which are lesions highly suggestive of protozoal abortion (Moeller 2012; Benavides et al. 2017). Molecular detection of *T. gondii* by PCR was important in these cases, since protozoan tissue forms were rare and solely observed in the placenta. Our findings highlight the importance of evaluating multiple tissues to mitigate the occurrence of false negative results, since PCR failed to detect *T. gondii* in several samples. This may be partly explained by the multifocal distribution of the lesions in tissues and variable detection rate in different organs from aborted fetuses (De Moraes et al. 2011).

N. caninum infection has been reported causing similar pathological findings of protozoal abortion in small ruminants; therefore, representing a major differential diagnosis in cases of abortion due to *T. gondii* infection in these species (González-Warleta et al. 2014). The recommended diagnostic approach in similar cases encompasses the observation of typical pathological findings of protozoal abortion in conjunction with agent confirmation by complementary tests, eg, molecular testing (González-Warleta et al. 2014), as carried out in our study.

Numerous studies on *T. gondii* genetic diversity using different techniques have emerged in the last decades, which has greatly improved the understanding of *T. gondii* genetic population structure across different geographical areas of the world (Dardé et al. 2014). Studies conducted in the 90's analyzing *T. gondii* strains from Europe and North America indicated a predominantly clonal population, in which most isolates could be grouped into three lineages, namely type I, II, and III (Shwab et al. 2014). This classification encompasses the conventional types, and the following discovered variants were considered atypical or exotic genotypes (Shwab et al. 2014). Subsequent studies demonstrated a more complex genetic population genetic structure in certain parts of the world, especially in Central and South America, in which great *T. gondii* genetic diversity occurs (Dubey and Su 2009). Currently, the conventional classification is employed along an additional numeric classification scheme, the ToxoDB PCR-RFLP genotype (Shwab et al. 2014).

The genotype identified in our study was classified as a Type III strain (ToxoDB genotype #2), which is a conventional type highly prevalent in samples from Europe, North America and Africa (Shwab et al. 2014). This genotype is also important and commonly detected in South America, although occurring at a lower frequency (Shwab et al. 2014). Limited information is available on *T. gondii* genotypes detected in cases of abortion in sheep worldwide (Dubey et al. 2009; Edwards and Dubey 2013). An atypical genotype (ToxoDB genotype #32) was detected in tissues of a stillborn lamb from an abortion storm in Texas, using 10 PCR-RFLP markers (Edwards and Dubey 2013). A study conducted in Spain analyzing sheep samples from abortion outbreaks and organs from adult sheep found a predominance of genotype ToxoDB#3 (type II PRU variant); nevertheless, other genotypes, including ToxoDB#3 (clonal type III) and ToxoDB#1 (clonal type II) were also detected at lower frequency. (Fernández-Escobar et al. 2020). No genotyping data from abortion outbreaks in Brazilian sheep flocks was found.

Therefore, this study highlights the importance of *T. gondii* infection as a cause of abortion outbreaks in sheep flocks in Brazil. Additionally, our molecular findings add to the rapidly expanding knowledge on *T. gondii* genotype diversity in Brazil.

Declarations

Conflicts of interest The authors have no competing interests to declare that are relevant to the content of this article.

Availability of data and material (data transparency): all data generated or analyzed during this study are included in this published article.

Author's contributions: All authors contributed to the study conception and design. The Fieldwork and pathological investigations were conducted by Luan Cleber Henker, Bianca Santana de Cecco, Igor Ribeiro dos Santos, Fernanda Genro Cony, Saulo Petinatti Pavarini, and David Driemeier. Serological assays and molecular analysis were performed by Fernanda Silveira Flores Vogel, Fagner D'ambroso Fernandes, and Isac Junior Roman. The first draft of the manuscript was written by Luan Cleber Henker and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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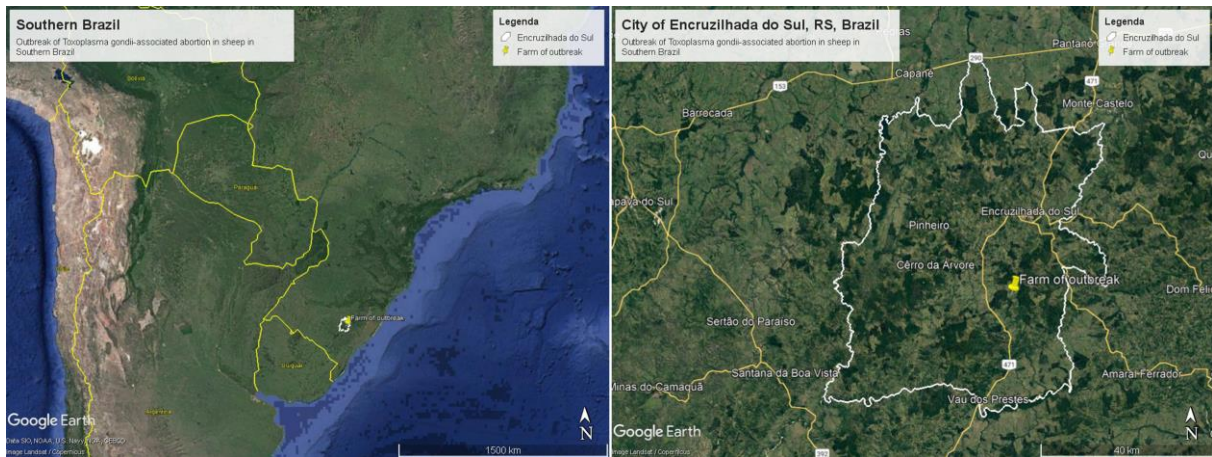
Fig. 1 Maps showing the location where the abortion outbreak due to *Toxoplasma gondii* infection occurred in a sheep flock in Southern Brazil. (Source: Google Earth).

Fig. 2 Abortion outbreak in sheep due to *Toxoplasma gondii* infection. (**A**) Ewe with retained placenta following abortion. (**B**) Gross examination of the placenta (chorioallantois, fetus 2). Multifocal white pinpoints in the cotyledons (arrows). (**C**) Histological examination of the placenta (cotyledon, fetus 2). The white foci in the cotyledons correspond microscopically to well-demarked areas of necrosis with deposition of necrotic debris affecting the chorionic epithelium (arrow). H&E, Bar 450 µm. (**D**) Brain, histological examination. Focal area of neuropil necrosis lined by a thin rim of glial cells (Fetus 2) (arrow). H&E, Bar 150µm.

Fig. 3 Sheep abortion due to *Toxoplasma gondii* infection. A *T. gondii* cyst (arrow) is observed in the cotyledonary placenta of fetus 2, H&E, Obj 100x, Bar 24µm.

6 MATERIAL TÉCNICO

Neste item é apresentado o material didático intitulado: **"Abortamentos e mortalidade perinatal em ruminantes e equinos: o exame de necropsia como ferramenta diagnóstica"**. Este conteúdo será distribuído primariamente na forma de livro eletrônico (*E-book*). Para facilitar a visualização do material, o conteúdo foi disponibilizado como um arquivo adicional.

7 PRODUÇÕES ADICIONAIS

Neste item são listados os demais trabalhos produzidos e publicados (autoria e coautoria) durante o período de desenvolvimento do doutorado (março de 2019 – junho de 2022), na área de diagnóstico em casos de abortamento.

HENKER, L.C.; LORENZETT, M.P.; KELLER, A.; SIQUEIRA, F.M.; DRIEMEIER, D.; PAVARINI, S.P. Fibrinonecrotic placentitis and abortion associated with *Pantoea agglomerans* infection in a mare. **Journal of Equine Veterinary Science**, v. 92, p. 103156-3, 2020. Doi: https://doi.org/10.1016/j.jevs.2020.103156

HENKER, L.C.; LORENZETT, M.P.; PIVA, M.M.; WRONSKI, J.G.; DE ANDRADE, D.G.A.; BORGES, A.S.; DRIEMEIER, D.; OLIVEIRA-FILHO, J.P.; PAVARINI, S.P. Alobar Holoprosencephaly in an Aborted American Quarter Horse Fetus. **Journal of Equine Veterinary Science**, v. 112, p. 103898, 2022. Doi: https://doi.org/10.1016/j.jevs.2022.103898

HENKER, L.C.; LORENZETT, M.P.; PAVARINI, S.P. Bovine congenital babesiosis. **Brazilian Journal of Veterinary Pathology**, v. 14, p. 70-74, 2021. Doi: https://doi.org/10.24070/bjvp.1983-0246.v14i1p70-74

MIONI, M.S.R.; HENKER, L.C.; TEIXEIRA, W.S.R.; LORENZETT, M.P.; LABRUNA, M.B.; PAVARINI, S.P.; DRIEMEIER, D.; ROUSSET, É.; SIDI-BOUMEDINE, K.; THIÉRY, R.; MEGID, J. Molecular detection of *Coxiella burnetii* in aborted bovine fetuses in Brazil. **Acta Tropica**, v. 227, p. 106258, 2021. Doi: https://doi.org/10.1016/j.actatropica.2021.106258

8 CONSIDERAÇÕES FINAIS

- Anaplasmose e babesiose devem ser consideradas diagnósticos diferenciais de abortamentos e mortes perinatais em bovinos, principalmente em regiões geográficas de ocorrência dessas doenças;

- Bezerros afetados (fetos e neonatos) apresentam alterações macroscópicas e microscópicas idênticas as alterações observadas em animais com anaplasmose e babesiose de diferentes faixas etárias;

- Testes moleculares realizados em animais afetados detectaram infecções transplacentárias causadas exclusivamente por um único agente, *Anaplasma* sp. ou *Babesia* sp., e infecções mistas causadas por ambos os hemoparasitas;

- Alterações macroscópicas foram incomuns em casos de abortamentos esporádicos de origem bacteriana e dois padrões microscópicos foram observados: 1) broncopneumonia primária com ocasional disseminação em casos de infecção por *Staphylococcus* sp., *Streptococcus* sp. e *Mannheimia haemolytica*; 2) doença sistêmica com septicemia em casos de infecções por *Escherichia coli* e *Listeria* sp.

- *Aspergillus* sp. foi o principal agente fúngico identificado, e casos de aborto micótico apresentaram placentite, dermatite e pneumonia.

- A placentite foi um achado constante em todos os casos de aborto fúngico/bacteriano em que as membranas fetais se encontravam disponíveis para avaliação;

- No caso de abortamento bovino por *Staphylococcus aureus*, inúmeros marcadores de genes de virulência foram detectados;

- *Toxoplasma gondii* pode estar associado a surtos significativos de abortamento em ovinos. A utilização de uma abordagem diagnóstica ampla envolvendo investigações epidemiológicas, sorológicas, patológicas e moleculares foi essencial para a melhor compreensão do surto de abortamento ovino acompanhado;

- A utilização da técnica de RFLP (Restriction fragment length polymorphism) com pesquisa de 10 marcadores possibilitou a identificação do genótipo de *T. gondii* detectado no surto de abortamento ovino;

- O material técnico ilustrado sobre diagnóstico anatomopatológico em casos de abortamentos em ruminantes e equinos foi elaborado com o propósito de servir como uma ferramenta adicional de consulta para estudantes de graduação, médicos

veterinários, estudantes de pós-graduação e patologistas veterinários. A literatura sobre diagnóstico anatomopatológico em casos de abortamentos é esparsa em língua portuguesa, assim, este material representa um compilado de informações básicas e de fácil acesso a respeito do tema.

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