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Cover illustration: Hippopotamus-like face due to Actinobacillus lignieresii infection in a steer. (Scheid H.V. et al., p.357)

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Original Article
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Causes of abortion in dairy cows in Uruguay

Melissa Macías-Rioseco[2,3], Caroline Silveira[2,3], Martin Fraga[2], Laura Casaux[2,3], Andrés Cabrera[4], María E. Francia[5], Carlos Robello[4], Leticia Maya[6], Leticia Zarantonelli[7,8], Alejandra Suanes[9], Rodney Colina[6], Alejandro Buschiazzo[8], Federico Giannitti[2] and Franklin Riet-Correa[2]*

ABSTRACT.- Macías-Rioseco M., Silveira C., Fraga M., Casaux L., Cabrera A., Francia M.E., Robello C., Maya L., Zarantonelli L., Suanes A., Colina R., Buschiazzo A., Giannitti F. & Riet-Correa F. 2020. **Causes of abortion in dairy cows in Uruguay**. *Pesquisa Veterinária Brasileira 40(5):325-332*. Instituto Nacional de Investigación Agropecuaria, Plataforma de Salud Animal, Estación Experimental INIA La Estanzuela, Ruta 50 Km 11, Colonia, 39173, Uruguay. E-mail: franklinrietcorrea@gmail.com

A case series study was conducted to determine the frequency of causes of abortion in dairy cattle in Uruguay. The sample size of 102 cases was composed of 53 fetuses, 35 fetuses with placentas, and 14 placentas without an associated fetus. All cases underwent gross and microscopic pathologic examinations as well as microbiological and serological testing. The etiology was determined in 54 (53%) of cases, 51 of which were caused by infectious agents. Within the observed 102 cases, 30 (29%) were caused by *Neospora caninum*, six (6%) by *Coxiella burnetii* and two (2%) by *Campylobacter fetus* subsp. *venerealis*. Bovine Parainfluenza-3 virus and *Salmonella enterica* serovar Newport caused one abortion each. Opportunistic bacteria (*Escherichia coli*, *Streptococcus* sp., *Staphylococcus* sp., *Mannheimia* sp., *Trueperella pyogenes*, and *Providencia stuartii*) were associated with 11 abortions. In two cases the fetal death was attributed to dystocia, and in one case the fetus had a congenital mesothelioma. Bovine viral diarrhea virus (BVDV) infection was identified in three fetuses; two of which were co-infected with and had typical lesions of *N. caninum*. No lesions were observed in the other fetus infected by BVDV. *Leptospira interrogans* was identified in one fetus without lesions. Despite the relatively low overall success rate in establishing an etiological diagnosis in cases of abortion in cattle, a systemic workup of bovine abortion is necessary to establish prevention and control strategies. This also facilitates monitoring and surveillance of reproductive diseases in dairy cattle, some of which represent a risk to public health.

INDEX TERMS: Dairy cattle, Uruguay, bovine abortion, *Campylobacter fetus*, *Coxiella burnetii*, *Neospora caninum*, cattle.

RESUMO.- [**Causas de aborto em bovinos de leite no Uruguai.**] Uma série de casos foi estudada para determinar a frequência de causas do aborto em bovinos leiteiros no Uruguai. A amostra, de 102 casos, foi composta por 53 fetos, 35 fetos com placentas e 14 placentas sem feto associado. Todos os casos foram submetidos a exames patológicos macroscópicos e microscópicos, além de testes microbiológicos e sorológicos. A etiologia foi determinada em 54 (53%) dos casos, 51 dos

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quais foram causados por agentes infecciosos. Nos 102 casos observados, 30 (29%) foram causados por *Neospora caninum*, seis (6%) por *Coxiella burnetii* e dois (2%) por *Campylobacter fetus* subsp. *venerealis*. O vírus da Parainfluenza-3 e *Salmonella enterica* serovar Newport causaram um aborto cada. Bactérias oportunistas (*Escherichia coli, Streptococcus* sp., *Staphylococcus* sp., *Mannheimia* sp., *Trueperella pyogenes* e *Providencia stuartii*) foram associadas a 11 abortos. Em dois casos, a morte fetal foi atribuída a distocia e, em um caso, o feto apresentava mesotelioma congênito. A infecção pelo vírus da diarreia viral bovina (BVDV) foi identificada em três fetos; dois dos quais foram co-infectados e apresentavam lesões típicas de *N. caninum*. Não foram observadas lesões no outro feto infectado pelo BVDV. *Leptospira interrogans* foi identificada em um feto sem lesões. Apesar da relativamente baixa taxa de sucesso no diagnóstico etiológico nos casos de aborto em bovinos, é necessário o diagnóstico sistemático dos abortos para estabelecer estratégias de prevenção e controle. Isso também facilita o monitoramento e a vigilância de doenças reprodutivas em bovinos leiteiros, algumas das quais representam um risco para a saúde pública.

TERMOS DE INDEXAÇÃO: Aborto bovino, bovinos de leite, Uruguai, *Campylobacter fetus*, *Coxiella burnetii*, *Neospora caninum*.

INTRODUCTION

In Uruguay, dairy production is one of the most important economic activities of the agricultural sector. Abortion in dairy cattle increases the cost of reproduction, medical treatments, feeding, and culling and replacement rates (De Vries 2006). Abortions are gestational losses that occur between day 45 of pregnancy and the end of gestation (Campero et al. 2018). There is little information available about the prevalence of abortion in dairy cattle in South America. A study from Brazil examined 161 aborted bovine fetuses from Rio Grande do Sul and the cause of abortion was determined in 51.5% of the cases (Corbellini et al. 2006). *Neospora caninum* was the most commonly detected agent (23% of the cases) followed by bacteria in 17.4%, fungi in 3.1% and viruses in 1.8% (Corbellini et al. 2006). The most recent study from Argentina, examined 150 bovine fetuses between 2004 and 2006 (Morrell 2010). The studied population was composed of dairy (23.4%) and beef (69.2%) cattle; the production class was unknown in the remaining cases (7.4%). The diagnosis was determined in 52% of the cases with *N. caninum* (14.7%), *Campylobacter fetus* (9.3%), *Leptospira* spp. (7.3%), and *Brucella abortus* (6.7%) being the most common agents. An etiological diagnosis was not achieved in the remainder 48% of the cases, although 25.3% of these had microscopic lesions suggestive of an infectious cause (Campero et al. 2018).

In Chile, a longitudinal study of 20 years examined a total of 494 bovine fetuses from 270 farms. Forty-eight percent of cases were from dairy farms, 10.1% from beef farms, and the rest from mixed production farms. The etiology was determined in 59.7% of the cases, and 52.2% of the cases had an infectious cause. The most commonly identified agent was *Leptospira* spp. in 25.2%. About 22% of the cases had microscopic lesions compatible with *N. caninum*, and *B. abortus* was detected in 14.3%. The two main viral agents reported were bovine herpesvirus-1 in 13.4% and bovine viral diarrhea virus (BVDV) in 7.7% of the infectious cases (Meyer Zarzar 2013).

Infectious etiologies are among the most commonly reported abortigenic agents in cattle in Uruguay. A study conducted from 2002 to 2004 analyzed 431 aborted bovine fetuses from dairy (54%) and beef (46%) farms. In 41% of the cases with diagnosis, the abortion was attributed to leptospirosis based on the detection of leptospiral maternal antibodies, and *N. caninum* was identified in 36%. *Campylobacter fetus* was reported in 13% of the cases, one of which, based on microscopic observation, was determined as coinfected with *Tritrichomonas foetus*. The bacterial etiology with the lowest reported frequency was *B. abortus* in 3% of the cases. Lastly, about 23% of the examined fetuses did not have any macroscopic or microscopic lesions and were of undetermined cause (Easton 2006).

The diagnosis of bovine abortion is complex, and the diversity of causes cannot be identified despite extensive laboratory testing (Antoniassi et al. 2013, Clothier & Anderson 2016). Bovine abortion has been considered a syndrome because of the complexity of their causes (Anderson et al. 1990, Campero et al. 2003). Fetal and placental autolysis is common, precludes the observation of lesions interfering with the successful identification of infectious agents. The circulation of more than one abortigenic agent in the same herd could represent another difficulty in the diagnosis of bovine abortion, particularly in epidemic outbreaks (Macías-Rioseco et al. 2019a). Likewise, bacterial cultures from fetal tissues and placentas are frequently contaminated with non-pathogenic or opportunistic bacteria (Clothier & Anderson 2016), making interpretation of results difficult, particularly in cases without lesions typical of bacterial infection. Nonetheless, a plethora of diagnostic approaches such as necropsy, histology, immunohistochemistry, bacteriology, virology, immunology and molecular biology assays, aid in the diagnosis of bovine abortion.

Achieving the etiologic diagnosis in bovine abortion is challenging mainly when adequate diagnostic tests are not available, particularly when caused by infrequent or opportunistic agents, or agents that are nonculturable by traditional microbiological methods. A study of 655 cases of bovine abortion in California, revealed that about 20% of the examined fetuses did not have any specific lesions (Clothier & Anderson 2016). Moreover, about 11.7% of these cases had macroscopic and/or microscopic lesions (abomasitis, pleuritis, peritonitis, hepatitis, splenitis, myocarditis, encephalitis and thymitis) with no pathogens identified. When a lesion is identified but there is no etiology found, infectious agents that are rarely detected in cases of abortion or difficult to isolate should be considered. The ideal diagnostic tests may be unavailable or perhaps the appropriate samples are not submitted to the laboratory for testing. Ideal samples for bovine abortion investigation are the aborted fetus, placenta, and serum from aborted and matched pregnant non-aborted dams. The objective of this work was to identify and determine the relative frequency of etiologies in cases of abortion in dairy cattle submitted to a veterinary diagnostic laboratory in Uruguay from 2015 to 2018.

MATERIALS AND METHODS

Case selection and case definition. From January 1st, 2015 to November 1st, 2018, bovine abortion cases were processed at the veterinary diagnostic laboratory of the Platform of Animal Health, at the National Institute of Agricultural Research in La Estanzuela experimental station, Colonia, Uruguay. Cases were either submitted to the laboratory by veterinary practitioners or collected directly from the dairy farms by our team upon the practitioner's request. Cases were defined as: 1) an aborted fetus; 2) an aborted fetus with its placenta; and 3) a placenta from an animal that aborted. Cases submitted from the same farm at the same time and composed of two or more fetuses and/or placentas from different dams were categorized as different cases. The etiologic diagnosis was determined by the association of the identified pathogen and the presence of compatible gross and/or microscopic lesions (see section on diagnostic tests below). Cases with an identified pathogen but with no lesions, and cases with lesions but with no causative agent identified were categorized as abortion cases of undetermined etiology. Information on sex, date of submission, geographic location of the farm, breed and age were obtained from the veterinarians and farmers. The fetal age in days was estimated based on the crown-to-rump length and other gross characteristics of the fetuses, and further categorized in first, second or third trimester of gestation. Cases were also categorized by the degree of autolysis as mild, moderate or severe.

Pathologic examination. Macroscopic evaluation of the placentas and fetuses were done by veterinary laboratory diagnosticians with pathology training. Samples of liver, kidneys, adrenal glands, spleen, lymph nodes, thymus, lungs, heart, brain, skeletal muscles, forestomachs, abomasum, cecum, spiral colon, duodenum, jejunum, ileum, and placenta (when available), were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4-6 µm, and stained with hematoxylin and eosin for microscopic examination. Depending on the initial microscopic examination and assessment of lesions and/or results of other diagnostic tests, and immunohistochemical (IHC) procedures for the detection of *Coxiella burnetii*, *Chlamydia* spp., BVDV, bovine parainfluenza-3 virus, and/or *N. caninum* were performed in selected tissues in some cases (see below). At necropsies, samples were collected for bacteriology, molecular biology, and serologic testing (see below).

Diagnostic tests. Placenta, liver, lung, and abomasal fluid from all cases were inoculated onto blood and MacConkey agars (Oxoid, Basingstoke, Hampshire, England) and incubated at 37°C in aerobic conditions. In addition, samples were also inoculated onto Skirrow agar (Oxoid, Basingstoke, Hampshire, England) and incubated in anaerobic jars (CampyGenTM, Oxoid, Basingstoke, Hampshire, England), at 37°C for 2 to 7 days in an atmosphere of 5-10% of oxygen and 5-10% carbon dioxide (Chaban et al. 2013). In the cases where bacterial isolates were obtained, bacterial identification was performed either by routine biochemical test or by sequencing the16S rRNA. Placenta, liver, kidney, aqueous humor, and abomasal fluid were also spiked into Leptospira Medium Base Ellinghausen, McCullough, Johnson and Harris (EMJH) and incubated in aerobiosis at 29°C for up to 6 months (Zarantonelli et al. 2018). Fetal pericardial fluid (when available) was analyzed by microagglutination test (MAT) to detect antibodies against *Leptospira* serovars Grippotyphosa, Icterohaemorrhagiae, Pomona, Canicola, Hardjo Bovis, Hardjo Prajitno and Wolfii at a cutoff point of 1/10 (Zarantonelli et al. 2018).

PCR for the lipL32 gene of pathogenic *Leptospira* species was done from the 102 cases, as homogenates of liver, kidney and, placenta (when available). The primers LipL32F (5'-ATCTCCGTTGCACTCTTTGC-3') and LipL32R (5'-ACCATCATCATCATCGTCCA-3') were used. The mix was exposed at 95°C for 5 min, then 35 cycles of 30 sec at 95°C, for denaturation, and 30 sec at 58°C, 60 sec at 72°C, and 7 min at 72°C for annealing and extension (Zarantonelli et al. 2018).

Direct immunofluorescence (DIF) for *Campylobacter fetus* was done on impression smears from all placentas, abomasal fluid, lung, and liver. Smears were fixed in ethanol at room temperature and incubated with an anti-*Campylobacter* antibody conjugated with fluorescein isothiocyanate (FITC) (Biotandil, Tandil, Buenos Aires, Argentina) (Figueiredo et al. 2002). Similarly, DIF for *Leptospira* spp. was performed on impression smears from kidney, liver, aqueous humor, and placenta using LEP-FAC multivalent rabbit FITC-bound antibody (NVSL, Ames/IA, USA). The smears were visualized in a fluorescence microscope (AxioLab.A1, Carl-Zeiss, Germany) set at wavelengths of 495 nm excitation and 519 nm emission.

Additionally, abomasal fluid or placenta smears were examined directly under dark field microscopy to assess for agents morphologically compatible with *Campylobacter* spp., *Leptospira* spp. and *Tritrichomonas foetus*. PCR for bovine herpesvirus-1 (BHV-1) and RT-PCR for BVDV were performed in pools of liver, lung, spleen, kidney, thymus, heart, brain, placenta, lymph node, and adrenal gland obtained individually in all cases that included fetuses. For BVDV, the primers V324mod (5'- ATGCCCWTAGTAGGACTAGCA-3') and V326mod (5' -WCAACTCCATGTGCCATGTAC-3') were used based on Maya et al. (2016). Briefly, the mix was exposed at 95°C for 10 min, then 45 cycles of 10 sec at 95°C, for denaturation, and 60 sec at 50°C for annealing and extension. The primers gCBoHV F (5'-GCGGGGGCTCGCCGAGGA-3') and gCBoHV R (5'-GGAGCGCACGGTCAGGGGC-3') were used for BHV-1 PCR. The mix was exposed at 95°C for 5min, then 35 cycles of 30 sec at 95°C, for denaturation, and 30 sec at 60°C for annealing, 1 min at 72°C for extension and lastly, 10 min at 72°C (Silva et al. 2007).

The IHC for BVDV antigen detection was done only in cases with a positive RT-PCR result for this virus. Heat induced antigen retrieval was performed in a decloaking chamber (Biocare Medical). A mouse IgG anti-BVDV (VMRD, Pullman/WA) was applied as primary antibody, followed by anti-mouse IgG horseradish peroxidase (HRP)-labeled polymer (DAKO, Santa Clara/CA), and 3-amino-9-ethylcarbazole (AEC, DAKO, Santa Clara/CA) as the chromogen. In one fetus with pneumonia with syncytial cells, RT-PCR for bovine parainfluenza-3 (BPI-3) virus was done from frozen samples of lung and the amplification products were sequenced, as we have previously described for this same case (Macías-Rioseco et al. 2019b).

Based on the presence of compatible lesions (necrotizing and suppurative placentitis with intratrophoblastic bacteria), IHC for *C. burnetii* and *Chlamydia* spp. were done in selected cases (Dilbeck & McElwain 1994, Giannitti et al. 2016). When a positive result for *C. burnetii* by IHC was obtained, PCR was used for detection of this pathogen in the formalin-fixed paraffin-embedded (FFPE) blocks containing placenta (Lorenz et al. 1998). The IHC procedures for *Chlamydia* spp. and *C. burnetii* and the PCR in FFPE placenta in these same cases has recently been published by our group (Macías-Rioseco et al. 2019c).

Immunohistochemistry and PCR for detection of *N. caninum* antigen and DNA, respectively, were done only in cases with compatible histologic lesions in the brain, heart, placenta and/or liver. For the IHC, a goat polyclonal antibody (VMRD, Pullman, WA) against *N. caninum* was used as a primary antibody, anti-goat IgG horseradish peroxidase (HRP)-labeled polymer (Vector polymer enzyme detection kit, Burlingame/CA) as the secondary antibody, and 3-amino-9-ethylcarbazole as the chromogen (DAKO, Santa Clara/CA). The PCR for *N. caninum* was done following the

procedure described by Yamage et al. (1996). PCR products were visualized on 1% agarose gel electrophoresis stained with SYBR safe (Invitrogen, USA), purified with QIAquick PCR Purification Kit and sequenced in house at the sequencing service of the "Institut Pasteur de Montevideo", Uruguay. All tests were performed with appropriate positive and negative controls for each run.

Statistical methods. The results of the pathologic examinations and diagnostic tests, as well as the fetal gestational age, degree of autolysis and the department where the dairy farm was located were recorded in a database using Microsoft Excel, and descriptive statistics were obtained.

RESULTS

The series was composed of 102 cases; 53 of the cases were only fetuses, 35 were placentas with fetuses, and 14 were placentas only. The 102 cases were submitted from 45 different farms. The maximum number of cases submitted from the same farm was 28. Most of the cases were submitted from the department of Colonia with 58 cases, followed by San Jose (17), Canelones (6), Lavalleja (4), Soriano (2), Florida (2), and Rio Negro (2). The department was not recorded in 11 cases. The degree of autolysis was recorded in 95 cases: 59 had mild autolysis, 28 had moderate autolysis, and eight were severely autolyzed, five of these were mummified fetuses for which no etiology was determined. Forty-two fetuses were in the second trimester of gestation at the time of the abortion, followed by 33 cases in the third trimester and five in the first trimester.

The etiology of the abortion was determined in 54 (53%) cases, while the cause was undetermined in 48 (47%). Of the cases with undetermined etiology, 11 (23%) had inflammatory and/or necrotizing lesions in various tissues suggesting an infectious process, although no agent could be identified in the tissues by the set of diagnostic tests performed in the study. Of the 54 cases that had an etiologic diagnosis, 51 (94.4%) were caused by infectious agents. Thirty-eight of the 51 (74.5%) were caused by agents that are primarily recognized as reproductive pathogens, one was caused by BPI-3 virus and another by *Salmonella enterica* serovar Newport. The remainder 11 cases (21.5%) were associated with opportunistic bacteria. The 38 cases caused by reproductive pathogens included *N. caninum* (30 cases), *C. burnetii* (6 cases), and *C. fetus* subsp. *venerealis* (2 cases). Of the 11 cases caused by opportunistic pathogens, *Escherichia coli* was identified in 4 cases, *Streptococcus* spp. in 2 cases, and *Streptococcus pyogenes*, *Staphylococcus* sp., *Trueperella pyogenes*, *Providencia stuartii*, and *Mannheimia* sp. were identified in one case each. Placentitis, bronchopneumonia, and/or hepatitis were the main histologic lesions observed in association with these opportunistic agents. Regarding the three cases with non-infectious causes (5.6% of the 54 cases with an identified cause), in two cases the death of the fetuses was due to dystocia and in one case the abortion was caused by a congenital mesothelioma affecting the abdominal and pelvic viscera that was extensive enough to be considered incompatible with life. The percentage of diagnosis was variable according to the material sent to the laboratory. When only the fetus was sent, the diagnosis was made in 50.9% of the cases; when the fetus and placenta were received the diagnosis was made in 62.8% of the cases, and when only the placenta was available the diagnosis was made in 37.7% of the cases (Table 1).

The 30 abortions caused by *N. caninum* (55.6% of the 54 cases with determined etiology) were from different dairy farms, accounting for 53.3% of the 45 farms included in the study. Of the six abortions caused by *C. burnetii*, five were from the same farm. *Campylobacter fetus* subsp. *venerealis* was the etiology in 2 cases from different farms.

The number of cases submitted per farm varied from one to 28, and the number of causes of abortion diagnosed on each farm varied from one to four (Fig.1). In farm 42, four different etiologic agents causing abortion were identified: *N. caninum* in two cases, *C. burnetii* in five, *Salmonella enterica* serovar Newport in one, and BPI-3 virus in another. In farm 43, five aborted fetuses were sent to the laboratory during an abortion outbreak, one abortion was caused by *C. fetus* subsp. *venerealis* and two by *N. caninum*; in the other two fetuses the cause of abortion was undetermined (Macías-Rioseco et al. 2019a). In farm 1, one abortion was caused by *N. caninum*, another by *E. coli*, and one was due to dystocia. In farm 17, an etiologic diagnosis could only be confirmed in one of nine examined cases.

The cases caused by *N. caninum* corresponded to abortions within the second trimester of gestation. The 30 fetuses aborted by neosporosis had typical lesions including non-suppurative encephalitis and gliosis (29 fetuses), myocarditis (24), myositis (diaphragm or tongue) (20) hepatitis (15), interstitial nephritis (10) and/or interstitial pneumonia (6). Additionally, placenta was submitted along with the fetus in 10 of these 30 cases, 7 of which had placentitis. The six abortions caused by *C. burnetii* corresponded to full-term gestations and had moderate to severe multifocal necrotizing and suppurative placentitis with intralesional and intratrophoblastic bacteria, and only one case had mild neutrophilic alveolitis. No cases of coxiellosis were diagnosed in cases where no placenta was available for examination. One case of abortion due to *C. fetus* subsp. *venerealis* had a non-suppurative fibrinous epicarditis and myocarditis; the placenta was not available for examination. The other case of *C. fetus* subsp. *venerealis* abortion had suppurative placentitis with arteriolitis and fibrinoid necrosis, neutrophilic bronchiolitis and alveolitis along with neutrophilic and histiocytic portal hepatitis. Suppurative and necrotizing placentitis was observed in cases associated with *E. coli*, *T. pyogenes*, and *S. enterica* serovar Newport. The latter also had intralesional coccobacilli in the placenta, along with minimal to mild neutrophilic lymphadenitis. In one case caused by *Staphylococcus* sp., the agent was isolated from the skin and from abomasal fluid, the fetus had diffuse hyperkeratosis, neutrophilic, histiocytic and fibrinous synovitis, along with moderate non-suppurative interstitial pneumonia

Table 1. Percentages of cases with diagnosis and without diagnosis within type of sample submitted

Sample submitted	With diagnosis	Without diagnosis	Total
Fetus	27 (50.9%)	26 (49.1%)	53
Fetus and placenta	22 (62.8%)	13 (37.2%)	35
Placenta	5 (35.7%)	9 (66.3%)	14
Total	54	48	102

with neutrophilic alveolitis and mild non-suppurative meningoencephalitis. One case caused by *Mannheimia* sp. had a moderate lymphohistiocytic and neutrophilic placentitis with multifocal trophoblastic necrosis. BPI-3 virus caused multifocal neutrophilic and histiocytic alveolitis (pneumonia) with a moderate number of syncytial cells in the lungs and intestines in one case (Macías-Rioseco et al. 2019b). In cases with lesions but without an identified etiology, the lesions consisted of myocarditis, myositis, glossitis, cerebral gliosis, nephritis, pneumonia, and hepatitis.

Brucella abortus was not isolated in any case. Other bacterial agents such as *Acinetobacter lwofii*, *Aerococcus urinae*, *Providencia* sp., *Yersinia* sp., *E. coli*, *Enterobacter* sp., *Corynebacterium* sp. and *Serratia* sp. were isolated on bacterial cultures, but due the absence of associated lesions expected for bacterial infections, these abortions were classified as of undetermined etiology. The gene lipL32 of *Leptospira* spp. was detected in one sample of liver by PCR, the causality of the abortion was not attributed to this agent based on the lack of fetal lesions generally associated with leptospirosis. None of the tested fetal pericardial and/or thoracic fluids were reactive at the cutoff reference point for MAT for *Leptospira* spp. antibodies, including the case that was PCR-positive for this agent. *Leptospira* spp. culture was negative in all cases. PCR for BHV-1 was negative in all cases. RT-PCR for BVDV was positive in three cases; two of them were in co-infection with *N. caninum*, and the protozoon was determined as the causal agent based on the presence of typical lesions and the positive results for *N. caninum* PCR and IHC. Due the absence of lesions, the etiology on the third BVDV-positive case was categorized as undetermined. Two of the three BVDV PCR-positive fetuses were aborted in the second trimester and the other in the third trimester of gestation. To assess whether these fetuses congenitally infected with BVDV harbored high antigenic viral loads and thus were persistently infected, IHC for BVDV antigen detection was performed in several tissues (brain, lung, heart, thymus, liver and small intestine), with negative results in all three cases (data not shown). Based on these results, we interpreted that the fetuses were suffering from transient BVDV infections.

DISCUSSION

In this case series, the most common cause of abortion was *N. caninum*. A previous study in Uruguay, showed a seroprevalence of *N. caninum* of 22% in dairy cows and 92% of the herds (Piaggio 2006). Our series was composed mainly of fetuses of gestational age in the second trimester, which may be explained in part by the high frequency of neosporosis. Abortions due to *N. caninum* are most commonly seen during the second and third trimesters of gestation (McAllister 2016). Our results and the high proportion of farms with seropositive cattle to *N. caninum* suggest that abortions caused by this agent potentially occur in most farms (Silveira 2019).

Coxiella burnetii is rarely reported as a cause of abortion in cattle (Agerholm 2014). In our study, this agent was observed as a sporadic cause of abortion (only one case in one dairy farm) and as a cluster of five cases within a period of five months in another farm (Macías-Rioseco et al. 2019c). We were able to identify abortions due to coxiellosis based on the microscopic

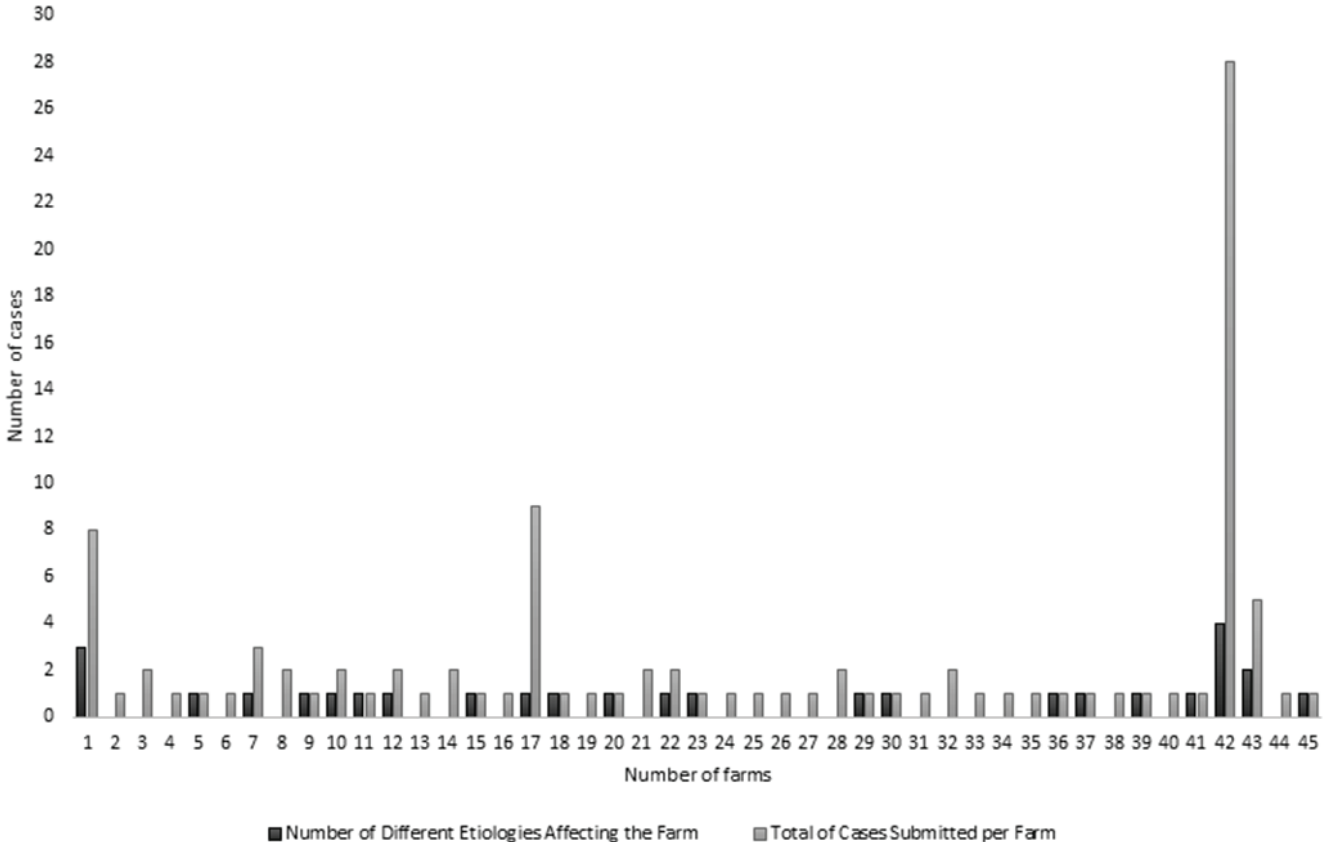


Fig.1. Frequency of different etiologies affecting each farm and total number of cases submitted per farm.

evaluation of the placenta followed by IHC along with PCR for *C. burnetii*. In all cases, the diagnosis of coxiellosis was based on the presence of necrotizing and suppurative placentitis with intralesional and intratrophoblastic bacteria identified as *C. burnetii*. The presence of lesions associated with the bacteria is key to the diagnosis of coxiellosis (Bildfell et al. 2000, Hazlett et al. 2013) since *C. burnetti* can be identified by PCR in the placenta of ruminants without being the cause of abortion (Hazlett et al. 2013).

These results suggest that *C. burnetii* is a previously undiagnosed cause of abortion in dairy cattle in Uruguay and highlight the importance of examining the placenta in aborted cattle to achieve this diagnosis. The presence of antibodies against *C. burnetii* in slaughterhouse workers from Uruguay has been associated with history of clinical signs, and a clinical case of endocarditis (Moreira et al. 1987), showing that this agent is an occupational hazard for veterinarians and slaughterhouse workers. Also, antibodies against *C. burnetii* have been found in different animal species in this country, including cattle (Moreira et al. 1987).

Campylobacter fetus subsp. *venerealis* was observed in relative low frequency (2 cases of 54 with an identified etiology), which could be due the fact that this venereal transmitted agent is usually a cause of infertility and causes abortion only sporadically (Michi et al. 2016, Silva et al. 2007). Abortions are more commonly detected between the fourth and sixth months of gestation (Silveira et al. 2018). A national survey that evaluated 340 dairy farms identified that about 50% of the farms use only natural breeding with bulls, and an additional 20% use a combination of natural breeding and artificial insemination (INALE 2014). This suggest that campylobacteriosis may still be a problem in dairy farms in Uruguay.

As seen in this study, several causes of abortion were diagnosed in some farms, which indicates that the diagnosis of a primary cause of abortion, including neosporosis, campylobacteriosis, or coxiellosis, does not exclude other causes of abortion. Hence, it is recommended to attempt the diagnosis in as many cases as possible in order to increase the chances of detecting other abortifacients and better understand the situation of each farm.

In our case series, only one of the tested fetuses was positive for *Leptospira* spp. by PCR on a liver sample Sequencing confirmed that the infection corresponded to the species *L. interrogans*. Fetal pericardial and/or thoracic fluids were antibody-negative by MAT in all fetuses, including the PCR-positive one. DIF and/or dark field microscopy for the detection of leptospires were also negative in all fetal imprints and abomasal fluids. While *Leptospira* spp. cannot be excluded as a relevant abortigenic pathogen in cattle in Uruguay, our study did not allow for confirmation of *Leptospira* spp. as a cause of abortions following the diagnostic criteria and case definition we established. Many *Leptospira* species and serovars have recently been isolated from bovine urine and blood samples in Uruguay, including *L. interrogans* serogroup Pomona serovar Kennewicki (20 strains), *L. interrogans* serogroup Canicola serovar Canicola (1 strain), *L. borgpetersenii* serogroup Sejroe serovar Hardjo (10 strains) and *L. noguchii* (9 strains, belonging to a variety of serogroups) (Zarantonelli et al. 2018). According to that study, 20% of the almost 1,000 sampled cows were eliminating *Leptospira* spp. in the urine (Zarantonelli et al. 2018). It is striking that with such a high number of animals eliminating leptospires in the urine, the present study did not find abortions caused by leptospirosis. It cannot be ruled out that *Leptospira*-induced abortions might occur with no detectable infection of the fetus or placenta and be caused by other mechanisms including the pathogen-triggered inflammatory cascade (Raghupathy 2001, Zi et al. 2015). Examples of such abortigenic scenarios include subclinical infections in the mother due to urinary tract infection (Schieve et al. 1994), periodontitis (Zi et al. 2015), bacterial vaginosis (Giakoumelou et al. 2016), among other pathologies. In animals such inflammatory processes linked to premature birth or miscarriage have also been described e.g. in bovine viral diarrhea (Moennig & Liess 1995), suggesting that different pathogen strains can produce different clinical outcomes. The role of the different *Leptospira* species found in the urine of healthy cattle as a cause of sporadic abortions and other reproductive failures should thus be further investigated (Zarantonelli et al. 2018). In the present series of abortions, most cases corresponded to sporadic abortions and only few outbreaks were studied. It is possible that infections by some *Leptospira* species and/or specific serovars may cause abortion outbreaks in herds, which probably were not subjected to confirmatory laboratory investigation during the period of this study. Establishing a surveillance system to study abortion outbreaks in Uruguay seems important to better understand the pathogenesis, epidemiology and best diagnostic techniques for leptospirosis and other abortive diseases in the country.

Viral abortions in cattle are reported in low frequencies in several studies (Vaucher et al. 2011, Clothier & Anderson 2016). In this case series, one abortion was caused by BPI-3 virus. This agent had been previously isolated from a bovine fetus and the case diagnosed in our study has been published elsewhere (Macías-Rioseco et al. 2019b). Abortions due to BHV-1 and BVDV were not observed in this series, although BVDV RNA was detected in three fetuses. None of the three cases had lesions compatible to BVDV, and IHC reactivities for BVDV antigen in liver, small intestine and brain were negative. The molecular detection of the pathogen confirms the circulation of the virus in these herds. Even though it seems that BVDV was not responsible for the abortions in these cases, it is important to test affected herds to identify persistently infected animals and the possibility of the occurrence of other forms of BVDV-associated diseases in the farms. Two of the positive BVDV animals were also positive for *N. caninum* by PCR and/or IHC and in fact had tissue lesions consistent with neosporosis, that was considered the primary cause of the abortions. It has been suggested that BVDV infections allow other pathogens to easily cross the fetoplacental barrier, increasing the risk of abortion (Murray 1991, Quinn et al. 2004).

One abortion in our series was due to *Salmonella* Newport, which is rarely reported causing abortions (Campero et al. 2018). In this farm the serovar Newport also caused neonatal calf diarrhea and neonatal mortality due to septicemia during the same period (data not shown), indicating that the abortion was part of the spectrum of diseases typically associated with salmonellosis in dairy cattle and not an isolated event. In cattle, *Salmonella enterica* serovars Typhimurium, Dublin, and Newport are the most commonly cause of salmonellosis.

The clinical disease can be enteritic and/or septicemic, the latter can result in abortion in pregnant cattle (Uzal et al. 2016, Campero et al. 2018, Costa et al. 2018). Abortions due to *Salmonella* are mostly associated with *Salmonella* Dublin (Campero et al. 2003), and less frequently, with *S*. Typhimurium (Easton 2006).

Other bacteria such us *E. coli*, *Streptococcus* spp., *T. pyogenes*, *Staphylococcus* spp. and *Mannheimia* spp. have previously been recognized as sporadic abortifacients in cattle, and as in other reports, were associated with suppurative lesions in the placenta, lungs, and occasionally other fetal tissues (Anderson et al. 1990, Campero et al. 2003, Clothier & Anderson 2016, Campero et al. 2018). One case was attributed to a congenital neoplasia (mesothelioma). While congenital neoplasia in cattle is very uncommon, mesothelioma is within the most frequently diagnosed cancers in bovine fetuses. Although mesotheliomas are of mesenchymal origin, they should be differentiated from disseminated metastatic adenocarcinomas. In adults the occurrence of mesotheliomas has been associated with exposure to asbestos, while this is less clear in fetuses (Peli et al. 2018).

CONCLUSIONS

Neosporosis is the main cause of abortions in the dairy cattle population that we studied.

Coxiellosis is a cause of outbreaks or sporadic cases of abortions in Uruguay.

Campylobacteriosis is still a cause of abortions, and most likely infertility in dairy cattle in the country.

It is important to further investigate the pathogenesis mechanisms, epidemiology and diagnosis of leptospirosis to determine the importance of this disease as a cause of abortion in Uruguay.

The systematic diagnosis of bovine abortion is necessary to set possible strategies of prevention and control, besides monitoring and surveillance of reproductive diseases in dairy cattle, some of which can represent a risk to public health.

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Causes of death of beef cattle raised in feedlots[1]

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ABSTRACT.- Estima-Silva P., Oliveira P.A., Bruhn F.R.P., Scheid H.V., Marques L.S., Ribeiro L.S. & Schild A.L. 2020. **Causes of death of beef cattle raised in feedlots.** *Pesquisa Veterinária Brasileira 40(5):333-339.* Laboratório Regional de Diagnóstico, Faculdade de Veterinária, Universidade Federal de Pelotas, Campus Universitário, Pelotas, RS 96010-900, Brazil. E-mail: alschild@terra.com.br

The causes of death of cattle kept in pre-export feedlots (PEFs) and in feedlot for finishing for slaughter are described. Two studies were conducted: a retrospective study of mortality cases in feedlots from 2000 to 2017 registered at the "Laboratório Regional de Diagnóstico" of the "Faculdade de Veterinária" of the "Universidade Federal de Pelotas"; and a prospective study from January 2018 to August 2019, following up 22 feedlots for finishing and six PEFs for the export of live cattle. From January 2000 to August 2019 samples of 150 cases of diseases that affected feedlot cattle were received from 22 feedlots for finishing and 115 of the six PEFs followed. Mortality considering all diagnosed diseases was significantly higher in feedlots for finishing ($p<0.05$), than in PEFs for the export of live cattle, of 1% and 0.12%, respectively. Diseases of the digestive system were the most important causes, of death in feedlots regardless of its purpose. Acidosis presented the highest mortality rates both in feedlot for finishing (3.33%) as in PEFs for export (0.95%). In all cases the disease occurred due to failure in the adaptation of animals to the ingestion of concentrated foods. Bovine tick fever and pneumonia presented mortality rates of 0.13% and 0.09%, respectively in PEFs. In the feedlot for finishing seneciosis was the second cause of death due to cattle coming from areas with high infestation by the plant. In the present study, it was possible to identify the main diseases that occur in cattle feedlots for finishing or for the export of live animals in the southern region of Rio Grande do Sul. These diseases are known in other systems of cattle breeding and can be prevented or controlled through management, chemoprophylaxis or vaccination, minimizing losses due to mortality.

INDEX TERMS: Beef cattle, feedlot, pre-export feedlots, acidosis, bloat, respiratory deseases, tick fever, cattle.

RESUMO.- [**Causas de morte de bovinos de corte criados em confinamentos**.] Descrevem-se as causas de morte de bovinos mantidos confinados em estabelecimentos pré-embarque (EPEs) para exportação de animais vivos e em estabelecimentos de terminação para abate. Foram realizados dois estudos: um retrospectivo dos casos de mortalidade em confinamentos de 2000 a 2017 registrados no Laboratório Regional de Diagnóstico da Faculdade de Veterinária da Universidade Federal de Pelotas; e um estudo prospectivo de janeiro de 2018 a agosto de 2019, acompanhando-se 22 confinamentos de terminação e seis EPEs para exportação de bovinos vivos. No total, de janeiro de 2000 a agosto de 2019 foram recebidos no LRD-UFPel amostras de 150 casos de enfermidades que afetaram bovinos confinados, 35 provenientes de 22 confinamentos de terminação e 115 dos seis EPEs acompanhados. A mortalidade considerando-se todas as enfermidades diagnosticadas foi significativamente maior nos confinamentos para terminação ($p<0,05$), do que nos EPEs para exportação de bovinos vivos, de 1% e 0,12%, respectivamente. As doenças do sistema digestivo foram

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as causas de morte mais importante nos confinamentos, independente da finalidade. Acidose apresentou as maiores taxas de mortalidade tanto nos confinamentos para terminação (3,33%) como nos EPEs para exportação (0,95%). Em todos os casos a doença ocorreu devido a falha na adaptação dos animais à ingestão de alimentos concentrados. Tristeza parasitária bovina e pneumonias apresentaram taxas de mortalidade de 0,13% e 0,09, respectivamente em EPEs de exportação. Nos confinamentos para terminação a seneciose foi a segunda causa de morte devido aos bovinos serem provenientes de áreas com alta infestação pela planta. Este estudo permitiu identificar as principais enfermidades diagnosticadas em confinamentos para terminação de bovinos ou para exportação de bovinos vivos na região Sul do Rio Grande do Sul. Observou-se que são doenças que ocorrem em outros sistemas de criação de bovinos e que podem ser prevenidas ou controladas por meio de manejo, quimioprofilaxia ou vacinação, minimizando prejuízos por mortalidade de animais.

TERMOS DE INDEXAÇÃO: Bovinos de corte, confinamento, estabelecimento pré-embarque, acidose, timpanismo, doenças respiratórias, tristeza parasitária.

INTRODUCTION

In recent years, there has been an increase in the demand for the diagnosis of diseases in cattle in the area of influence of the "Laboratório Regional de Diagnóstico", of the "Faculdade de Veterinária", of the "Universidade Federal de Pelotas" (LRD/UFPel), especially in feedlot cattle. The main purpose of feedlots in Brazil is cattle finishing, i.e., to produce animals ready for slaughter (Malafaia et al. 2016). In 2014, 2.4% of the Brazilian cattle herd (198.7 million heads) was finished in feedlots, representing 10.8% of the 43.3 million heads officially slaughtered across the country. This livestock rearing system has expanded due to the appreciation of the dollar and consequently, the increased export of live cattle (Bailone 2019). These animals are maintained in pre-export feedlots (PEFs) for periods predetermined by the importing countries. Despite this increase in feedlot cattle, little is known about the diseases affect cattle that occur in this rearing system in southern Brazil, as well as their impacts on animal health, production and costs (Malafaia et al. 2016).

Respiratory diseases are the main causes of morbidity and mortality in cattle feedlots in the United States, despite advances in operational management and health protocols in this sector. The cumulative incidence of these diseases is estimated at 16.2% (Avra et al. 2017), impacting the profitability of farms that use this system for cattle finishing (Cernicchiaro et al. 2013). It has been mentioned that approximately 75% of morbidity and 50% of mortality in cattle feedlots in the United States is caused by respiratory diseases (Kelly & Jansen 1986, Edwards 2010). In Brazil, in addition to pneumonia, digestive disorders are also cited as important diseases diagnosed in feedlot cattle (Malafaia et al. 2016). In the area of influence of LRD-UFPel, which corresponds to the southern region of Rio Grande do Sul, surveys and previous retrospective studies have shown that diseases of the respiratory tract are important causes of death in calves, mainly of dairy breeds, up to one year of age raised in feedlots (Assis-Brasil et al. 2013). However, little is known about the causes of death in cattle reared in feedlots for finishing or exportation. It is, therefore, fundamental to know the main causes of death in this breeding system to identify control strategies, which could prevent economic damage to beef cattle ranching in the region.

The goal of this paper was to determine the main diseases that cause mortality in feedlot cattle in the area of influence of the LRD-UFPel, verify their prevalence and develop control strategies according to the current reality of this breeding system.

MATERIALS AND METHODS

A retrospective study of diagnosed diseases in feedlot cattle in the area of influence of the LRD-UFPel was carried out, and the mortality rates and epidemiological characteristics of each disease were verified. For this study, a survey of necropsy reports from the LRD-UFPel from January 2000 to December 2017 was conducted.

A prospective study was also carried out on feedlot cattle farms in the southern region of Rio Grande do Sul state. The feedlots were visited to identify the sanitary condition, food management practices and the age of the cattle on each farm since protocols differ and depend on the ultimate goal of each farm. This study was conducted in feedlot cattle intended for export to Turkey and Venezuela as well as feedlots dedicated to slaughtering.

Necropsies were performed at the feedlots or at the laboratory to diagnose the diseases that caused the death of the cattle. The organs of the cattle subjected to necropsy by the veterinarians responsible for the feedlots were also sent to the LRD-UFPel. Complementary analyses (histopathological, bacteriological, parasitological) were performed when necessary to confirm the diagnosis. Outbreaks were considered when several animals with similar clinical signs reported by the responsible veterinarians died within a short period of time. As many necropsies as possible were performed for each outbreak.

Export to Turkey. The cattle destined for export to Turkey remained in quarantine after the number of animals to be exported was reached (minimum 8000 heads). After the entry of all cattle in PEF, which could take up to 15-20 days due to the significant number of cattle, quarantine conditions were initiated by "Ministério da Agricultura Pecuária e Abastecimento" (MAPA). During this period (21 days), the entry or exit of animals was prohibited (MAPA 2018). The total duration of stay in the PEFs could reach 45 days.

Cattle were submitted to an incoming sanitary protocol involving endectocidal drugs, vaccines against infectious bovine rinotracheitis (IBR), bovine viral diarrhea (BVD), bovine respiratory syncytial virus (BRSV), bovine parainfluenza (PI), *Pasteurella multocida* and *Mannheimia haemolytica*, and clostridiosis, in addition to a dose of oxytetracycline. In these operations, chemoprophylaxis for bovine tick fever was performed with imidocarb (1,2mg/kg of body weight) or oxytetracycline (6,7mg/kg of body weight) and diminazene diaceturate (1.17mg/kg of body weight). Animals intended for export were noncastrated males aged up to 18-month-old, of European breed standard, and with a maximum body weight of 285kg. During the quarantine period, blood was collected from all the cattle; the blood samples were required to be negative for IBR, BVD, paratuberculosis and leukosis. Animals positive for any of these diseases were isolated and considered unfit for export but released into domestic trade after the embarkation of the others. In the same period, the animals were also tested for tuberculosis and brucellosis. If they were positive for either of these diseases, they were isolated and referred for sanitary slaughter after the end of quarantine and the boarding of the other animals.

Export to Venezuela. For those intended for export to Venezuela, the quarantine period, also supervised by MAPA, was 24 hours; however, cattle could remain on site for approximately 15 days to complete the lot to be exported. Cattle in this case had to be males, and there were no requirements for age or breed pattern or sanitary protocols because slaughtering was performed shortly after arrival at the destination. Body weights above 400kg were required. In these operations, chemoprophylaxis for bovine tick fever was performed only in cattle from Santa Vitória do Palmar/RS due to the absence of the tick (*Rhipicephalus microplus)*, which is the only vector of babesiosis and the main vector of anaplasmosis, in that region.

Finishing feedlots. In the finishing feedlots, only cattle aged over 12 months were included. These animals were reared in pastures before arriving at the feedlots. Vaccinations were not required, although owner generally administered preventive vaccinations for clostridiosis and performed endectocidal treatments and preventive chemoprophylaxis for bovine tick fever. The animals remained in the feedlot for approximately 90-120 days.

Statistical analysis. The program OpenEpi was used for all the statistical analyses (Dean et al. 2006), and a minimum confidence of 95% was considered ($p<0.05$). Comparisons of the specific mortality rates (%) of the different diseases observed within and between each feedlot system were made using Fisher's exact tests or chi-square tests. Fisher's exact test was performed when there were less than five observations in the contingency table for the test. The mortality rate was calculated considering the number of dead cattle as a result of each disease during each feedlot cycle. The quantification of the risk of occurrence of diseases was performed by calculating the relative risk with a confidence interval of 95%.

RESULTS

From January 2000 to August 2019, 150 materials were received at the LRD-UFPel for the diagnosis of diseases that affected feedlot cattle, including cadavers for necropsies and refrigerated or formalized organs. Six PEFs located in the municipalities of Capão do Leão, Rio Grande, Turuçu, Cristal and Eldorado do Sul that exported cattle lots to Turkey and/or Venezuela were followed. Out of the 150 materials received, 35 came from 22 feedlots for finishing cattle, and 115 came from the six PEFs.

Diseases diagnosed in PEFs for cattle intended for export to Turkey and in feedlots for finishing are presented in Table 1 and 2, respectively. In a population of 8,000 at-risk

Table 1. Diseases diagnosed in beef feedlot cattle for export to Turkey, population at risk and specific mortality rate in the southern region of Rio Grande do Sul, from January 2000 to August 2019

Diagnosis	No. of dead	Population at risk	Mortality rate (%)*
Acidosis	95	10000	0.95a
Bloat	70	36000	0.19b
Tick fever	30	24000	0.13c
Pneumonia/bronchopneumonia	27	30000	0.09c
Starvation	4	16000	0.03d
Trauma	3	16000	0.02d
Abomasum ulcer	3	16000	0.02d
Senecio spp. poisoning	2	16000	0.01d
Pericarditis	2	16000	0.01d
Peritonitis	2	16000	0.01d
Ceratoconjuntivitis	1	8000	0.01d
Coenurosis	1	8000	0.01d
Meningitis	1	8000	0.01d

* Different letters in column indicate statistical difference ($p<0.05$) by chi-square test.

Table 2. Diseases diagnosed in beef feedlot cattle to finishing, population at risk and specific mortality rate in the southern region of Rio Grande do Sul, from January 2000 to August 2019

Diagnosis	No. of dead	Population at risk	Mortality rate (%)*
Acidosis	10	300	3.33a
Senecio spp. poisoning	28	1170	2.39ab
Pneumonia/bronchopneumonia	13	870	1.49bc
Bloat	22	1600	1.38c
Tick fever	23	2000	1.15c
Abomasum ulcer	1	348	0.29cd
Tetanus	5	2500	0.20d
Rabie	1	1200	0.08d

* Different letters in the column indicate statistical difference ($p<0.05$) by the chi-square test.

animals in a PEF for export to Venezuela, five animals died of bovine tick fever, and three died of trauma. The mortality rates in this case were 0.063% and 0.038%, respectively. The comparison of mortality rates of all the diseases diagnosed in PEFs intended for Turkey and in feedlots for finishing is presented in Table 3. The mortality rate was significantly higher ($p<0.05$) in feedlots for finishing than in feedlots for export.

Deaths by acidosis occurred in both PEF and finishing feedlot. This disease presented the highest mortality rates both in feedlots for finishing (3.33%) and in PEFs for export to Turkey (0.95%). Acidosis occurred in two PEFs and in a feedlot for finishing. In one PEF, cattle were fed concentrate in a quantity equivalent to 3% of their body weight to increase bulk. In the other, the cattle received a commercial mixture of oatmeal with concentrate. In the finishing feedlot, cattle received corn silage and concentrate, and there was no prior adaptation of the animals to the food. In these cases, anorexia, dehydration, ruminal atony and recumbency were observed. The necropsies showed that the ruminal content was liquified, yellow-green in color and acidic. Histologically, the rumen showed increased papillae and the vacuolization of epithelial cells, forming vesicles. There was also multifocal neutrophil infiltrate in the mucosa and rumen submucosa, forming small pustules.

Bloat caused the death of 92 animals in two PEFs and in a feedlot for finishing (Table 1 and 2). In a PEF, the diet contained concentrate (30%) and corn silage (70%), and a consumption rate of 0.5% of the body weight of the animals was calculated. There was gradual adaptation of the cattle to the food until a proportion of 50% concentrate with consumption of 2% to 2.2% of their body weight was achieved. The outbreak in this case occurred in cattle that arrived at the feedlot after the beginning of adaptation and, therefore, did not participate in the adaptation process. In another PEF, cattle were maintained for a period without a diet of concentrate plus oats. The manufacturer indicated that diet should be provided daily *ad libitum* in covered troughs. The feed was removed after consumption and administered again at variable intervals up to 10 hours later due to the absence of coverage in the troughs. In a single feedlot for finishing, the protocol indicated by the manufacturer was followed. This protocol consisted of feeding the animals the diet in the trough alternated with feeding in the pasture, which was gradually decreased until only concentrated feed was administered on the basis of 2% of their body weight. In one outbreak, there was no history of adaptation of cattle to food or the composition of the diet.

In cases of bloat, clinical signs were characterized by marked rumen distension, respiratory difficulty, salivation and protrusion of the tongue. During the necropsies, ruminal dilation, tongue and rectum protrusion and the presence of foam mixed with ruminal content were observed. In the cadavers, congestion of the musculature and organs in the thoracic cavity and pallor of the tissues in the caudal portion was also observed. This difference was eventually observed in a marked line in the caudal third of the esophagus, called the bloat line.

Bovine tick fever was diagnosed on 58 occasions in four PEFs and in four feedlots for finishing, and the disease manifested in both isolated cases and outbreaks. Table 4 shows cases of bovine tick fever according to the etiological agent. Five outbreaks of bovine tick fever diagnosed in feedlots for finishing occurred, with mortality reaching 2.5% in feedlots that did not administer chemoprophylaxis for the disease. In a PEF in which two export operations to Venezuela took place, cattle from Santa Vitória do Palmar did not get sick. In PEFs in which all animals were submitted to chemoprophylaxis for bovine tick fever, the mortality rate was 0.1%.

Anaplasmosis was clinically characterized by jaundice or anemia and fever. In cases of cerebral babesiosis, incoordination and eventual aggression were observed. The necropsies revealed

Table 3. Mortality rate comparison and quantification of disease risk between cattle finishing feedlot and pre-export feedlots (PEF)

Diagnosis	Finishing*	PEF Turkey*	RR**	CI***
Acidosis	3.33a	0.95b	3.51	1.85 - 6.66
Senecio spp. poisoning	2.39a	0.01b	191.5	45.67 - 802.60
Pneumonia/bronchopneumonia	1.49a	0.09b	11.95	6.26 - 22.84
Bloat	1.38a	0.19b	7.07	4.39 - 11.39
Tick fever	1.15a	0.13b	9.20	5.36 - 15.81
Abomasum ulcer	0.29a	0.02b	15.33	1.60 - 146.90
All diseases	1.00a	0.12b	8.69	7.01- 10.770

*Different letters in the line indicate statistical difference ($p<0.05$) by the chi-square test, ** RR = relative risk, *** CI = 95% confidence interval.

Table 4. Number of cases of tick fever according to etiological agent in beef cattle feedlot in the southern region of Rio Grande do Sul, from January 2000 to August 2019

Etiological agent	No. of dead	Population at risk	Mortality rate*
Anaplasma marginale	20	34000	0.06a
Babesia bovis	13	34000	0.04ab
Mixed infection	6	34000	0.02b
Babesia bigemina	5	34000	0.02b

* Different letters on the column indicate statistical difference ($p<0.05$) by the chi-square test.

that the liver was yellowish and enlarged, and the biliary vesicle was distended with lumpy contents. Splenomegaly with protrusion of red pulp was observed, and the kidneys appeared dark. In cases of cerebral babesiosis, the cerebral cortex presented cherry-red coloration. In two cases of cerebral babesiosis, no clinical signs were observed. The diagnosis confirmation was made by observation of Giemsa-stained organ smears in which f parasitic structures within the red blood cells compatible with *Anaplasma marginale*, *Babesia bigemina* and/or *Babesia bovis* were identified.

Pneumonias occurred in three PEFs and in two feedlots for finishing, affecting a total of 40 cattle, all under 18 months of age. In PEFs for export to Turkey, a vaccination against the respiratory diseases IBR, BVD, bovine respiratory syncytial virus, bovine parainfluenza, *Pasteurella multocida* and *Mannheimia haemolytica* from different manufacturers was administered at the time of entry of the animals into the feedlot.

Cattle diagnosed with respiratory diseases presented dyspnea, sialorrhea and, eventually, uni- or bilateral purulent nasal discharge. Gross lesions ranged from mild anteroventral consolidation accompanied by edema and pulmonary emphysema to irregular areas of atelectasis with pulmonary hepatization and fibrin deposition, according to the gravity of the case. Histologically, edema, congestion and hemorrhage, inflammatory infiltrate of mononuclear cells, necrosis of alveolar walls and syncytial cells that sometimes presented necrosis were observed. There were also mixed inflammatory infiltrates in the bronchi, bronchioli and alveoli as well as hyperplasia due to type II pneumocytes and marked edema in the interlobular septa and alveoli in addition to oat grain cells. There was growth of *M. haemolytica* in bacterial culture performed from lung samples collected at necropsy in two cases and *P. multocida* in three cases.

Cases of bovine tick fever, pneumonia and bloat also occurred without obvious clinical signs.

Thirty cases of *Senecio* spp. poisoning were diagnosed, two of which occurred in animals less than 18 months old who were confined to two PEFs. The other 28 cases occurred in five different feedlots for finishing, and the animals were over 18 months of age. The clinical signs included progressive weight loss and diarrhea. The liver was firm, whitish, with nodules on the surface. There was also edema of the vesicle wall, ascites and edema in the mucosa of the abomasum. Histologically, fibrosis, megalocytosis and proliferation of the bile ducts was observed.

The other diseases occurred sporadically, with low mortality (Table 1 and 2).

DISCUSSION

The results of the present study showed that cattle mortality was significantly higher in feedlots for finishing than in PEFs for the export of live cattle. Cattle destined for export receive greater care in feedlots due to the requirements of the importing countries; this care does not occur in animals in feedlots for finishing. Other factors, such as cattle age and time of stay in the feedlot, could also influence the mortality of animals on these farms.

In the present study, the diagnosis of diseases that caused mortality in feedlots in the southern region of Rio Grande do Sul was performed with relative ease. Although many clinical signs of diagnosed diseases are nonspecific, diagnosis was possible by examining macroscopic and histological lesions to confirm the clinical suspicion of each disease. Death without obvious clinical signs in cases of ruminal bloat, pneumonia and cerebral babesiosis in confined cattle described in this study has also been reported by other authors (Glock & DeGroot 1998). Cerebral babesiosis is described as one of the leading causes of death without obvious clinical signs in cattle in the region (Estima-Silva et al. 2016).

Diseases of the digestive system, mainly acidosis, caused the highest mortality in feedlot cattle in the southern region of Rio Grande do Sul from 2000 to 2019. This is not surprising since when cattle reach the feedlot, regardless of the system, they start ingesting carbohydrate-rich rations, often without prior adaptation or with insufficient adaptation. In the present study, it was observed that all cases of acidosis occurred in export and termination feedlots, in which cattle were not adapted to the new feed or the adaptation was inadequate or late. Cattle fed excessive amounts of highly fermentable carbohydrates associated with inadequate amounts of fiber can lead to ruminal acidosis (Valente et al. 2017). A gradual adaptation to this type of food can be effective in preventing the condition (Snyder & Credille 2017).

Additionally, with regard to diseases affecting the digestive system, bloat is as an important disease in feedlots, regardless of their purpose for finishing or export. This disease occurs in cattle fed diets rich in grains and is related to factors such as the physical form of the diet and changes in the populations of ruminal bacteria and protozoa and their fermentation products (Meyer & Bryant 2017). In an outbreak described in this paper, the lack of adaptation to food was identified as a cause of the outbreak. In another outbreak, the cattle were fasted for up to 10 hours, which predisposes them to the occurrence of bloat due to the eagerness with which animals begin to feed when they have access to food again. This has been described by other authors who noted that hunger led to the ingestion of excessive amounts of food after a period of fasting (Abdisa 2018). The individual case observed in a feedlot for finishing demonstrated that previous adaptation is effective in avoiding the disease, since in this feedlot, the animals were adapted, and only one case occurred, probably due to individual susceptibility of the animal.

Another disease of importance in the feedlots in the region during the study period was bovine tick fever. There was no significant difference between the occurrence of anaplasmosis and babesiosis in both types of feedlots. In an epidemiological study on bovine tick fever in the southern region of the state, approximately 60% of the diagnosed cases were babesiosis (Almeida et al. 2006). In feedlots in general, there is no favorable environment for the tick to complete its cycle; therefore, in the case of babesiosis, which is transmitted exclusively by this ectoparasite, cattle probably entered in feedlot during the incubation period of the disease. On the other hand, babesiosis can occur be due to imbalance between the inoculum and the immune state of the animal that may decrease due to stress. This fact would explain the occurrence of the disease throughout the feedlot stay (Gonçalves 2000). Another important fact is that most animals reared in feedlots for finishing or export are less than 24 months old and are therefore included in the age group most susceptible to bovine tick fever in the southern region of the Rio Grande do Sul (Almeida et al. 2006). In addition, European breed of

cattle, which are most susceptible to bovine tick fever, are in demand for export feedlots and are predominant in feedlots for finishing in certain regions.

Cattle tick fever prophylaxis was efficient in preventing the occurrence of the disease in feedlots in this study. It has been shown that animals receiving chemoprophylaxis are nine times less likely to die as a result of this disease than those who don't receive chemoporphylaxis. In addition, it should be noted that PEF cattle from Santa Vitória do Palmar that were treated did not get sick. This region of RS, which is located south of parallel 32, is free from the vector ticks and therefore cattle are more susceptible to bovine tick fever (Almeida et al. 2006). This suggests that the use of anaplasmicides and babesicides can be used to control these diseases in feedlot cattle. It has been shown that in addition to preventing the disease, these drugs also increase weight gain (Silva et al. 2015).

Pneumonia was also a significant cause of death among feedlot cattle, especially in those in finishing feedlots. Respiratory disorders have been identified as the leading causes of death in cattle in Brazil (Malafaia et al. 2016) and in other countries (Avra et al. 2017). All cases of pneumonia occurred in cattle under 18 months of age. The age of cattle is a risk factor for the occurrence of pneumonia (Avra et al. 2017). The disease was the leading cause of death in calves under one year in a retrospective study of mortality in young animals conducted in the area of influence of the LRD-UFPel (Assis-Brasil et al. 2013). The results of this study showed that unvaccinated finishing feedlot cattle were approximately 12 times more likely to die than vaccinated cattle. In PEFs for export to Turkey, cattle were vaccinated against respiratory diseases, and mortality was significantly lower than mortality due to these diseases in finishing cattle. This indicates that the practice is effective in preventing deaths from this disease. Other studies have reported that the vaccine is effective, especially if administered before the animals enter the feedlot (Magalhães 2017). Another fact that should be mentioned is that even with epidemiological conditions favorable for the occurrence of disease in PEFs, such as age and stress, mortality due to this disease was lower in these feedlots, which also reinforces the idea that vaccination is effective in the control of this disease.

Senecio spp. poisoning was the second cause of death in feedlots for finishing. Intoxication by this species is one of the most important causes of death in cattle reared in the field in the southern region of Rio Grande do Sul and mainly affects animals older than 18 months (Grecco et al. 2010, Panziera et al. 2018). In the feedlots for finishing, cattle were older than 18 months and came from this region; thus, cases of intoxication were expected. It should be noted that this is not a disease that occurs as a result of the feedlot itself or its management. The acquisition of cattle for feedlots from areas where there is no *Senecio* spp. or where the plant is controlled is the only way to prevent deaths from intoxication during the feedlot period. In PEFs exporting to Turkey, the chances of intoxication are substantially decreased. This is probably because the cattle in these feedlots are usually newly weaned calves that go straight to the feedlot without sufficient contact with the plant.

Other diseases that caused deaths in feedlot cattle include abomasum ulcer, which has been previously reported as a disease that generates economic losses in feedlots. This disease has a multifactorial etiology, including stress and changes in feeding characteristics (Marshall 2009), factors observed in the studied feedlots. However, in the feedlots in this study, mortality from this disease was low. Starvation and trauma also had little importance as a cause of death. In general, starvation occurs due to a lack of adaptation to the feed due to temperament, which prevents it from feeding from the trough (Macitelli et al 2018). Trauma occurs due to sodomy and is very frequent when intact male cattle are kept in agglomerations (Macitelli et al 2018), which was observed in this study. Rabies was diagnosed in a bovine in a feedlot for finishing; it was likely that the affected bovine was already in the incubation period of the disease when it reached the feedlot. Attention should be drawn to the possibility of outbreaks when the disease is diagnosed in places near confinement areas where there is crowding of animals, which is a risk factor for the spread of the disease. Other diseases, such as pericarditis, peritonitis, meningitis, listeriosis, tetanus and coenurosis, occurred sporadically and did not greatly affect the mortality rate.

In the present study, it was possible to demonstrate the main diseases that occur in cattle feedlots for finishing or for the export of live animals in the southern region of Rio Grande do Sul. These diseases are known in other systems of cattle breeding and can be prevented or controlled through management, chemoprophylaxis or vaccination, minimizing losses due to mortality.

CONCLUSIONS

Cattle mortality is significantly higher in feedlots for finishing than for export in the southern region of Rio Grande do Sul.

Ruminal acidosis, bloat, pneumonia and bovine tick fever were the main causes of death in cattle feedlot both for export and finishing in the southern region of Rio Grande do Sul.

The gradual introduction of high fermentable carbohydrate-rich diet to feedlot cattle, in general, is efficient to prevent deaths from acidosis and bloat.

Cattle over 18 months old, when originating from areas with *Senecio* spp. can enter feedlots already intoxicated by the plant.

Chemoprophylaxis for bovine tick fever proved to be efficient for the control of outbreaks of the disease in feedlots.

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Conflict of interest statement.- The authors declare that there are no conflicts of interest.

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Original Article
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Pythiosis in cattle in Northeastern Brazil[1]

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ABSTRACT.- Maia L.A., Souto E.P.F., Frade M.T.S., Pimentel L.A., Azevedo E.O., Kommers G.D., Riet-Correa F. & Dantas A.F.M. 2020. **Pythiosis in cattle in Northeastern Brazil.** *Pesquisa Veterinária Brasileira 40(5):340-345.* Laboratório de Patologia Animal, Hospital Veterinário Universitário, Centro de Saúde e Tecnologia Rural, Universidade Federal de Campina Grande, Campus de Patos, Av. Universitária s/n, Bairro Santa Cecília, Patos, PB 58708-110, Brazil. E-mail: erickplatini@gmail.com

The epidemiological, clinical and pathological aspects of cutaneous pythiosis occurring in cattle from three farms in the Northeastern of Brazil are described. A biopsy of the lesions of one bovine from each farm was performed. In two cases, the affected cattle had contact with water accumulated in dams during the dry season in the semiarid region. Another case occurred in the coastal tropical region in cattle grazing around irrigation channels. Clinically, lesions were observed mainly on the skin of the thoracic and/or pelvic limbs, characterized by flat and irregular ulcerated areas or nodules of varying sizes, some with fistulous tracts penetrating deep into the subcutaneous tissue. In one case the regional lymph nodes were affected. Histologically, in all cases, pyogranulomatous dermatitis associated with negative hyphae images, in hematoxylin-eosin stained sections, were observed. In sections stained by Grocott methenamine silver, the hyphae measured 2-8μm and had irregular ramifications and rare septations. Immunohistochemistry technique demonstrated strong immunolabeling for *Pythium insidiosum*. Pythiosis should be included in the differential diagnosis of dermatopathies in cattle in the Northeastern of Brazil.

INDEX TERMS: Pythiosis, Brazil, cattle diseases, pyogranulomatous dermatitis, oomycete.

RESUMO.- [Pitiose em bovinos no Nordeste do Brasil]. Descrevem-se os aspectos epidemiológicos, clínicos e patológicos da pitiose cutânea em bovinos de três propriedades do Nordeste do Brasil. Uma biópsia das lesões de um bovino de cada propriedade foi realizada. Em dois casos, os bovinos afetados tiveram acesso à água acumulada em açudes durante a estação seca da região semiárida. O outro bovino acometido estava a pastoreio próximo a canais de irrigação na região litorânea. Clinicamente, as lesões foram observadas principalmente na pele dos membros torácicos e/ou pélvicos e caracterizavam-se por áreas planas e irregulares de ulceração ou nódulos de tamanhos variados, alguns com trajetos fistulosos penetrando profundamente no tecido subcutâneo. Em um caso, os linfonodos regionais foram afetados. Histologicamente, em todos os casos, observou-se dermatite piogranulomatosa associada a imagens negativas de hifas, em secções corados por hematoxilina e eosina. Em seções coradas por metenamina de prata de Grocott, as hifas mediam 2-8μm e possuíam ramificações irregulares com raras septações. A imuno-histoquímica demonstrou forte imunomarcação para *Pythium insidiosum*. A pitiose deve ser incluída como diagnóstico diferencial de dermatopatias de bovinos no Nordeste do Brasil.

TERMOS DE INDEXAÇÃO: Pitiose, bovinos, Brasil, doenças de bovinos, dermatite piogranulomatosa, oomiceto.

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INTRODUCTION

Pythiosis is caused by *Pythium insidiosum*, an aquatic oomycete of the kingdom *Straminipila* that affects several species of domestic animals, wild animals and man (Santurio et al. 2006, Gaastra et al. 2010). The occurrence of the disease is related to the contact of the animals with water containing mobile zoospores that constitute the infecting form of the agent (Santurio et al. 2006, Gaastra et al. 2010).

Pythiosis in cattle is considered infrequent and has been described in the United States (Miller et al. 1985), Venezuela (Pérez et al. 2005), Colombia (Cardona et al. 2012) and Brazil (Grecco et al. 2009, Santos et al. 2011). The low occurrence of the disease in this species has been attributed mainly to the spontaneous recovery of the lesions (Santurio et al. 1998, Grecco et al. 2009). In Brazil, although the disease occurs more frequently in horses, cases of pythiosis in cattle have been diagnosed in the Pantanal region (Santurio et al. 1998, Santos et al. 2011) and in the West (Gabriel et al. 2008) and South of Rio Grande do Sul (Grecco et al. 2009).

In a study on skin diseases in horses in the Northeastern of Brazil, pythiosis represented 24.38% of the cases studied (Pessoa et al. 2014). In addition, several clinical forms of the disease have also been diagnosed in sheep, including cutaneous (Tabosa et al. 2004), nasal (Portela et al. 2010) and digestive (Pessoa et al. 2012) forms. Cases of cutaneous pythiosis had also been reported in a goat (Do Carmo et al. 2015), in three mules (Tabosa et al. 1999, Pessoa et al. 2014), and recently in a donkey (Maia et al. 2016). However, there are no reports of the disease affecting cattle in this region. The objective of this study was to describe the epidemiological, clinical and pathological findings of pythiosis in cattle in the Northeastern region of Brazil.

MATERIALS AND METHODS

The information included in this study was obtained through visits to farms with suspected cases of cutaneous pythiosis in cattle. Epidemiological and clinical data were collected and skin biopsies were performed on one animal from each farm visited. Lymph node biopsy was also performed on a bovine.

The collected material was fixed in 10% buffered formalin, included in paraffin wax, cut at 4-6mμ, and stained by hematoxylin and eosin (HE). For the morphotintorial characterization of hyphae selected sections were stained by Grocott's methenamine silver (GMS) and periodic acid of Schiff (PAS). The photographed images and the diameter of the hyphae were obtained through an Axio Scope A1 digital camera, Axio Cam MRc5, controlled by the software Zen 2012 (Carl Zeiss MicroImaging GmbH).

Immunohistochemistry (IHC) was performed for identification of the agent. The IHC protocol was performed according to Martins et al. (2012) using a polyclonal antibody (anti-*Pythium insidiosum*) produced in rabbits (Gabriel et al. 2008). Briefly, the primary antibody at a dilution of 1 in 1,000 was detected by use of streptavidin-biotin-alkaline phosphatase complex and labelling was 'visualized' with Permanent Red (Dako, Glostrup, Denmark) as a substrate chromogen. We analyzed a previously confirmed positive control (equine cutaneous pythiosis) simultaneously with the tested samples. The negative control included tissue samples incubated with phosphate buffered saline instead of primary antibody.

RESULTS

Three cases of pythiosis were diagnosed in cattle in the Northeastern region of Brazil. The first occurred in November 2007, in the municipality of São Mamede, in the mesoregion of the Sertão of the State of Paraíba, with semi-arid climate. In this farm the herd was composed of 50 cattle and the disease was observed in only one animal. The herd was raised in semi-extensive regime, fed with elephant grass and had access to a weir pond.

The second case occurred in January 2014 in the municipality of Japaratuba, in the eastern mesoregion of the State of Sergipe, with a tropical sub-humid climate. The region is characterized by coastal vegetation and Atlantic forest. From a herd of 90 cattle, two animals had cutaneous lesions. The animals grazed in a low land area crossed by an irrigation channel draining water continuously. The area was flooded during the rainy season and was the only source of food. The owner mentioned that in previous years other cattle had similar skin lesions and recovered spontaneously.

The third case occurred in October 2014, in the municipality of Lavras da Mangabeira, in the mesoregion of the Center-South of the state of Ceará, with semi-arid climate. The affected animal belonged to a herd of 33 cattle and 9 horses. These animals were raised extensively in native pasture (caatinga) and had access to an area adjacent to a pond where the vegetation remained green throughout the year.

The cutaneous lesions presented different characteristics regarding the location, form of presentation and clinical evolution. In Bovine 1, multifocal nodules were observed in the cervical, tibial, metacarpal and gluteal regions. Most of these nodules presented ulcerated, irregular, reddish surface with raised edges (Fig.1A) and others were covered by skin or showed depressed and alopecic fistulous tracts in the skin. In the biopsies, the lesion affected the skin, the subcutaneous tissue and sometime infiltrated the adjacent musculature. The cut surface showed irregular yellowish and reddish areas, delimited by whitish, smooth and shiny tissue (Fig.1C). In addition to the cutaneous lesions, the superficial cervical and pre-crural lymph nodes were enlarged and at the cut showed multifocal coalescent yellow nodules interspersed with discrete reddish areas (Fig.1D).

In Bovine 2 and 3, the lesions, measuring 8-15cm, were characterized by ulcerated areas, with slightly elevated irregular borders and slightly depressed, moist and reddish center (Fig.1B). Bovine 2 were located in the metacarpal region and in Bovine 3, in the humeral and tibial regions. In Bovine 3, ulcerated and parched areas with dark halo-delimited borders were also observed. In the cut surface, these lesions were restricted to the epidermis and dermis and were characterized by a whitish, firm tissue with multifocal coalescent areas ranging from 0.5 to 1.0cm.

In all cases, no treatment was prescribed, but spontaneous regression of the lesions occurred in 7 months in Bovine 1, 1 month in Bovine 2 and 2 months in Bovine 3.

The microscopic lesions observed in all cases were similar, varying only in the intensity of the cellular or tissue response, and were characterized by ulcerative pyogranulomatous dermatitis associated with negative hyphae images (Fig.2A). In Bovine 1, a marked cellular inflammatory response extending from the superficial dermis to the deep dermis, subcutaneous tissue and adjacent musculature, with multifocal areas of

necrosis, was observed. This inflammatory response was characterized by the presence of multinucleated giant cells, epithelioid macrophages, lymphocytes and plasma cells, as well as neutrophils and eosinophils associated with diffuse connective tissue proliferation. Multifocal areas of hemorrhage and mineralization were also observed. The lymph nodes showed similar pyogranulomas with negative images of hyphae in the cortex and medulla.

In Bovine 2 and 3 the lesion was observed mainly in the superficial dermis and was characterized by multifocal to coalescent pyogranulomas composed of central areas containing discrete neutrophils and degenerate eosinophils, surrounded by macrophages, epithelioid macrophages and multinucleated giant cells, peripherally delimited by fibrous connective tissue. Less conspicuous granulomas consisted mainly of epithelioid macrophages and few multinucleated giant cells.

In all cases, transverse and longitudinal hyphae, sometimes surrounded by radiated eosinophilic material forming Splendore-Hoeppli phenomenon, were observed inside some pyogranulomas and in giant cells cytoplasm (Fig.2A and 2B). In the sections impregnated by GMS, were observed hyphae

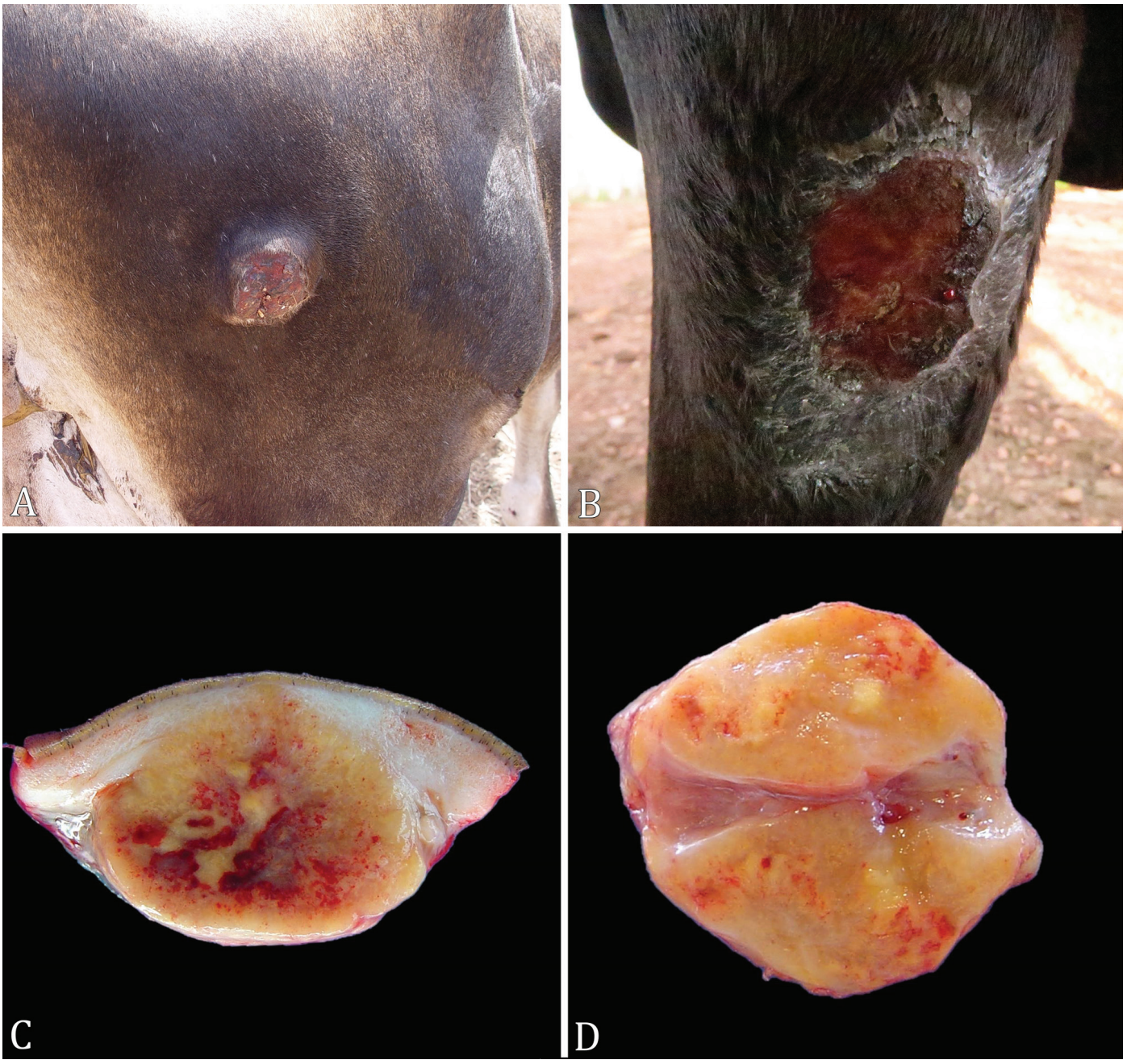


Fig.1. Pythiosis in cattle in Northeastern Brazil. (**A**) Skin, gluteal region. Nodular lesion with raised edges, and ulcerated, irregular, reddish surface. (**B**) Skin, humeral region. Ulcerated lesion with slightly elevated irregular borders, and depressed, moist and reddish center. (**C**) Nodular lesions, cut surface. Irregular yellowish and reddish areas delimited by whitish, smooth and shiny tissue. (**D**) Diffusely enlarged regional lymph node, cut surface. Multifocal to coalescent yellow nodules interspersed with discrete reddish areas.

with a diameter varying from 2 to 8μm showing thick and parallel walls, with irregular ramifications and rare septations (Fig.2C). These hyphae were not stained by PAS and by IHC had strong immunolabeling for *Pythium insidiosum* (Fig.2D).

DISCUSSION

The diagnosis of pythiosis was made based on the epidemiological, clinical and pathological aspects, associated to the morphotintorial characteristics of the agent and the IHC. Although the three cases reported in this study occurred in the Northeastern region of Brazil, different epidemiological characteristics were observed according to the place of occurrence of the disease. Two cases occurred in semi-arid regions, from October to November, during the dry season, where the disease has been diagnosed in other species of animals, attributed to favorable environmental conditions and the scarcity of forage. The regional habit of maintaining water reserves, in the form of dams where the animals have access, especially during the dry season when pasture around these ponds stays green and the rest of the pasture is dry, favored the occurrence of the disease. In these conditions

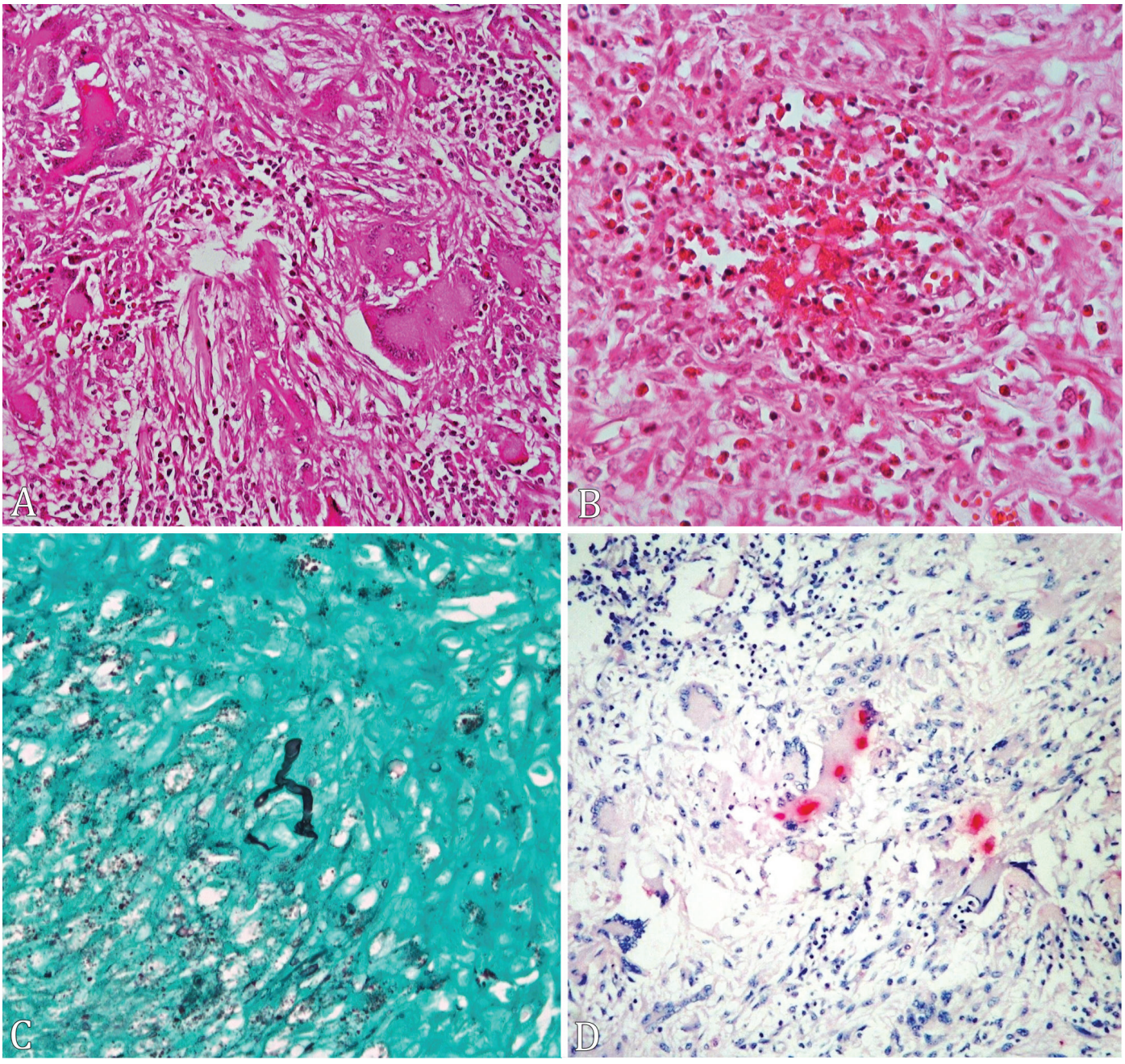


Fig.2. Pythiosis in cattle in Northeastern Brazil. (**A**) Skin. Pyogranulomatous dermatitis with numerous multinucleated giant cells and negative hyphae images in their cytoplasm (arrow). HE, bar = 100μm. (**B**) Skin. Hyphae surrounded by Splendore-Hoeppli phenomenon. HE, bar = 50μm. (**C**) Skin. Hyphae impregnated in black. GMS, bar = 50μm. (**D**) Skin. Strong immunolabeling of hyphae in red for *Pythium insidiosum*. IHC, bar = 50μm.

the animals should stay long periods in the shallow around the dams which are potential sources of infection by *Pythium insidiosum* (Tabosa et al. 2004, Pessoa et al. 2012, 2014).

The disease was also diagnosed in a region of humid coastal tropical climate of the Brazilian Northeastern, in an area flooded during the rainy season, crosses by an irrigation channel with running water for the all year. These environmental conditions differ from what is normally observed in the Northeastern region and resembles what has been described in other regions of Brazil (Gabriel et al. 2008, Grecco et al. 2009).

Recently, two outbreaks of bovine pythiosis were reported in the West and South regions of Rio Grande do Sul, affecting respectively 76 and 16 cattle that were placed in flooded pastures during the summer (Gabriel et al. 2008, Grecco et al. 2009). In the Western region of this state, pasture flooding was attributed to irrigation channels (Gabriel et al. 2008), while in the Southern region where equine pythiosis is endemic, it was associated with low fields and flat areas subject to frequent flooding during the rainy season (Grecco et al. 2009).

In the Brazilian Pantanal, the majority of pythiosis cases occur during the rainy season (February-May) when the region is flooded, in animals grazing on these native pastures with slow drainage, abundant aquatic vegetation and high temperatures (Santos et al. 2011). A similar situation was observed in Case 2, in the coastal region, in cattle grazing in flooded areas around and inside an irrigation channel which facilitates the contact of the skin with water at high temperatures. It is important to note that these epidemiological conditions had not been reported previously in Northeastern of Brazil, since all cases of pythiosis described in horses (Tabosa et al. 1999), sheep (Tabosa et al. 2004, Portela et al. 2010, Pessoa et al. 2012), goats (Do Carmo et al. 2015) and donkeys (Maia et al. 2016) occurred in the semi-arid region during the dry season in animals grazing in and around dams when there was shortage of forage in the pastures.

In two cattle, macroscopic lesions were similar to those described previously in this species (Santurio et al. 1998, Gabriel et al. 2008, Grecco et al. 2009, Santos et al. 2011). These lesions corresponded microscopically to the intense tissue response with a marked presence of fibroplasia, neovascularization and rare hyphae, and in these animals the clinical course of the disease was short with spontaneous healing of the lesions. However, attention was drawn to the characteristic of the lesion observed in Bovine 1, which were macroscopically nodular and sometimes infiltrated the subcutaneous and adjacent musculature, in addition to involvement of regional lymph nodes, characterizing metastasis.

Besides the nodular aspect, it was observed that the clinical course of the disease in this animal was longer, compared to the other animals in this study, as well as other reports in the literature (Santurio et al. 1998, Gabriel et al. 2008, Grecco et al., 2009, Santos et al. 2011). It is believed that the difference in the microscopic lesion characterized by an intense inflammatory response with marked presence of giant cells and hyphae and little tissue response, associated with the infiltrative character of the lesion and the longer clinical course may have contributed to the involvement of regional lymph nodes observed only in this animal, suggesting, apparently, an individual response.

The way one type of response overlaps and dominates the other *in vivo* is extremely complex and determined by several factors, including the type of antigen presenting cell (antigen presenting cell, APC), (dendritic cell versus macrophage and versus lymphocyte), the cytokine involved at the time of antigen presentation, which may be influenced by the nature of the pathogen or the antigen the regulation of cytokine-differentiated T cells; the dose and affinity of the antigen for the T cell receptor; The timing and level of co-stimulatory signals expressed from both APC to T cell to T cell to APC during primary and secondary responses and the cessation of specific T and B responses through activation-induced cell death (Martins et al. 2012). This set of factors probably guides the different types of inflammatory response observed between the same species and between different species.

Dissemination of *P. insidiosum* to other organs has been reported, however is considered rare, and may occur hematogenously by direct extension of the lesions or by lymphatic route (Gaastra et al., 2010). It is possible that the lymph node metastasis observed in only one bovine had occurred via the lymphatic route, since only the superficial lymph nodes responsible for the drainage of the cutaneous lesion were affected. Metastases to regional lymph nodes have already been described in sheep (Tabosa et al. 2004) and horses (Miller & Campbell 1984, Chaffin et al. 1995, Martins et al. 2012), but to date it was not reported in cattle.

Pythiosis in cattle is considered an infrequent disease. However, in studies that described the disease in cattle in Brazil, the authors report that the farmers are familiar with the disease and mentioned the occurrence of animals with similar disease in previous years and in neighboring properties (Pérez et al. 2005, Gabriel et al. 2008, Grecco et al. 2009, Santos et al. 2011). In this work, this fact was also reported in two of the farms visited, suggesting that as in the South and Pantanal Matogrossense, in the Northeastern region, pythiosis in cattle is underdiagnosed and occurs more frequently than reported. This is possibly associated with several factors, such as the spontaneous regression of the lesions, a short clinical course (when compared to equines), non-invasive lesions, and absence of systemic involvement. Due to these characteristics is possible that the farmers do not consult veterinary practitioners for the diagnosis and treatment of the disease.

CONCLUSIONS

Pythiosis in cattle occurs in a semi-arid and tropical coastal region of Northeastern Brazil, and should be included in the differential diagnosis of chronic infectious and neoplastic diseases affecting the skin of cattle.

The disease may present as an ulcerative and flat cutaneous lesion but also a nodular lesion with a prolonged course and in these cases the lymph nodes may be affected in addition to the skin.

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Cerebrospinal fluid analysis in 58 ruminants showing neurological disorders[1]

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ABSTRACT.- Câmara A.C.L., Gonzaga M.C., Ziober T.M., Queiroz C.R.R., Fino T.C.M., Castro M.B., Borges J.R.J. & Soto-Blanco B. 2020. **Cerebrospinal fluid analysis in 58 ruminants showing neurological disorders.** *Pesquisa Veterinária Brasileira 40(5):346-354.* Hospital Escola de Grandes Animais, Universidade de Brasília, Área Especial SRB, Galpão 4, Granja do Torto, DF 70636-200, Brazil. E-mail: aclcamara@yahoo.com.br

Ruminants may be affected by a wide variety of central nervous system (CNS) diseases. Cerebrospinal fluid (CSF) analysis forms the basis for *ante mortem* diagnostic evaluation of ruminants with clinical signs involving the CNS. Despite its importance as a tool to aid diagnosis, data regarding CSF examinations in spontaneous cases of CNS diseases in ruminants from Brazil are limited, and most reports involve experimental studies. Therefore, this study aimed to report the results of CSF analysis in 58 ruminants showing signs of neurological disorders. CSF samples for analysis were obtained from 32 cattle, 20 sheep, and 6 goats by cerebello-medullary cistern (n=54) or lumbosacral space (n=4) puncture. These ruminants showed neurological signs related to viral (n=13), mycotic (n=3), or bacterial (n=15) infections, and toxic (n=21), traumatic (n=4), or congenital disorders (n=2). CSF analysis from ruminants with viral infections presented lymphocytic pleocytosis, even though CSF showed no changes in several cases of rabies. Neutrophilic pleocytosis, cloudiness, presence of fibrin clots, and abnormal coloration were evident in the CSF of most cases of CNS bacterial infection, such as meningoencephalitis, meningitis, abscesses, myelitis, and a case of conidiobolomycosis. On the other hand, CSF was unchanged in most cases of toxic disorders, as botulism and hepatic encephalopathy. Elevated CSF density was observed in 60% of ruminants diagnosed with polioencephalomalacia. Our findings show that evaluation of CSF is a valuable diagnostic tool when used in association with epidemiological, clinical and pathological findings for diagnosis of CNS diseases in ruminants.

INDEX TERMS: Cerebrospinal fluid, ruminants, neurological disorders, central nervous system, cytology, liquor, livestock, neurology.

RESUMO.- [**Análise do líquido cefalorraquidiano em 58 ruminantes com distúrbios neurológicos.**] Os ruminantes podem ser afetados por uma grande variedade de doenças do sistema nervoso central (SNC). A análise do líquido cefalorraquidiano (LCR) constitui a base da avaliação diagnóstica *ante mortem* de ruminantes com sinais clínicos envolvendo o SNC. Apesar de sua importância como ferramenta para auxiliar no diagnóstico, os dados referentes aos exames do LCR em casos espontâneos de doenças do SNC em ruminantes no Brasil são limitados, e, a maioria dos relatos envolve estudos experimentais. Portanto, este trabalho teve como objetivo relatar os resultados da análise do LCR em 58 ruminantes com distúrbios neurológicos. Amostras do LCR foram obtidas de 32 bovinos, 20 ovinos e 6 caprinos por punção da cisterna cerebelo-medular (n=54) ou espaço lombossacro (n=4) para posterior análise. Esses ruminantes apresentaram

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sinais neurológicos relacionados a infecções virais (n=13), micóticas (n=3) ou bacterianas (n=15), e desordens tóxicas (n=21), traumáticas (n=4) ou congênitas (n=2) A análise do LCR de ruminantes com infecções virais apresentou pleocitose linfocítica, embora, em vários casos de raiva, o LCR não tenha apresentado alterações. Pleocitose neutrofílica, turbidez, presença de coágulos de fibrina e coloração anormal foram evidentes no LCR da maioria dos casos de infecções bacterianas do SNC, como meningoencefalites, meningites, abscessos, mielite e um caso de conidiobolomicose. Por outro lado, o LCR não foi alterado na maioria dos casos dos distúrbios tóxicos, como botulismo e encefalopatia hepática. A densidade elevada no LCR foi observada em 60% dos ruminantes diagnosticados com polioencefalomalácia. Nossos resultados mostram que a avaliação do LCR é uma valiosa ferramenta de diagnóstico, quando usada em associação com os achados epidemiológicos, clínicos e patológicos para o diagnóstico de doenças do SNC em ruminantes.

TERMOS DE INDEXAÇÃO: Líquido cefalorraquidiano, ruminantes, distúrbio neurológico, animais de produção, citologia, líquor, neurologia, sistema nervoso central.

INTRODUCTION

Ruminants may be affected by a wide variety of central nervous system (CNS) diseases, including bacterial, viral, and protozoal infections, poisonings, metabolic disorders, trauma, and neoplasms (Scott 2004, Mayhew 2009). Neurological disorders have a noteworthy incidence in ruminants, as observed in retrospective studies conducted in cattle presenting clinical signs of neurological disorders from the Brazilian states of Rio Grande do Sul, Mato Grosso do Sul, and Paraíba, achieving prevalence of 9.16% (552/6,021 cases), 31.5% (341/1,082 cases), and 33.8% (139/411 cases), respectively (Sanches et al. 2000, Galiza et al. 2010, Ribas et al. 2013).

Cerebrospinal fluid (CSF) analysis forms the basis for *ante mortem* diagnostic evaluation of ruminants with clinical signs involving the CNS. This complementary test can assist in determining a conclusive diagnosis, obtaining samples for other diagnostic tests (microbiological culture or viral isolation), or as an aid to monitor treatment response (Mayhew 2009, Stokol et al. 2009, Nagy 2017). In ruminants, CSF is easily and safely obtained, in most cases, through cerebellar-medullary (cisterna magna) or lumbosacral puncture (Scott 2010, Nagy 2017).

Despite its importance as a tool to aid diagnosis, data regarding CSF examinations in spontaneous cases of CNS diseases in ruminants from Brazil are limited (Lisbôa et al. 1996, Della Libera et al. 2004, Câmara et al. 2009, 2011, 2014a, 2014b, 2014c, Queiroz et al. 2018, Moreira et al. 2018), and most reports involve experimental studies (Lisbôa et al. 2009, Batista et al. 2011, Cunha et al. 2011, Isernhagen et al. 2011). Therefore, this study aimed to report the results of CSF analysis in 58 ruminants showing signs of neurological disorders.

MATERIALS AND METHODS

Clinical records of ruminants from two referring hospitals (Veterinary Hospital of the "Universidade Federal Rural do Semi-Árido", Mossoró/RN, and Large Animal Veterinary Teaching Hospital of "Universidade de Brasília", Brasília/DF, Brazil) with CSF analysis records were evaluated. Information retrieved included epidemiological, laboratory, and conclusive diagnosis data. Thirty-two cattle, 20 sheep, and 6 goats were included in the study, totaling 58 ruminants. Criteria for inclusion of cases was a conclusive diagnosis based on a combination of epidemiological, clinical, laboratory, and pathological evaluation. Cases of rabies were also confirmed by gold-standard methods (direct immunofluorescence or mouse inoculation test) in an official government laboratory ("Diretoria de Vigilância Ambiental em Saúde", DIVAL, DF).

CSF samples were collected aseptically by puncturing the cerebellum-medullar cistern (n=54) or lumbosacral space (n=4), and analyzed according to the methodology proposed by Scott (2004, 2010). Erythrocyte and leukocyte counts were performed in a Neubauer chamber. For cytological examination and leukocyte differential counts, a smear of a cell concentrate obtained by centrifugation was stained with Panotic stain (NewProv InstantProv, Pinhais/PR, Brazil). CSF density was obtained by using a clinical refractometer (Quimis, Diadema/SP, Brazil), and protein concentration was determined by a colorimetric method (Katal, Belo Horizonte/MG, Brazil) using a semi-automated biochemical analyzer (Bioplus BIO-2000, Barueri/SP, Brazil).

CSF aliquots were seeded on defibrinated sheep blood agar (5%) for microbiological culture, followed by incubation in a bacteriological incubator at 37°C for 48h. Thereafter, we analyzed the macroscopic and morphotintorial characteristics (by Gram staining method), and biochemical profile (MacFaddin 2000). In cases of conidiobolomycosis, CSF and CNS fragments were seeded on Sabouraud Chloramphenicol Agar and incubated at 28°C for 7 days (Câmara et al. 2011).

From the 58 ruminants, 31 (53.4%) died spontaneously and 27 (46.6%) were submitted to *euthanasia in extremis*. For pathological evaluation, organ samples were collected according to clinical suspicion and were fixed in 10% formalin, and then routinely processed for histology and stained using the hematoxylin and eosin (HE) technique.

RESULTS AND DISCUSSION

Epidemiological data, clinical evolution and conclusive diagnosis from 58 ruminants showing neurological signs are presented at Table 1. Results of the CSF analyses according to the etiology are summarized in Tables 2 to 5. Microbiological assays were performed on every CSF sample from each case, but, as expected, only bacterial CNS diseases yielded microbiological growth (Table 3).

Eleven cases (18.9%) of rabies were diagnosed, including one goat (Case 1) and 10 cattle (Cases 2-11). In all rabies cases, the common epidemiological background was the absence of preventive vaccination and the presence of hematophagous bats on the farms (Moreira et al. 2018). A conclusive diagnosis of rabies was obtained by histology (Negri's bodies inclusions), immunohistochemistry, or gold-standard methods. Rabies is a viral disease with important zoonotic potential and high lethality (Moreira et al. 2018). In our cases, CSF analysis revealed pleocytosis in 6 of 11 ruminants (54.5%), with counts of 16-333 leukocytes/μL, mainly involving mononuclear cells. The other five ruminants (45.5%) had no CSF changes (leukocytes counts peaked 6/μL). Despite few reports on CSF findings in rabid ruminants, when mononuclear pleocytosis (mainly lymphocytic) is present, CSF analysis presents as an important ancillary tool for diagnosis and allows exclusion of potential CNS bacterial infections that cause similar

Table 1. Epidemiological data, clinical evolution, and conclusive diagnosis from 58 ruminants showing neurological signs

Case	Species	Breed	Age	Sex	Evolution (days)	Conclusive diagnose
1	Goat	Saanen	10 months	M	5	Rabies
2	Cattle	Nelore	NI	F	NI	Rabies
3	Cattle	Crossbreed	3 years	F	4	Rabies
4	Cattle	Crossbreed	Adult	F	7	Rabies
5	Cattle	Crossbreed	4 months	F	5	Rabies
6	Cattle	Girolando	6 years	F	10	Rabies
7	Cattle	Crossbreed	Adult	F	NI	Rabies
8	Cattle	Nelore	4 months	F	6	Rabies
9	Cattle	Crossbreed	2.5 months	M	NI	Rabies
10	Cattle	Nelore	5 months	M	6	Rabies
11	Cattle	Crossbreed	1 year	M	6	Rabies
12	Cattle	Crossbreed	13 months	F	7	BoHV-5
13	Cattle	Nelore	1 year	M	3	BoHV-5
14	Sheep	Santa Inês	5 years	F	30	Conidiobolomycosis
15	Sheep	Santa Inês	4 years	F	15	Conidiobolomycosis
16	Sheep	Santa Inês	5 years	F	20	Conidiobolomycosis
17	Sheep	Santa Inês	2 years	F	< 1	Listeriosis
18	Sheep	Crossbreed	18 months	F	4	Listeriosis
19	Sheep	Crossbreed	ND	M	10	Listeriosis
20	Goat	Saanen	9 years	F	12	Listeriosis
21	Sheep	Dorper	5 months	F	20	Suppurative meningitis
22	Cattle	Crossbreed	Adult	M	10	Suppurative meningitis
23	Cattle	Crossbreed	6 days	F	4	Suppurative meningitis
24	Goat	Crossbreed	2 months	M	3	Suppurative meningitis
25	Sheep	Somalis	35 days	M	2	Suppurative meningitis
26	Sheep	Santa Inês	1 month	F	1	Suppurative meningitis and plexus choroiditis
27	Cattle	Crossbreed	6 months	F	13	C7-C8 abscess
28	Cattle	Holstein	11 months	F	10	Pituitary abscess
29	Cattle	Tabapuã	10 months	M	20	Pituitary abscess
30	Goat	Crossbreed	5 years	F	2	Leptomeningitis and thalamus abscess
31	Goat	Crossbreed	6 months	F	10	T1-T2 abscess
32	Cattle	Crossbreed	2 years	M	2	Polioencephalomalacia
33	Cattle	Nelore	2 years	F	NI	Polioencephalomalacia
34	Sheep	Crossbreed	Adult	F	5	Polioencephalomalacia
35	Cattle	Nelore	2 years	F	3	Polioencephalomalacia
36	Sheep	Crossbreed	6 months	F	2	Polioencephalomalacia
37	Sheep	Crossbreed	18 months	F	7	Polioencephalomalacia
38	Sheep	Crossbreed	4 years	F	7	Polioencephalomalacia
39	Sheep	Morada Nova	3 years	F	1	Polioencephalomalacia
40	Goat	Crossbreed	4 years	F	1	Polioencephalomalacia
41	Sheep	Santa Inês	1 year	M	1	Polioencephalomalacia
42	Cattle	Crossbreed	2 years	F	1	Botulism
43	Cattle	Crossbreed	6 years	F	4	Botulism
44	Cattle	Crossbreed	5 months	F	4	Botulism

Case	Species	Breed	Age	Sex	Evolution (days)	Conclusive diagnose
45	Cattle	Crossbreed	5 years	F	3	Botulism
46	Cattle	Crossbreed	7 years	F	3	Botulism
47	Cattle	Crossbreed	22 months	M	2	Botulism
48	Cattle	Crossbreed	18 months	M	3	Botulism
49	Sheep	Santa Inês	Adult	M	NI	Botulism
50	Sheep	Crossbreed	15 months	M	8	Botulism
51	Sheep	Crossbreed	ND	F	10	Botulism
52	Sheep	Santa Inês	3 years	F	90	Poisoning by *Brachiaria*
53	Sheep	Santa Inês	Adult	F	6	Atlanto-axial dislocation
54	Cattle	Nelore	9 months	M	6	C2-C3 fracture
55	Cattle	Nelore	3 years	F	4	T6-T8 fracture
56	Cattle	Nelore	5 months	F	7	Spinal cord compression
57	Cattle	Gir	2 months	F	7	Spinal lipomatosis
58	Cattle	Crossbreed	75 days	F	NI	Cerebellar hypoplasia

NI = not informed, BoHV-5 = bovine herpesvirus 5, M = male, F = female.

neurological signs (Moreira et al. 2018), since this finding is typically associated to viral infections (Stokol et al. 2009). Recently, a study evaluating 17 rabid cattle concluded that CSF may not change or may exhibit discrete mononuclear pleocytosis and/or mildly elevated protein within the CSF (Queiroz et al. 2018). Our results reinforce the wide variation of CSF findings reported in rabid ruminants (Queiroz et al. 2018), and reiterate the difficulty of *ante mortem* diagnosis of this condition. We reinforce rabies zoonotic potential, and suspected cases should be isolated and contact kept to a minimum with only vaccinated humans. Gloves and masks should be worn during examination, samples collection or when performing necropsies (Moreira et al. 2018).

Two sporadic cases of meningoencephalitis caused by bovine herpesvirus 5 (BoHV-5) (Cases 12 and 13) were identified in two 1-year-old Nelore steers. These cases were from large beef herds, mainly feedlot steers, with history of sporadic deaths. Herd management included semiannual deworming and vaccination against foot and mouth disease, rabies, and clostridiosis. Epidemiological background and pathological features are essential for differential diagnosis of BoHV-5 meningoencephalitis vs. rabies, since CSF results may be similar (Lisbôa et al. 2009, Isernhagen et al. 2011, Queiroz et al. 2018). Herein, CSF analysis varied from unchanged (Case 12) to a marked mononuclear pleocytosis (304 leukocytes/μL) (Case 13). CSF results in BoHV-5 meningoencephalitis are well correlated with the intensity and severity of the inflammatory process in the CNS (Queiroz et al. 2018), which agreed with the clinical evolution and pathological findings in our cases.

CSF analysis from three sheep diagnosed with conidiobolomycosis (Cases 14-16) revealed a range of results, since no CSF changes (Case 14) to the presence of fibrin clots (Case 16), and even alterations normally associated with bacterial infections (cloudy appearance, high protein levels, light yellow coloration, and neutrophilic pleocytosis) (Case 15). Three outbreaks of conidiobolomycosis occurred in the semiarid region of Rio Grande do Norte, Brazil, and affected 22 sheep. Most animals showed the rhinopharyngeal form of the disease, while the rhinocerebral form was diagnosed in these three sheep, which presented neurological signs, macroscopic lesions infiltrating the cribiform plate, frontal and temporal bones, as well as cerebral cortex lesions, and histological findings of meningoencephalitis and malacia (Câmara et al. 2011). However, only the sheep with the most severe lesions (Case 15) presented CSF alterations similar to cases of bacterial meningoencephalitis (Mayhew 2009, Stokol et al. 2009, Câmara et al. 2014a). Although the protein concentration was not determined in two cases, the presence of fibrin clots without CSF color or leukocyte changes in one sheep (Case 16) is strongly associated with high protein levels (Scott 2004). Thus, evaluation of CSF is recommended for determining CNS involvement in cases of conidiobolomycosis (Câmara et al. 2011).

Listeriosis was diagnosed in four cases (Cases 17-20). Three adult sheep showed meningoencephalitis (Cases 17-19), while a 9-year-old Saanen doe presented myelitis (Case 20). In all cases, microbiological assays from CSF samples revealed the etiological agent as the Gram-positive bacillus *Listeria monocytogenes*. One adult sheep (Case 18) was part of a meningoencephalitis outbreak in a large sheep flock receiving silage (Câmara et al. 2014c). The other two affected sheep were sporadic cases from farms located in Distrito Federal (Case 17) and Ceará (Case 19), in Midwestern and Northeastern Brazil, respectively. In two sheep, CSF results revealed a cloudy appearance, yellowish coloration, presence of fibrin clots, slight protein increase, and neutrophilic pleocytosis with 92 (Case 18) and 66 (Case 19) leukocytes/μL, of which 65% and 80% consisted of polymorphonuclear cells, respectively. In the other sheep (Case 17), despite the colorless and clear appearance of the CSF, pleocytosis was the highest among cases, but was mainly monocytic. In about 49% of cattle with listeriosis, mononuclear pleocytosis is observed (Stokol et al. 2009), but this is less common in sheep and goats (Mayhew 2009). In listeriosis, neutrophilic pleocytosis is the most frequently observed change in sheep (Scott 2010). This feature was also observed in listerial myelitis (Case 20), that

Table 2. Results of cerebrospinal fluid analyses of 16 ruminants showing neurological signs caused by viral (n=13) and mycotic (n=3) diseases

Disease	Case	Aspect	Color	Density	pH	Proteins (mg/dl)	Glucose (mg/dl)	Erythrocytes (/µL)	Nucleated cells (/µL)	Pleocytosis
Rabies	1	Clear	Colorless	1.005	8	46.7	146	1,242	29	Lymphocytic (69%) and monocytic (25%)
	2	Clear	Colorless	1.008	8	34.8	95	2	32	Lymphocytic
	3	Clear	Colorless	1.006	8	24.69	108	86	16	Neutrophilic
	4	Clear	Colorless	1.004	8	50	110	3	6	Absent
	5	Clear	Colorless	1.006	7	38.6	105	310	70	Monocytic
	6	Clear	Colorless	1.004	8	18.8	53	20	5	Absent
	7	Clear	Colorless	1.008	8	16.2	28	3	1	Absent
	8	Clear	Colorless	1.006	8	30.5	68	7	3	Absent
	9	Cloudy	Light yellow	1.006	8	45.15	84	1,311	333	Lymphocytic
	10	Clear	Colorless	1.006	6.5	39.13	80	70	24	Monocytic (55%) and lymphocytic (45%)
	11	Clear	Colorless	1.006	8	23.4	69	9	2	Absent
BoHV-5	12	Clear	Colorless	1.006	8	15.89	61	8	9	Absent
	13	Cloudy (++)	Whitish	1.008	8	117.5	50	58	304	Monocytic
Conidiobolomycosis	14	Clear	Colorless	1.010	8	ND	50	2	5	Absent
	15	Cloudy	Light yellow	1.006	ND	200	ND	1	20	Neutrophilic
	16	Clear	Colorless	1.000	ND	ND	ND	6	4	Absent

ND = not determined, BoHV-5 = bovine herpesvirus 5.

Table 3. Results of cerebrospinal fluid analyses of 15 ruminants showing neurological signs due to bacterial diseases

Disease	Case	Aspect	Color	Density	pH	Proteins (mg/dl)	Glucose (mg/dl)	Erythrocytes (/µL)	Nucleated cells (/µL)	Pleocytosis	Bacterial culture
Listeriosis	17	Clear	Colorless	1.008	ND	ND	ND	9	213	Monocytic	ND
	18	Cloudy	Light yellow	1.005	8	400	ND	176	92	Neutrophilic	*Listeria monocytogenes*
	19	Cloudy	Yellow	1.008	8	360	ND	95	66	Neutrophilic	*L. monocytogenes*
	20	Clear	Colorless	1.034	8	480	50	48	75	Neutrophilic	*L. monocytogenes*
Suppurative meningitis	21	Cloudy (++)	Opalescent	1.012	8	107.1	27	5	7,470	Neutrophilic	*Escherichia coli*
	22	Cloudy	Reddish	1.008	8	135	18.6	2,000	720	Neutrophilic	ND
	23	Purulent	Milky	ND	ND	ND	ND	ND	ND	ND	*Stenotrophomonas* sp.
	24	Cloudy	Light yellow	1.030	ND	300	ND	2,150	598	Neutrophilic	*Escherichia coli*
	25	Cloudy	Light yellow	1.020	ND	200	ND	58	453	Neutrophilic	ND
	26	Cloudy (++)	Colorless	1.016	ND	ND	ND	555	333	Monocytic and neutrophilic	ND
Abscesses	27	Clear	Colorless	1.006	7	18.5	20	13	3	Absent	*Proteus* sp.**
	28	Cloudy	Reddish	1.014	ND	500	ND	72,000	50	Neutrophilic	*Trueperella pyogenes*
	29	Clear	Colorless	1.008	ND	412	ND	11	17	Absent	
	30	Cloudy	Light yellow	1.010	7.5	300	ND	235	997	Neutrophilic	ND*
	31	Clear	Colorless	1.010	8	200	ND	3	1	Absent	*Streptococcus* sp.**

ND = not determined; * Gram positive bacilli type bacteria observed in histology, ** bacteria cultured from vertebrae abscesses.

presented increased density (1,034 kg/m^3) and protein (480 mg/dL), presence of fibrin clots, and neutrophilic pleocytosis (75 leukocytes/μL), despite no cranial nerve alterations. The difficulty in isolating the bacteria from CSF samples is notorious, and it is necessary to refrigerate the samples for several days to reduce false negative results (Mayhew 2009). Recently, culturing CSF samples or CNS fragments has been presented as a useful laboratory tool for making a conclusive diagnosis of *L. monocytogenes* meningoencephalitis in sheep (Câmara et al. 2014c).

Suppurative meningitis was diagnosed in six ruminants (Cases 21-26). In four young animals (two lambs - Cases 25 and 26; one calf - Case 23; and one goat kid - Case 24), the disease was associated with immunosuppression, which may be due to inadequate colostrum intake, malnutrition, or viral infection (Câmara et al. 2014a). The other cases occurred in a 5-month-old sheep (Case 21), as a consequence of a contaminated tail-docking wound, and an adult cattle (Case 22) in which the etiology remained undetermined. Cerebellar–medullary puncture of a 6-day-old crossbred calf with 4-day evolution of nervous signs contained a purulent material, and CSF analysis was impossible (Case 23). A CSF sample and CNS fragments were microbiologically cultured, revealing *Stenotrophomonas* sp. This bacterial genus is mainly responsible for meningitis in immunosuppressed or debilitated humans (Reddy et al. 2006), reinforcing the immunosuppression possibility (Fecteau & George 2004). Three other young ruminants (Cases 24-26) presented cloudy-

Table 4. Results of cerebrospinal fluid analyses of 21 ruminants showing neurological signs caused by toxic disorders

Disease	Case	Aspect	Color	Density	pH	Proteins (mg/dl)	Glucose (mg/dl)	Erythrocytes (/μL)	Nucleated cells (/μL)	Pleocytosis
Polioencephalomalacia	32	Clear	Colorless	1.008	7.5	13.43	44.83	2	3	Absent
	33	Clear	Colorless	1.006	8	25.1	15	15	3	Absent
	34	Clear	Colorless	1.006	8	30	50	150	50	ND
	35	Clear	Colorless	1.010	9	ND	50	105	4	Absent
	36	Clear	Colorless	1.010	8	ND	ND	44	6	Absent
	37	Clear	Colorless	1.005	8	ND	70	21	2	Absent
	38	Clear	Reddish	1.005	8	ND	60	1,005	3	Absent
	39	Clear	Yellowish	1.030	8	ND	500	450	9	Absent
	40	Clear	Colorless	1.020	ND	ND	50	220	6	Absent
	41	Clear	Colorless	1.012	8	61.5	ND	1,623	3	Absent
Botulism	42	Clear	Colorless	1.006	8	3.2	70	22	0	Absent
	43	Clear	Colorless	1.010	8	ND	50	6	2	Absent
	44	Clear	Colorless	1.005	8	ND	150	7	1	Absent
	45	Clear	Colorless	1.010	8	ND	50	4	2	Absent
	46	Clear	Colorless	1.010	8	ND	150	3	1	Absent
	47	Clear	Colorless	1.020	9	ND	ND	2	2	Absent
	48	Clear	Colorless	1.010	9	ND	150	72	2	Absent
	49	Clear	Colorless	1.006	8	20.3	60	0	1	Absent
	50	Clear	Colorless	1.005	8	ND	20	3	2	Absent
	51	Clear	Colorless	1.015	8	ND	ND	38	4	Absent
Poisoning by *Brachiaria*	52	Clear	Colorless	1.006	7	ND	50	ND	1	Absent

ND = not determined.

Table 5. Results of cerebrospinal fluid analyses of 6 ruminants showing neurological signs caused by traumatic (n=4) and congenital disorders (n=2)

Disease	Case	Aspect	Color	Density	pH	Proteins (mg/dl)	Glucose (mg/dl)	Erythrocytes (/μL)	Nucleated cells (/μL)	Pleocytosis
Atlanto-axial dislocation	53	Clear	Colorless	1.006	8	43.9	45	85	26	Monocytic
C2-C3 fracture	54	Clear	Colorless	1.008	7	35.9	93	645	12	Monocytic
T6-T8 fractures	55	Clear	Colorless	1.006	8	21.0	53	3	2	Absent
Spinal cord compression	56	Cloudy (+++)	Reddish	1.012	8	118.3	31	Countless	3,555	Monocytic
Spinal lipomatosis	57	Clear	Colorless	1.012	8.5	47.5	116	35	11	Lymphocytic
Cerebellar hypoplasia	58	Clear	Colorless	1.008	7	17.77	95.45	39	3	Absent

looking CSF with a light yellow coloration, presence of fibrin clots, increased density, and neutrophilic pleocytosis. These findings are consistent with those reported in sheep (Scott 2010) and cattle (Scott 2004, Stokol et al. 2009) with bacterial meningoenchephalitis, except for the protein concentration that is, in most cases, above 1g/dL (Fecteau & George 2004, Mayhew 2009, Scott 2010). Another relevant aspect is to observe the presence of toxic or degenerate neutrophils, common in cases of bacterial infections; while parasitic infections may present non-degenerate and hypersegmented neutrophils, in addition to eosinophilia (Mayhew 2009). In two cases (Cases 21 and 24), microbiological assays yielded *Escherichia coli*, considered to be the most common Gram-negative meningeal pathogen in neonate cattle (Fecteau & George 2004). In sheep and goats, *E. coli, Pasteurella* sp., *Corynebacterium pseudotuberculosis*, *Streptococcus* sp., and *Trueperella pyogenes* have already been cultured from clinical cases (Mayhew 2009).

Pituitary abscess syndrome (PAS), or basilar empyema, is a neurological disease characterized by the formation of abscesses in the vascular complex over the basosphenoid bone (Fernandes et al. 2000, Braun et al. 2017a). PAS was diagnosed in two calves (Cases 28 and 29) aged 8 and 11 months, associated with the use of a controlled suckling device and hematogenous dissemination from bronchopneumonia (Câmara et al. 2009). Herein, CSF analysis showed increased protein and mild neutrophilic pleocytosis (17 and 50 leukocytes/μL), while 12 cases of cattle with CNS abscesses averaged 12 leukocytes/μL and protein below 425mg/dl. In other ruminants, CSF analysis may, however, be normal, indicating a non-suppurative response, or the CSF may be clearly purulent, with bacteria present (Mayhew 2009). Recently, a 22-month-old bull was diagnosed with PAS and revealed markedly increased, mixed pleocytosis (2,437 cells/μL) and high protein concentration (3.1g/L) (Braun et al. 2017a). In one of the CSF aliquots (Case 28), microbiological assays yielded *T. pyogenes,* the most common bacteria in chronic suppurative lesions of the brain in cattle (Fernandes et al. 2000, Mayhew 2009). The treatment for PAS is usually considered ineffective, but favorable results have also been reported (Câmara et al. 2009, Braun et al. 2017a).

A sporadic case of leptomeningitis and thalamus abscess (Case 30) in a doe was recorded. CSF analysis revealed turbidity, yellowish coloration, and marked neutrophilic pleocytosis (997 leukocytes/μL). This doe presented parotideal caseous lymphadenitis one month earlier. Brain abscesses manifest as a chronic process, while suppurative leptomeningitis has an acute onset (Mayhew 2009), strongly suggesting that this case was secondary to caseous lymphadenitis, since CNS histology presented Gram-positive bacilli (Câmara et al. 2014a). Other cases presenting medullary signs secondary to abscesses compression were observed in a 6-month-old crossbreed calf (Case 27) and a goat (Case 31). CSF analysis in both cases were unremarkable. Necropsy revealed the presence of spinal cord compression by vertebral abscesses. Microbiological culture from the abscess contents yielded *Proteus* sp. and *Streptococcus* sp., respectively. CSF changes depend on the location of the abscess(es), but when the abscess(es) does not infiltrate the dura mater (pachymeningitis), the CSF may be unchanged (Braun et al. 2017b).

Of the 58 ruminants, 10 animals (17.2%) were diagnosed with polioencephalomalacia (PEM). The disease occurred as sporadic cases (Cases 32-34, 36, and 41) and as outbreaks (Cases 35 and 37-40) affecting small ruminants and cattle. In most ruminants (Cases 32-38 and 41), PEM was correlated with high-fermentation carbohydrate diets. Such food management promotes the reduction of rumen pH, leading to lactic acidosis, and consequent inhibition of thiamin-producing microorganisms, favoring the multiplication of thiaminase-producing bacteria (Haven et al. 1983), resulting in PEM clinical manifestations (Sant'Ana et al. 2009). The remaining animals (Cases 39 and 40) presented PEM lesions correlated with sulfur poisoning from water contaminated by petroleum (Batista et al. 2013). In CSF samples from thiamine-deficiency PEM, most ruminants presented all CSF parameters within reference values for cattle (Scott 2004) and sheep (Scott 2010). In five cases (50%; Cases 35, 36, 39, 40, and 41), CSF analysis showed density above the normal or in the upper limit (≥1.010) of the physiological parameters. This finding is considered non-specific and of low precision for investigation of the cause of neurological diseases in ruminants (Scott 2004, 2010). Furthermore, the presence of increased density is an unusual finding in ruminants with PEM, since only the presence of normal protein concentrations and mild lymphocytic pleocytosis have been reported (Scott 2004, Mayhew 2009, Nagy 2017). As in the cases reported here, 75% of cattle with PEM in a previous report also showed no pleocytosis (Stokol et al. 2009); this finding was also described for PEM experimentally induced in calves by sulfur administration (Cunha et al. 2011). Ruminants poisoned by petroleum presented physical CSF alterations, such as a yellowish coloration (xanthochromia) and the presence of fibrin clots (Case 39). The former feature may be observed within hours of subarachnoid hemorrhage (Scott 2010), possibly caused by trauma; and it is consistent with a history of falls and the macroscopic findings of subdural hemorrhages in the CNS (Batista et al. 2013). Moreover, the presence of moderate protein increase, mild mononuclear pleocytosis, and xanthochromia are also described, particularly if there is already a CNS lesion (Mayhew 2009). Fibrin clots in the CSF are indicative of bacterial contamination (Câmara et al. 2014a); however, microbiological assays resulted negative, which may be explained by the increase in protein concentration (Stokol et al. 2009).

Seven cattle (Cases 42-48) and three sheep (Cases 49-51) were diagnosed with botulism (10 ruminants; 17.2%). These 10 outbreaks occurred on different farms from Northeastern (Câmara et al. 2014b) and Midwestern Brazil. In these ruminants, CSF analysis revealed a clear, colorless appearance, absence of fibrin clots, increased density (≥1.010) in 6 of 8 cases (75%), while leukocyte counts peaked at 4 leukocytes/μL. Such results are within the physiological limits for both species (Scott 2004, 2010). This finding was similar to those previously reported in cattle affected by botulism in São Paulo, Brazil (Lisbôa et al. 1996). These features are to be expected, since *Clostridium botulinum* toxins promote functional disorder at nerve synapses, where they compete with acetylcholine (Mayhew 2009). Conclusive diagnosis of these outbreaks of botulism was based on epidemiological and clinical findings and the absence of macro and microscopic lesions (Dutra et al. 2005), confirming the economic importance of the disease

in Brazilian farms (Câmara et al. 2014b). The CSF findings found here presented similar results among ruminants with PEM and botulism. Therefore, we highlight the importance of correlating CSF findings with epidemiological, clinical, and pathological findings to ensure that a conclusive diagnosis is reached correctly.

One sheep (Case 52) was poisoned by *Brachiaria* sp. grass; the animal showed photosensitization and neurological signs, probably as a consequence of hepatic encephalopathy. CSF analysis showed no changes. Pathological examination revealed moderate jaundice distributed diffusely throughout the carcass, and the liver was enlarged (hepatomegaly), with brownish coloration and multifocal whitish spots. *Brachiaria* grasses are widely used in Brazil as the main food source for ruminants, even though it may cause hepatogenic photosensitization. The compounds responsible for *Brachiaria* toxicity are saponins, mainly protodioscin (Diamantino et al. 2018).

Four cases of spinal cord trauma were diagnosed: one sheep (Case 53) and three cattle (Cases 54-56). The sheep was attacked by dogs, and suffered cervical vertebral dislocation, whereas the cattle were found recumbent in the paddocks and the cause of trauma remained undetermined. A calf diagnosed with spinal cord compression (Case 56) presented cloudy and reddish CSF, with increased protein content (118.3mg/dL), the presence of countless erythrocytes, and monocytic pleocytosis. Although CSF from trauma patients may present these features (Mayhew 2009), it is worth emphasizing the importance of not allowing blood contamination of the CSF. Such an error could change the composition of the CSF, especially the protein concentration and cellularity (Câmara et al. 2011). Nevertheless, monocytic pleocytosis was also present in two other cattle (Cases 53 and 54), in which CSF collection was more successful. Therefore, monocytic pleocytosis might be observed in ruminants with spinal compression after short-term survival. On the other hand, a cow diagnosed with a vertebral fracture (Case 55) did not show any change in the CSF. Collection and analysis of CSF from animals suffering from spinal cord trauma is valuable for ruling out other causes of acute spinal cord disease, such as viral, verminous, and protozoal myeloencephalitis (Mayhew 2009). In fact, increases in proteins concentrations (>0.39g/L) and nucleated cell counts (>4.5 cells/μL) in the CSF of recumbent cows have been linked to spinal cord injury (Achard et al. 2017) and to decreased short-term survival (Bilodeau et al. 2018). Accordingly, firm evidence of subarachnoid hemorrhage may not always be found on analysis of CSF in spinal cord trauma (Mayhew 2009), as in our cases.

Two cases of congenital diseases in calves were evaluated; one was a case of spinal lipomatosis (Case 57) and another was a case of cerebellar hypoplasia (Case 58). These cases occurred on different dairy farms, and no other congenital diseases had previously been reported for these herds. The first case (Case 57) presented increased protein concentration (47.5mg/dL) and slight lymphocytic pleocytosis. On the other hand, the CSF findings of the latter (Case 58) was unremarkable. Increased protein and nucleated cell count in the CSF have also been reported earlier in a calf showing neural tube defects (Cagnotti et al. 2019). Congenital malformations in ruminants can have various causes, such as teratogenic chemicals (Panter & Stegelmeier 2011), genetic factors (Cagnotti et al. 2019), and some viruses (Agerholm et al. 2015). It was not possible to determine the etiology in these two cases in this study.

CONCLUSIONS

The findings of cerebrospinal fluid (CSF) analysis from ruminants with signs of neurological disease described here confirmed that pleocytosis (an increase in CSF cellularity) was associated with infectious diseases. However, no CSF changes may be observed in rabies, as seen in several cases in the present study.

The changes described in the number of nucleated cells and differential cell count are usually signs of bacterial infection, with an elevation of polymorphonucleated neutrophilic granulocytes in diseases involving purulent processes (Scott 2004, Câmara et al. 2014a), as observed here in cases of meningoencephalitis, suppurative meningitis, abscesses, myelitis, and in a case of conidiobolomycosis. On the other hand, CSF usually remains unchanged in toxic, metabolic, degenerative, and neoplastic diseases (Stokol et al. 2009), as in the reported cases of botulism and *Brachiaria* sp. poisoning.

In PEM, protein increases and mild mononuclear pleocytosis have been observed in CSF (Scott 2004, Mayhew 2009), while 60% of our cases presented elevated density as the only alteration.

Conflict of interest statement.- The authors declare that there are no conflicts of interest.

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Actinobacillosis outbreak in cattle with clinical manifestation of hippopotamus-like face[1]

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ABSTRACT.- Scheid H.V., Estima-Silva P., Monteiro F.L., Marques L.S., Coelho A.C.B Lima M., Ladeira S.R.L. & Schild A.L. 2020. **Actinobacillosis in cattle with clinical manifestation of hippopotamus-like face**. *Pesquisa Veterinária Brasileira 40(5):355-359*. Laboratório Regional de Diagnóstico, Faculdade de Veterinária, Universidade Federal de Pelotas, Campus Universitário s/n, Capão do Leão, RS 96010-900, Brazil. E-mail: alschild@terra.com.br

Actinobacillosis outbreak with clinical manifestation of hippopotamus-like face observed in a property located in the municipality of Capão do Leão, Southern Brazil, in September 2016, is described. The cattle herd remained for most of the year in rice stubble. When these areas were occupied with new crops, they were transferred to areas where there were small native forests. Three cattle were affected. They presented a volume increase in the nasolabial and maxillary region, and there was also regional lymph node swelling. The evolution of the disease occurred in approximately six months. In tissue fragments collected for culture, *Actinobacillus lignieresii* was isolated. The diagnosis was based on clinical findings, histopathological evaluation characterized by the presence of piogranulomas with Splendore Hoeppli reaction in its center, bacterial isolation, and identification of *A. lignieresii* by polymerase chain reaction (PRC) and genetic sequencing.

INDEX TERMS: *Actinobacillus lignieresii*, diseases of cattle, hippo face, molecular diagnostics, cattle.

RESUMO.- [**Actinobacilose em bovinos com manifestação clínica de cara de hipopótamo.**] Descreve-se um surto de actinobacilose com manifestação clínica de cara de hipopótamo diagnosticado em uma propriedade localizada no município do Capão do Leão, Rio Grande do Sul em setembro de 2016. Os bovinos permaneciam durante a maior parte do ano em restevas de arroz e quando as áreas eram ocupadas com novas lavouras eram transferidos para áreas onde havia pequenas matas nativas. Foram afetados três bovinos adultos que apresentavam aumento de volume na região nasolabial e maxilar e havia, também, tumefação dos linfonodos regionais. A evolução da enfermidade era de aproximadamente seis meses. Nos fragmentos coletados para cultura houve isolamento de *Actinobacillus lignieresii*. O diagnóstico foi baseado nos achados clínicos, na avaliação histopatológica caracterizada pela presença de piogranulomas com reação de *Splendori Hoeppli* no centro, no isolamento bacteriano, identificação de *Actinobacillus lignieresii* por reação em cadeia da polimerase (PRC) e sequenciamento genético.

TERMOS DE INDEXAÇÃO: *Actinobacillus lignieresii*, bovinos, cara de hipopótamo, diagnóstico molecular.

INTRODUCTION

Actinobacillosis is a cosmopolitan disease of sporadic occurrence characterized by the inflammatory piogranulomatous reaction. The disease affects cattle and less commonly sheep, pigs and horses (Andreazza et al. 2013, Caffarena et al. 2017). In ruminants, it is caused by *Actinobacillus lignieresii*, gram-negative aerobic bacterium, present in the oral cavity, upper respiratory tract, and rumen (Andreazza et al. 2013).

Actinobacillus infection occurs due to mucosal and epidermis lesions allowing the bacteria to penetrate in injured tissues.

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The bacteria can affect regional lymph nodes and other organs by lymphatic or hematogenic route (Tessele et al. 2014). In the southern region of Brazil, cattle are placed in areas of rice and soybean cultivation after harvest, providing appropriated epidemiological conditions to the appearance of cases, since in the stubbles remain fibrous and dry stalks that can injure the oral mucosa of animals (Andreazza et al. 2013, Margineda et al. 2013). The disease is often diagnosed in cattle in the southern region of Brazil, especially in the subclinical form in slaughter animals, and is often confused with tuberculosis (Tessele et al. 2014, Schild et al. 2018).

Actinobacillosis, in its classical form, presents with lesions in the tongue and regional lymph nodes of the head and neck (Andreazza et al. 2013, Caffarena et al. 2017). Atypical cases are described by several authors and characterized by skin lesions (Margineda et al. 2013), respiratory system lesions (Peli Agnelo et al. 2009), lymph nodes lesions (Caffarena et al. 2017) and generalized lymph nodes and organs lesions (Kasuya et al. 2017). Lesions on the lips, palate, pharynx, nasal pits and face, when diffuse lead to a clinical picture called hippo-like face. This form of the disease, although mentioned by some authors, is not often described (Mondadori et al. 1994).

The diagnosis of actinobacillosis is based on lesions characteristic of the disease such as the presence of multiple firm and yellowish nodules surrounded by fibrous tissue and deposition of immunocomplexes known as a Splendore Hoeppli (Margineda et al. 2013, Tessele et al. 2014). However, molecular identification and genetic sequencing is important in the elaboration of the diagnosis especially in atypical cases of the disease (Caffarena et al. 2017).

The aim of this paper was to describe the epidemiology and pathology of an outbreak of actinobacillosis with clinical manifestation of hippo-like face, discussing the possible routes of infection, as well as performing the molecular identification of the *A. lignieresii*.

MATERIALS AND METHODS

In September 2016, three cattle belonging to a property located at the municipality of Capão do Leão, southern Brazil, showed clinical signs of increased face volume, mainly in the nasolabial and maxillary region. Epidemiological and clinical data from the veterinarian responsible for the property were obtained. Biopsies in two cattle for histopathological examination and microbiological culture were performed. An affected steer was euthanized and necropsied. Fragments of all organs were fixed in 10% buffered formalin, routinely processed and stained with hematoxylin and eosin (HE). Tissue samples were sent to the bacteriology laboratory of Laboratório Regional de Diagnóstico (LRD-UFPel), being sown in blood agar culture media and MacConkey.

Paraffin blocks containing biopsy and necropsy material and bacterial culture isolated from clinical specimens were sent to the virology and immunology laboratory of the Faculdade de Veterinária da Universidade Federal de Pelotas (LabVir UFPel) for molecular identification of the agent and genetic sequencing.

Construction and analysis of primers. Primers for amplification of an 180 base pair (bp) fragment of *Actinobacillus lignieresii* 16S ribosomal RNA were constructed in the OligoPerfect™ software (Thermo Fischer). The partial sequence *A. lignieresii* strain NCTC 4189 16S ribosomal RNA gene, deposited in GenBank under code AY362892, was used for the construction. Primers Al_F: 5 'ACG GGT GAG TAA TGC TTG GG 3' and Al_R: 5 'GCT AGA GAT CGT CGG CTT GG 3' were tested on the BLAST primer[6] and target the microorganisms *A. lignierisii, Actinobacillus pleuropneumiae, Actinobacillus suis, Haemophilus haemolyticus, Haemophilus influenzae, Glaesserella parasuis* and *Conservatibacter flavescens*. Alignment of these sequences was performed in BioEdit Sequence Alignment Editor Software version 7.0.5.3, with nucleotide difference between the sequences being observed (Fig.1). By using this strategy, it was possible to differentiate *A. lignieresii* from other microorganisms at the molecular level, by analysis of the amplified fragment with the Al_F and Al_R primers.

DNA extraction and PCR. DNA extraction from bacterial colonies isolated from animal tissue samples and paraffin blocks containing biopsy and necropsy tissue using DNAzol™ reagent (Thermo Fischer) and ReliaPrep™ FFPE gDNA MiniPrep System kit (Promega), respectively, both according to the manufacturer's recommendations was performed. The extracted DNA was used in the polymerase chain reaction (PCR) for partial amplification of the Actinobacillus lignieresii 16S ribosomal RNA gene. PCR was performed using 100-200ng extracted DNA, 1x GoTaq® Colorless Master Mix (Promega) and 0.4µM of each primer. The conditions were: 94°C for 5 minutes, 35 cycles of 94, 50 and 72°C for 30 seconds each, and 72°C for 7 minutes. The PCR product was developed on 2% agarose gel and visualized in UV light after electrophoresis. Product purification was performed using the PureLink® Quick Gel Extraction and PCR Purification Kit (Thermo Fischer).

Sequence analysis. The purified PCR product in quadruplicate using BigDye Terminator v3.1 Cycle Sequencing (Thermo Fischer) was sequenced. The results were analyzed using the Staden program (Staden 1996) to obtain the quadruplicate consensus sequence.

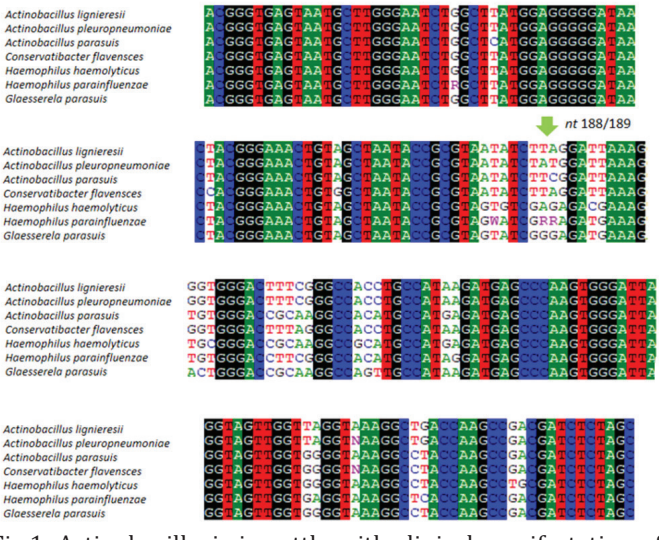


Fig.1. Actinobacillosis in cattle with clinical manifestation of hippopotamus-like face. Alignment of target sequences (primers Al). *Actinobacillus lignieresii* LRD16855 (sequenced sample), *A. lignieresii* strain NCTC 4189 (AY362892), *Actinobacillus pleuropneumoniae* strain Shope 4074 (NR_04475), *Actinobacillus suis* strain JCM 2433 (LC383928), *Haemophilus haemolyticus* strain CIP 103290 (NR_104930), *Haemphilus parainfluenzae* ATCC 33392 strain CCUG 12836 (NR_042878), *Glaesserella parasuis* strain CCUG 3712 (NR_042879) e *Conservatibacter flavenscens* strain CCUG 24852 (NR_159347).

[6] Available at <https://www.ncbi.nlm.nih.gov/tools/primer-blast/>

Alignment with GenBank deposited sequences and the identity matrix were performed in the BioEdit Sequence Alignment Editor Software.

RESULTS

The three cattle affected out of 321 animals were Montana breed, approximately two years old. Cattle remained for most of the year in rice stubbles, and when the areas were occupied with new crops, cattle were transferred to small native forest areas. A gradual increase in the volume of the face, which stretched from the lips to the lateral portion of the jaw and snout were observed (Fig.2). There was also tumefaction of regional lymph nodes. The skin of the nostril region was thick, peeled, and hairless. The time of evolution of the lesions was approximately six months, and progressive weight loss was observed. The enlarged lymph nodes of the head and neck often observed in cattle at the farm improve with treatment with potassium iodide and penicillin. It is noteworthy that a case of actinobacillosis in the tongue (wood tongue) and lymph nodes of the head and neck was diagnosed at the same farm in the previous year (Schild et al. 2017).

At necropsy lips, nostrils and face were increased in volume and the skin was thick. There was an intense proliferation of connective tissue interspersed by small yellow nodules in the buccinator muscle. In the anterior area of the palate, one centimeter of diameter ulcer was observed (Fig.3). The submandibular, retropharyngeal, and parotid lymph nodes were enlarged and had yellowish nodules wrapped with whitish fibrous tissue.

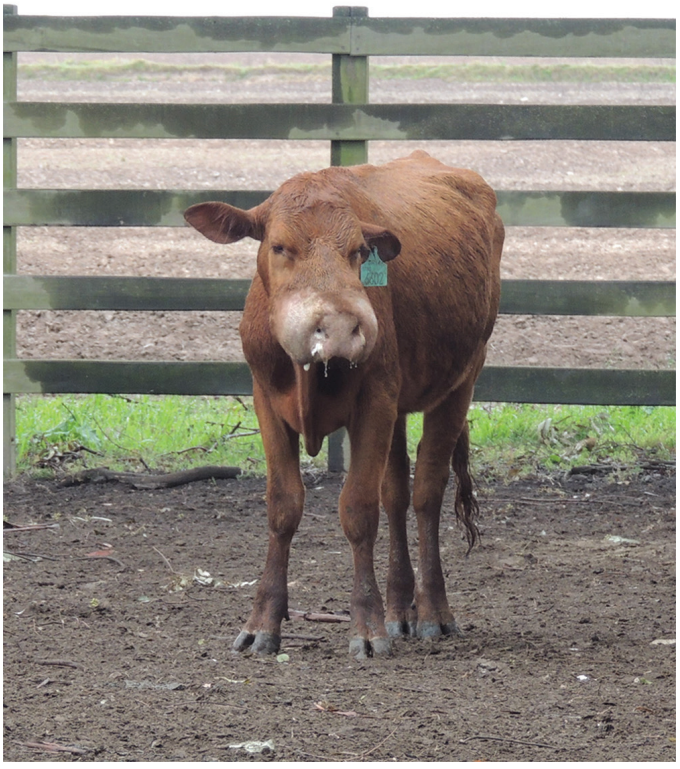


Fig.2. Actinobacillosis in cattle with clinical manifestation of hippopotamus-like face. There is an enlargement of the face affecting the nose and buccinator muscle. There are alopecia and peeling of the skin and serous to mucous nasal discharge.

Histologically, in the subcutaneous tissue and between muscle fibers of the affected region there were numerous pyogranulomas with central necrosis and presence of eosinophilic material forming rosettes (Splendore Hoepli) (Fig.4). These areas were surrounded by neutrophils, plasmocytes, macrophages, and giant cells. Proliferated fibrous connective tissue involved all pyogranulomas. The lesions extended to the nostrils and lips, in which the epidermis was thick and peeled. Small colonies of gram-negative bacteria, whose biochemical tests resulted inconclusively were isolated in bacteriological cultures. This allowed the identification only of the genus as *Actinobacillus* spp.

Paraffin material and bacterial colonies isolated were positive in PCR for ribosomal RNA gene 16S de *Actinobacillus lignieresii* (primers Al). The sequence obtained was compared with sequences deposited in GenBank that amplify with the



Fig.3. Actinobacillosis in cattle with clinical manifestation of hippopotamus-like face. An ulcer is in the anterior portion of the palate.

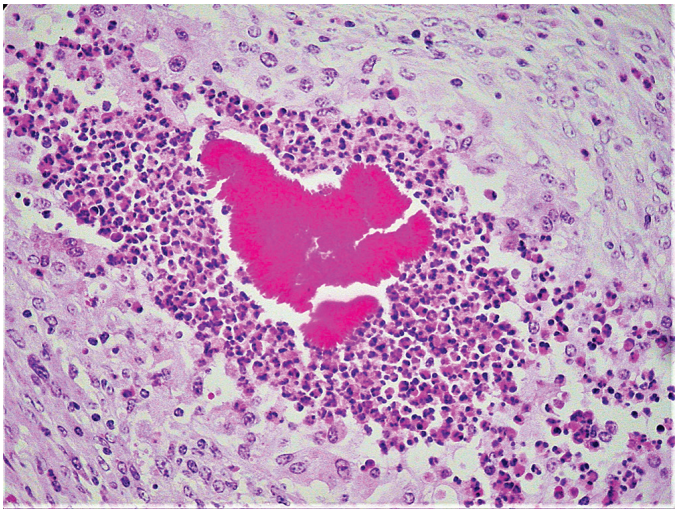


Fig.4. Actinobacillosis in cattle with clinical manifestation of hippopotamus-like face. Pyogranulomatous lesion in subcutaneous tissue with central necrosis, neutroplhils, macrophages and lynfocites and eosinophilic material forming rosettes (Splendore Hoeppli). HE, obj.40x.

Table 1. Identity percentage of target genetic sequencing with *Actinobacillus lignieresii* (primers Al)

	Strains (% identity)							
Strains	A*	B	C	D	E	F	G	H
A*	ID**	100	98.3	92.3	86.8	92.3	89.6	95.6
B	100	ID	98.3	92.3	86.8	92.3	89.6	95.6
C	98.3	98.3	ID	91.2	86.8	91.8	89.0	95.0
D	92.3	92.3	91.2	ID	92.8	90.1	93.4	93.4
E	86.8	86.8	86.8	92.8	ID	89.6	93.4	87.9
F	92.3	92.3	91.8	90.1	89.6	ID	91.2	89.6
G	89.6	89.6	89.0	93.4	93.4	91.2	ID	90.7
H	95.6	95.6	95.0	93.4	87.9	89.6	90.7	ID

* A = *A. lignieresii* (sequenced sample LRD 16855), B = *A. lignieresii* strain NCTC 4189 (AY362892), C = *Actinobacillus pleuropneumoniae* strain Shope 4074 (NR_04475), D = *Actinobacillus suis* strain JCM 2433 (LC383928), E = *Haemophilus haemolyticus* strain CIP 103290 (NR_104930), F = *Haemphilus parainfluenzae* ATCC 33392 strain CCUG 12836 (NR_042878), G = *Glaesserella parasuis* strain CCUG 3712 (NR_042879, H = *Conservatibacter flavenscens* strain CCUG 24852 (NR_159347); ** ID= identity.

primers Al_F and Al_R. The identity of 100% was obtained with the *A. lignieresii* (Table 1). The identity of 98.3% was obtained with the *Actinobacillus pleuropneumoniae,* due to differences in nucleotides 80 and 81. The identity of the samples with the other microorganisms ranged from 86.8% to 95.6%, and a difference can be observed between several nucleotides of the sequences (Fig.1).

DISCUSSION

Actinobacillosis, in the clinical form of a hippo-like face, described in the present study, is rare. The disease was characterized mainly by diffuse lesions on the lips, nostrils and face. In the present case, this clinical manifestation likely is related to the ulcerative lesion observed on the necropsied cattle palate. This lesion probably occurred as a result of the permanence of cattle in a native forest area. Small shrubs and fragments of branches, which due to stiffness and size could cause more easily injuries in this region of the oral cavity. Ulcers around the molar teeth were the entry route of infection in hippo-like face cases (Anderson et al. 1990, Turni et al. 2019), which was not present in cattle of this report, suggesting a different port of entry for the agent. The fact that the histological lesions were observed mainly in the subcutaneous tissue of the face and snout, does not rule out the possibility that the lesion was started by penetration of the agent into small lesions in the skin and/or nasal mucosa. In the property, the form of the disease affecting the head lymph nodes is frequent and, therefore, the environment is contaminated by the microorganism. Other authors describing the cutaneous form of the disease, mention environmental contamination as an important factor for the occurrence of different manifestations of the disease (Cahalan et al. 2012).

It has been mentioned that younger animals would be more predisposed to the development of the disease, especially due to the exchange in dentition that would favor infection (Rycroft & Garside 2000), however, several outbreaks are reported in adult animals that have complete dentition (Dhand et al. 2003). There were no lesions in dental alveoli or the adjacent gums in necropsied cattle, which would rule out the possibility of infection by this route. In a case of atypical lymphatic actinobacillosis described, the possibility of penetrating the agent through the intact mucosa and infection of regional lymph nodes from where the bacteria would spread via the lymphatic due to suppuration is mentioned (Caffarena et al. 2017).

DNA sequenced from the bacterial colony isolated from the tissues of affected cattle presented 100% identity with the *Actinobacillus lignieresii,* unequivocally proving the association of this bacterium in the etiology of the disease. It has been described that bacterial identification of the agent in chronic lesions or in animals treated with antibiotics can be difficult, requiring the use of molecular diagnostic techniques for the detection of *Actinobacillus* (Milne et al. 2001). The methodology developed in the present study is an auxiliary tool and indispensable for the diagnosis and precise identification of the agent in new outbreaks, especially in atypical cases of the disease.

A differential diagnosis should include other diseases that cause an increased volume of the face, such as infection by *Mannheimia granulomatis*, bacterium a condition colloquially known as lechiguana described in the southern region of Rio Grande do Sul, Minas Gerais and São Paulo (Ladeira et al. 1996, Birgel et al. 1999). In this case, however, the swelling is very firm, and occurring mainly over the scapula and its surroundings, and the histological lesion is characterized by extensive fibrosis in the subcutaneous tissue (Riet-Correa et al. 1992). Other diseases such as nocardiosis, which causes pyogranulomatous lesions in the skin and actinomycosis characterized by pyogranulomatous osteomyelitis, especially in the mandible (Relun et al. 2019), should also be differentiated from actinobacillosis.

CONCLUSIONS

Actinobacillosis with clinical manifestation of hippopotamus-like face, although unusual, occurs with epidemiological conditions similar to the others forms of the disease, and the entry route to the infection by the agent may be determinant in its clinical manifestation.

We draw attention to the importance of molecular diagnosis for the identification of *Actinobacillus lignieresii*, especially in atypical cases of the disease.

Conflict of interest statement.- The authors declare that there are no conflicts of interest.

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Pathogenesis of *Bovine alphaherpesvirus 2* in calves following different routes of inoculation[1]

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ABSTRACT.-Amaral B.P., Jardim J.C., Cargnelutti J.F., Martins M., Weiblen R. & Flores E.F. 2020. **Pathogenesis of *Bovine alphaherpesvirus 2* in calves inoculated by different routes.** *Pesquisa Veterinária Brasileira 40(5):360-367.* Setor de Virologia, Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria, Av. Roraima 1000, Rua Z, Prédio 63A, Santa Maria, RS 97105-900, Brazil. E-mail: eduardofurtadoflores@gmail.com

Bovine alphaherpesvirus 2 (BoHV-2) is the agent of herpetic mammilitis (BHM), a cutaneous and self-limiting disease affecting the udder and teats of cows. The pathogenesis of BoHV-2 is pourly understood, hampering the development of therapeutic drugs, vaccines and other control measures. This study investigated the pathogenesis of BoHV-2 in calves after inoculation through different routes. Three- to four-months seronegative calves were inoculated with BoHV-2 ($10^7 TCID_{50}.mL^{-1}$) intramuscular (IM, n=4), intravenous (IV, n=4) or transdermal (TD) after mild scarification (n=4) and submitted to virological, clinical and serological monitoring. Calves inoculated by the IV route presented as light increase in body temperature between days 6 to 9 post-inoculation (pi). Virus inoculation by the TD route resulted in mild inflammatory lesions at the sites of inoculation, characterized by hyperemia, small vesicles, mild exudation and scab formation, between days 2 and 8pi. Virus or viral DNA was detected by PCR in the crusts/swabs collected from lesions of 3 out of 4 animals inoculated TD from day 2 to 8pi. Viremia was detected in 3/4 animals of the IM group (from day 4 to 8pi); in 2/4 animals of the IV group (days 6 and 8pi) but not in the TD group. Calves from all inoculated groups seroconverted to BoHV-2 in titers from 4 to 64, as indicated by virus-neutralizing (VN) assays performed in sera collected at day 15pi. Administration of dexamethasone (Dex) to the inoculated calves at day 48pi, did not result in virus reactivation as indicated by lack of virus detection in the blood and/or in inoculation sites and no increase in VN antibody titers. These results demonstrated that BoHV-2 was able to replicate efficiently in calves following different routes of exposure, produced viremia after IM and IV inoculation and was not reactivated by Dex treatment.

INDEX TERMS: Pathogenesis, *Bovine alphaherpesvirus 2*, calves, cattle, acute infection, latency.

RESUMO.- [**Patogênese do alfaherpesvírus bovino 2 em bezerros inoculados por diferentes vias.**] O alfaherpesvírus bovino 2 (BoHV-2) é um agente etiológico da mamilite herpética (BHM), uma doença cutânea e autolimitante do úbere e tetos de vacas. Pouco se sabe sobre a patogênese do BoHV-2, dificultando o desenvolvimento de medicamentos terapêuticos e vacinas. Este estudo investigou a patogênese do BoHV-2 em bezerros após a inoculação por diferentes

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vias. Bezerros soronegativos de três a quatro meses foram inoculados com BoHV-2 (10^7TCID50.mL^{-1}) por via intramuscular (IM, n=4), por via intravenosa (IV, n=4) ou transdérmica (TD, n=4) após escarificação leve e submetidos a monitoramento virológico, clínico e sorológico. Os bezerros inoculados pela via IV apresentaram aumento leve da temperatura corporal entre os dias 6 a 9 pós-inoculação (pi). A inoculação do vírus pela via TD resultou em lesões inflamatórias leves nos locais de inoculação, caracterizadas por hiperemia, pequenas vesículas, exsudação leve e formação de crostas, entre os dias 2 e 8pi. O vírus ou DNA viral foi detectado por PCR nas crostas/swabs coletados de lesões de 3 de 4 animais inoculados TD do dia 2 ao 8pi. Viremia foi detectada em 3/4 dos animais do grupo IM (do dia 4 ao 8pi); em 2/4 animais do grupo IV (dias 6 e 8pi), mas não no grupo TD. Bezerros de todos os grupos inoculados soroconverteram o BoHV-2 em títulos de 4 a 64, conforme indicado por ensaios de vírus-neutralização (VN) realizados em soro coletado no dia 15pi. Administração de dexametasona (Dex) nos bezerros inoculados no dia 48pi, não resultou em reativação do vírus, como indicado pela falta de detecção de vírus no sangue e/ou nos locais de inoculação e pela ausência de aumento nos títulos de anticorpos. Estes resultados demonstraram que o BoHV-2 foi capaz de replicar eficientemente em bezerros seguindo diferentes vias de inoculação, produziu viremia após a inoculação IM e IV e não foi reativado pelo tratamento com Dex.

TERMOS DE INDEXAÇÃO: Patogênese, alfaherpesvírus bovino 2, bezerros, bovinos, infecção aguda, latência.

INTRODUCTION

Bovine alphaherpesvirus 2 - formerly bovine herpesvirus type 2 (BoHV-2) - is an enveloped, double-stranded DNA virus belonging to the family Herpesviridae, subfamily Alphaherpesvirinae, genus *Simplexvirus* (ICTV 2018). BoHV-2 is genetically and antigenically related to *Human alphaherpesviruses* 1 and 2 (HSV-1, HSV-2) (Sterz et al. 1974, Borchers et al. 1990, Ehlers et al. 1999). BoHV-2 is the agent of bovine herpetic mammillitis (BHM), a vesicular, erosive and necrotic cutaneous disease of the udder and teats of cows. The distribution and prevalence of BoHV-2 infection are poorly understood, but serologic data indicate that the virus is widespread in cattle worldwide (Rweyemamu et al. 1966, Dardiri & Stone 1972, Gibbs & Rweyemamu 1977).

Historically, BoHV-2infection has been described in several countries, including the United States (US) (Dardiri & Stone 1972), Great Britain (Johnston & Scott 1971), Brazil (Alice 1977, Campos et al. 2014), Kenya (Martin & Gwynne 1968), Australia and Japan (Turner et al. 1974, Imai et al. 2005). In addition to the classic BHM, BoHV-2 has also been associated with a generalized nodular skin condition called pseudo-lumpy skin disease (PLSD) (Gibbs & Rweyemamu 1977). Antibodies to BoHV-2 have been detected in wild ruminants (Plowright & Jesset 1971) and the infection has been experimentally reproduced in goats (Westbury 1981) and sheep (Torres et al. 2009b). However, a potential role of these species in the epidemiology of BoHV-2 remains uncertain. Likewise, there is no evidence of human infection, in spite of attempts to reproduce the infection upon inoculation of volunteers (Martin et al. 1966). Large outbreaks of BoHV-2-associated cutaneous disease occurring in a 10-year-period were described in dairy breed calves in California, US (Watanabe et al. 2017). These findings suggest that, like to other simplex viruses, BoHV-2 may circulate silently in susceptible host populations, occasionally producing clinical cases or outbreaks. This behavior may also explain findings of positive BoHV-2 serology in cattle populations with no evident, or scarce, history of compatible clinical disease (Almeida et al. 2008, Torres et al. 2009b).

In spite of its long story and several decades of scattered studies, several aspects of BoHV-2 biology and pathogenesis remain obscure. The pathogenesis of acute and latent infection remains poorly understood. The few pathogenesis studies in cattle were performed two or three decades ago, using old and nowadays obsolete techniques and resources. Experimental reactivation of the infection has not been consistently demonstrated (Probert & Povey 1975, Turner et al. 1976, Castrucci et al. 1980) and attempts to recover the virus upon explant cultures of sensory nerve ganglia have failed (Letchworth & Carmichael 1982). In some of the studies demonstrating latency and reactivation, biases may have been introduced by inoculating the virus intravenously (Castrucci et al. 1972, Scott & Martin 1978). Likewise, the sites in which the virus may remain latent remain unknown (Letchworth & Carmichael 1982). Pathogenesis studies were performed in animal models, with their inherent pitfalls and restrictions (Almeida et al. 2008, Torres et al. 2009a). Thus, a better understanding of BoHV-2 pathogenesis in their natural hosts is needed.

In addition, bovine herpesviruses have been proposed as viral vector for vaccine antigen delivery (Kanekiyo et al. 2019). As such, BoHV-2 may be also a candidate viral vector for vaccine use. Therefore, the present study investigated selected aspects of the pathogenesis of BoHV-2 in calves.

MATERIALS AND METHODS

Cell culture and virus. *Bovine alphaherpesvirus 2* (BoHV-2) strain New York 01 (NY01) was kindly provided by Dr. Fernando A. Osorio (Department of Veterinary and Biomedical Sciences, University of Nebraska, Lincoln, USA). All procedures of virus replication, quantitation and virus neutralizing assays (VN) were performed in CRIB cells (a MDBK-derived cell line resistant to BVDV). Cells were maintained in Eagle's minimum essential medium (MEM) (Vitrocell®, Nova Campinas, São Paulo, Brazil) supplemented with 10% fetal calf serum (Vitrocell®), penicillin (10,000 IU.mL-1), streptomycin (10mg/mL-1) and amphotericin B (250µg.mL-1) (Sigma - Aldrich®, Darmstadt, Hessen, Germany). The virus inoculum consisted of the supernatant of CRIB cells infected with BoHV-2 at passage # 12, containing 10^7 $TCID_{50}$.mL^{-1} (median tissue culture infectious doses).

Animal experiment. Sixteen holstein with ages ranging from 90 to 120 days, tested negative for BoHV-2 antibodies by virus neutralizing (VN) assays were used in the experiment. The animals were randomly allocated in four groups of four animals each and submitted to the following inoculation protocols: four calves were inoculated by the intramuscular route (IM group); four calves were inoculated by intravenous route (IV group); four calves were inoculated in the skin of the internal face of the left hind limb, after scarification with a needle (TD group) and application of the virus inoculum with the help of a cotton swab. Each animal was inoculated with 10^7 $TCID_{50}$.mL^{-1} of the virus. The four remaining animals (Control group) were inoculated with minimal essential médium (MEM) by the IM route (n=1), IV route (n=1) and by scarification of the skin,

as described by the TD group (n=2). The experimental groups were maintained in separated barns and given food and water ad libitum.

Experimental inoculated animals were monitored daily for local and systemic clinical signs; blood, nasal secretions and exudates of the inoculation sites were collected for virus detection. Clinical monitoring consisted of visual inspection and photographic registration of local signs (TD group); measurements of rectal temperatures, recording of appetite and alertness (all groups). Blood samples and nasal swabs were collected every two days from calves of the IM and IV groups. Nasal secretions and buffy coats were submitted to DNA extraction and PCR for the BoHV-2 genome. Swabs collected from the sites of virus inoculation (TD group) were collected daily and also submitted to PCR for BoHV-2 DNA. Serum samples were collected at days 0 and 14 pi and submitted to VN assays for BoHV-2 antibodies. Forty-eigtht days after virus inoculation, inoculated animals were submitted to dexamethasone (Dex) treatment (Azium–Merck Sharp and Dohme®, Kenilworth, Nova Jersey, USA), 0.1mg/kg/day for 5 consecutive days, and monitored thereafter for viremia, virus shedding, clinical signs and seroconversion. All procedures involving animals were approved by an institutional committee on ethics and animal welfare (CEUA-UFSM, approval number 6173221215).

Virus neutralizing assays. Virus-neutralizing (VN) assays were performed in duplicates in 96-well plates, incubating two-fold dilutions of the sera against 100-200 $TCID_{50}$.50μL^{-1} of the virus for 1h, followed by addition of a suspension of CRIB cells and incubation of the plates at 37°C in a CO_2 incubator. Readings were performed at 96h, by visualization of typical BoHV-2 cytopathic effect (cpe) in indicator cells. VN titers were considered as the reciprocal of the highest serum dilution that prevented the production of cpe. Sera positive and negative for BoHV-2 antibodies were used as controls in all assays.

Nucleic acid extraction and PCR. Total DNA was extracted from buffy coats, nasal secretions or cutaneous scabs/exudates by thephenol-chloroform protocol and submitted to final elution in 50µl of Tris-EDTA solution. A semi-nested PCR targeting a sequence within the BoHV-2 glycoprotein B gene was used for viral DNA detection. The first round targeted a 624bp sequence and used the primers F (forward) - CTCCAGCGACGATCCTAATTTC (position 6528) and R (reverse) TATGCGTTGTGCTCTGAGTG (position 7151). The second reaction targeted a 347pb sequence and used the same forward primers and the reverse primer CGGTGGTCTCAAGGTTGTTC (position 6874). PCR reactions were performed in a 25µl volume, using 3µl of DNA template, 0.4µM of each primer, 2mM $MgCl_2$, 8mM of dNTPs, 1 × reaction buffer, 10% of reaction dimetilsulfoxide and 1.5 unit of Taq polymerase (Life Technologies®, Carlsbad, Califórnia, USA). PCR conditions were: initial denaturation (95°C for 5min), followed by 30 cycles of 95°C for 45s; 56°C for 30s for primer annealing and 72°C for 45s for primer extension; and a final extension of 7 min at 72°C. Products were visualized in a 2% agarose gel, stained with Gel Red (Biotium®, Fremont, California, EUA) and visualized under UV light. Control included total DNA extracted from mock-infected and BoHV-2 infected CRIB cells.

Statistical analysis. Statistical analysis was performed using the Prism software (GraphPad; 6th version). Students T-test was performed on all groups. Statistical differences between groups were considered significant at P<0.01.

RESULTS

Acute infection

No overt systemic signs were observed in inoculated calves in the days following virus inoculation. Food intake and alertness remained unaltered upon detailed observation. The body temperature of animals of the IV group presented an increase between days 4 and 8-9pi; groups IM and TD presented a transient increase in body temperature at day 11pi. The temperature of control calves remained within normal limits (Fig.1). Comparing to the basal temperatures, animals from group IV presented the higher variation (P<0.01). Temperatures returned to pre-inoculation levels at day 11-12pi and remained at those levels up to the end of the monitoring period.

Virus inoculation after skin scarification (TD) resulted in mild inflammatory lesions within the inoculation sites. The observed changes were hyperemia, small vesicles, mild exudation and scab formation, recorded between days 2 and 8pi (Fig.2). At day 8pi, only residual and slight scabs were still present. Animals inoculated with MEM presented transient hyperemia (1-2 days) and very thin scab formation along the lines of scarification (Fig.2).

Table 1 presents the results of PCR performed in total DNA extracted from swabs/scabs of cutaneous lesions (or sites of virus inoculation) of the TD group. Viral DNA was detected in material collected from lesions of 3 out of 4 animals (75%). Virus shedding/presence was detected from day 2 to 8pi in two animals and from day 2 to 6pi in one calf (Table 1). No viral DNA was detected after day 10pi up to day 14pi, when the collection was discontinued. These results showed a transitory virus replication/detection (lasting 4 to 6 days) in the lesions associated with skin inoculation.

The presence of viral DNA as indicator of viremic spread was investigated in the blood of inoculated calves in the days following inoculation. For this, total DNA extracted from buffy coats was submitted to the same PCR described above. The results are presented in table 2. Viral DNA was detected in the buffy coats of 3/4 animals (75%) of the IM group, from

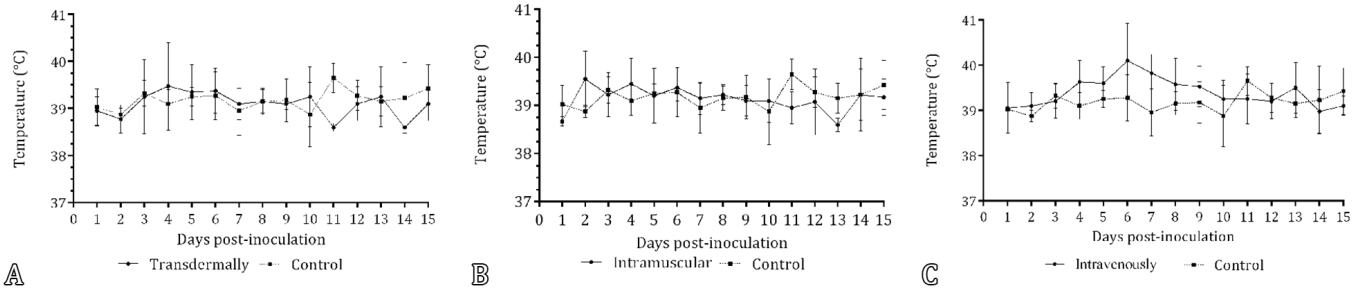


Fig.1. Mean body temperature (T°C) of calves inoculated with *Bovine alphaherpesvirus 2* (BoHV-2) by different routes: (**A**) transdermal, (**B**) intramuscular or (**C**) intravenous.

day 4 to 8pi; and in 2/4 animals (50%) of the IV group, at days 6 and 8pi. No viral DNA was detected in the buffy coats of TD group nor in control calves (Table 2). These results demonstrated a transient viremia in calves inoculated by the IM and IV routes and no detectable viremia in the TD group.

Calves from all inoculated groups seroconverted to BoHV-2 in titers from 4 to 64, as indicated by VN assays performed in serum samples collected at day 15pi. The mean VN titer developed by the IV group (GMT=38.05) was statistically higher than those from the IM (GMT=8) and TD (GMT=4.75) groups (P<0.01). Control animals remained seronegative throughout the experimental period (Table 2). The results of VN tests confirmed, indirectly, an efficient virus replication in calves of the three inoculated groups.



Fig 2. Skin lesions developed by calf 276 inoculated with (**A**, **B**, **D**, **E**, **G**, **H**) *Bovine alphaherpesvirus 2* (BoHV-2) transdermally and (**C**, **F**, **I**) by a control calf inoculated with culture medium, at different days after virus inoculation. (**A**-**B**) 276 and (**C**) control. Days 2 and 4pi, respectively, thin scabs and skin retraction derived from scarification. (**D**-**E**) Days 6 and 8pi. Thick, yellowish scabs and small disrupted vesicles. (**F**) Control. Day 8pi. A thin scab, advanced stage of healing. (**G**-**H**) 276. Days 10 and 12pi. Crust remnants, advanced stage of healing. (**I**) Skin completely healed (control).

Latent infection

In attempts to reactivate latent infection, inoculated calves were submitted to a protocol of Dex administration beginning at day 48pi (five daily IM doses, following by daily examination and specimen collection). Again, food intake and alertness remained unchanged. A slight and transient increase in body temperature was observed in groups TD and IM between days 6 and 9 post-Dex (pDex) and at day 7pDex in group IV (Fig.3). Control calves presented a slight increase in body temperature at day 8 pDex.

No evidence of virus reactivation was observed in the inoculated calves upon Dex administration. PCRs of the buffy coats (IM, IV groups) and from swabs collected from the inoculation sites (ID group) were negative for viral DNA.

Table 1. Results of PCR performed on DNA extracted from material obtained from swabs and lesions of calves inoculated transdermally with *Bovine alphaherpesvirus* 2 (BoHV-2)

Animal ID	Days post-inoculation							
	0	2	4	6	8	10	12	14
276	-	+	+	+	+	-	-	-
280	-	+	+	+	+	-	-	-
284	-	-	-	-	-	-	-	-
294	-	+	+	+	-	-	-	-

Likewise, the VN titers pre- (day 48pi) and post-Dex (day 60pi) remained unaltered in most animals, or with a single dilution difference (higher or lower), demonstrating absence of re-stimulation of the immune system upon Dex treatment. Taken together, these results demonstrated lack of detectable virus reactivation.

DISCUSSION

Bovine alphaherpesvirus 2 (BoHV-2) has been a neglected virus, probably due to its limited sanitary, economic importance and rare occurrence. The scarce knowledge about its biology, molecular biology and pathogenesis has somehow hampered its use as a model for human simplex viruses and as a vector for vaccine delivery, among others. Our group has long been interested in BoHV-2 epidemiology and pathogenesis (Almeida et al. 2008, Torres et al. 2010). More recently, we have focused our interest in the use of BoHV-2 for anti-viral drug testing and for development of vaccine platforms for antigen delivery. In this sense, a better understanding of the biology of BoHV-2 in its natural host would help future developments.

Early studies on BoHV-2 focused on clinic-pathological description of clinical cases/outbreaks (Weaver et al. 1972, Castrucci et al. 1978, Castrucci et al. 1979, Woods et al. 1996, Kemp et al. 2008) and/or experimental reproduction of clinical disease in cattle (Weaver et al. 1972, Turner et al.

Table 2. Virological and serological findings in calves inoculated with *Bovine alphaherpesvirus 2* (BoHV-2) by different routes

Group	Animal ID	PCR - days post inoculation								VN assays - days post inoculation			
		0	2	4	6	8	10	12	14	0	15	48	60
Intramuscular	274	-	-	+	+	-	-	-	-	< 2	< 2	< 2	< 2
	277	-	-	-	-	-	-	-	-	< 2	4	< 2	2
	281	-	-	+	+	+	-	-	-	< 2	16	16	8
	282	-	-	-	-	+	-	-	-	< 2	8	8	16
Intravenous	283	-	-	-	+	+	-	-	-	< 2	32	64	32
	287	-	-	-	+	+	-	-	-	< 2	32	64	32
	295	-	-	-	-	-	-	-	-	< 2	64	64	32
	299	-	-	-	-	-	-	-	-	< 2	16	32	32
Transdermal	276	-	-	-	-	-	-	-	-	< 2	8	4	8
	280	-	-	-	-	-	-	-	-	< 2	4	4	2
	284	-	-	-	-	-	-	-	-	< 2	4	2	<2
	294	-	-	-	-	-	-	-	-	< 2	4	4	4
Control	286	-	-	-	-	-	-	-	-	< 2	< 2	< 2	< 2
	289	-	-	-	-	-	-	-	-	< 2	< 2	< 2	< 2
	292	-	-	-	-	-	-	-	-	< 2	< 2	< 2	< 2
	293	-	-	-	-	-	-	-	-	<2	< 2	< 2	< 2

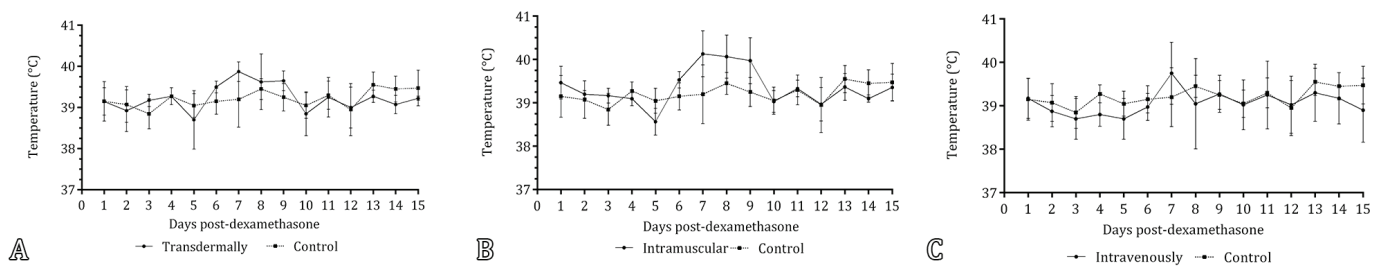


Fig.3. Mean body temperature (T°C) of calves inoculated with *Bovine alphaherpesvirus 2* (BoHV-2) by different routes: (**A**) transdermal, (**B**) intramuscular and (**C**) intravenous and submitted to dexamethasone administration at day 40pi.

1974, Castrucci et al. 1978, Woods et al. 1996, Kemp et al. 2008) and animal models (Westbury 1981, Almeida et al. 2008, Smee & Leonhardt 1994, Torres et al. 2009a, 2010). A few experimental studies focusing on acute disease (Castrucci et al. 1977, Castrucci et al. 1982) or latent infection Probert & Povey (1975), Scott & Martin (1978), Letchworth & Carmichael (1982) introduced probably biases on data interpretation due to IV virus inoculation, an unlikely natural route of exposure. For instance, the virus tropism and systemic dissemination were investigated after IV inoculation (Castrucci et al. 1977, 1978). Against this hypothesis, several lines of evidence point out for virus penetration/transmission by direct/indirect contact (Castrucci et al. 1982). Thus, the results of some early studies should be interpreted with caution since they may not reflect the natural route of inoculation. Likewise, results from studies in animal models might not necessarily reflect the events occurring in the natural hosts.

We performed BoHV-2 inoculation though different routes for the following reasons. The TD route was chosen because it would more closely resemble the natural route of infection. Using this protocol of virus inoculation, we have successfully reproduced udder and teat lesions typical of BHM in ewes (Almeida et al. 2008). The IV route was employed trying to reproduce the virological findings of early studies which used this route (Castrucci et al.1977, Castrucci et al. 1978, Martin & Scott 1979). Finally, the IM route aimed at stimulating the events following IM administration of BoHV-2 as a vaccine vector.

In our study, viremic dissemination was observed after IM inoculation and, to a lesser extent, after IV administration. In both groups, detection of viral DNA in the blood was transient, lasting 1 to 4 days. In contrast, virus inoculation after skin scarification did not result in viremic spread in spite of virus replication and detection in local sites for 4 to 6 days (Table 1). The localized nature of cutaneous BoHV-2 replication and pathology has been previously attributed to the route of virus penetration/exposure, specific virus tropism and, additionally, to the surface temperature which may favor virus replication (Letchworth et al. 1982a). In fact, early studies have demonstrated that BoHV-2 replicates more efficiently at lower temperatures (30-3°C), comparing to replication at physiological bovine temperatures (38-39°C) (Letchworth et al. 1982a, Letchworth & Carmichael 1984). The preference for replication at low temperatures might also explain the higher incidence and distribution of BoHV-2 in regions of temperate climate (Letchworth & Carmichael 1984). On the other hand, systemic viral spread has been demonstrated only after IV inoculation (Castrucci et al. 1977, 1978). In this sense, our results demonstrated that IM inoculation results in virus replication and viremic spread. These findings might be a safety concern towards the potential use of BoHV-2 as a vaccine vector for IM use. Thus, the possible consequences of BoHV-2 viremic spread upon IM administration should be properly addressed.

A major route of BoHV-2 penetration in natural infections seems to be throughout micro-lesions/abrasions in the epithelium of the udder and teats. As such, our protocol of TD inoculation aimed at mimetizing the natural events leading to virus penetration and replication. Virus inoculation after skin scarification resulted in mild inflammatory lesions characterized by hyperemia, small vesicles, mild exudation and scab formation between days 2 and 8pi (Fig.2). Contrasting with some previous studies (Gibbs et al. 1973, Letchworth et al. 1982b), no classical BHM lesions were reproduced upon skin inoculation. In a previous study, we reproduced similar BHM lesions in the udder and teats of ewes experimentally inoculated (Almeida et al 2008). The differences in the clinical outcome may attributed to the nature of the skin epithelium of the hind limbs versus udder/teats and/or to the procedure of virus inoculation. In any case, virus replication and mild, transient cutaneous lesions were produced upon TD virus inoculation. Virus replication seemed to be restricted to the cutaneous surface of the inoculation sites since viremic spread was not detected.

Although BoHV-2 is known to establish latent infections, recovery of infectious virus and/or demonstration of virus reactivation after corticosteroid treatment has not been consistently achieved either in natural or experimental infections (Probert & Povey 1975, Castrucci et al 1980, Letchworth & Carmichael 1982). A few attempts were successful in recovering virus and/or demonstrating reactivation upon corticosteroid treatment, yet mainly in animals inoculated by the IV route (Scott & Martin 1978, Martin & Scott 1979, Castrucci et al.1982), which is unlikely to be the natural route of infection. Otherwise, latency, reactivation and colonizing of sensory nerve ganglia with latent DNA have been demonstrated in experimental models (Almeida et al. 2008, Torres et al. 2009a). In our study, no virus shedding or seroconversion were observed upon Dex administration, in spite of virus replication in the sites of virus inoculation and presence of the virus in blood during acute infection. These results corroborate previous studies which failed in demonstrating virus reactivation in BoHV-2-infected cattle (Probert & Povey 1975, Castrucci et al 1980, Letchworth & Carmichael 1982). From the vaccine perspective, the failure/lack of reactivation would be a highly desirable property of a candidate vaccine viral vector. Unfortunately, we did not keep the calves for investigating the sites of latent infection upon the different routes of exposure.

Nearly 50 years after the initial identification of BoHV-2 as the agent of BHM (Gibbs et al. 1973) and, subsequently, the demonstration of its relationship with human simplex viruses (Snowden et al 1985, Castrucci et al. 1990, Ehlers et al. 1999), the pathogenesis of BoHV-2 in cattle is still poorly understood. Thus, our data contributes for the overall knowledge on the biology and pathogenesis of this simplex virus. Regardless its restricted sanitary/economic relevance, this bovine virus should be looked at from different perspectives. First, BoHV-2 infection offers a unique opportunity to study selected aspects the molecular biology, pathogenesis and immunology of a simplex virus in their natural host. HSV-1 and HSV-2 are among the most studied human viruses, yet the studies *in vivo* are largely restricted to animal models (Kollias et al. 2015). Thus, studying the biology of a simplex virus in its natural host may particularly attractive/fascinating since it may provide new and more realistic insights in the virus-host interactions. Second, the biological and genetic similarity of BoHV-2 with human simplex viruses may be exploited for the research and development of pharmaceuticals and vaccines as well. In this sense, BoHV-2 has been proposed as a potential target/model for the development of anti-herpetic drugs (Field et al. 2006, Dezengrini et al. 2010, Torres et al.

2010). Third, animal herpesviruses have been long proposed as potential platforms for vaccine delivery and a number of current animal vaccines are based on herpesvirus vectors (Verma & Weitzman 2005, Kanekiyo et al. 2019). Thus, more than a cattle pathogen of questionable clinical relevance, BoHV-2 may represent an attractive alternative to study the interactions of simplex viruses with their hosts, for research/development of anti-herpetic drugs and veterinary vaccines.

CONCLUSION

Inoculation of BoHV-2 in calves by different routes results in virus replication, yet viremic spread occurs after intramuscular or intravenous inoculation but not after transdermal inoculatio.

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Identification and characterization of pestiviruses isolated from individual fetal bovine serum samples originated in Rio Grande do Sul state, Brazil[1]

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ABSTRACT.- Merchioratto I., Monteiro F.L., Traesel C.K., Flores E.F., Weiblen R. & Brum M.C.S. 2020. **Identification and characterization of pestiviruses isolated from individual fetal bovine serum samples originated in Rio Grande do Sul state, Brazil.** *Pequisa Veterinária Brasileira 40(5):368-373.* Laboratório de Virologia, Curso de Medicina Veterinária, Universidade Federal do Pampa, BR-472 Km 585, Cx. Postal 118, Uruguaiana, RS 97508-000, Brazil. E-mail: mariobrum@unipampa.edu.br

The identification of diversity of bovine pestiviruses circulating in the field is fundamental for continuous evaluation of diagnostic tests and vaccine composition. In this article we performed the genetic and antigenic characterization of twelve bovine pestiviruses isolated in the western region of Rio Grande do Sul, Brazil. The viruses were isolated from sera of bovine fetuses or from animals with clinical presentations suggestive of pestivirus infection. Genetic characterization by sequencing and phylogenetic analysis of the 5'UTR region of the viral genome allowed for the identification of bovine viral diarrhea virus (BVDV-1a, 4/12, 33.3%), BVDV-1b (6/12, 50%) and BVDV-2 (2/12, 16.7%). The reactivity of the isolates with a panel of monoclonal antibodies raised against envelope proteins (Erns, E1 and E2) demonstrated a high antigenic variability among isolates. Thus, the active circulation of bovine pestivirus infection, with high genetic and antigenic variability, in cattle on the western border of RS was confirmed, demonstrating the importance of continuous characterization of the pestiviruses circulating in the cattle herds to keep the diagnostic and control measures up to date.

INDEX TERMS: Pestivirus, fetus, serum, bovine, Brazil, BVDV, diarrhea viral bovine, variability, monoclonal antibodies, cattle.

RESUMO.- [**Identificação e caracterização de pestivírus isolados de amostras individuais de soro fetal bovino originárias do Rio Grande do Sul, Brasil.**] A identificação da diversidade de pestivírus bovinos que circulam no campo é fundamental para a avaliação contínua dos testes de diagnóstico e composição de vacina. Neste artigo, realizamos a caracterização genética e antigênica de doze pestivírus bovinos isolados na região oeste do Rio Grande do Sul, Brasil. Os vírus foram isolados de soros de fetos bovinos ou de animais com apresentações clínicas sugestivas de infecção por pestivírus. A caracterização genética por sequenciamento e análise filogenética da região 5'UTR do genoma viral permitiu a identificação do vírus da diarréia viral bovina (BVDV-1a, 4/12, 33,3%), BVDV-1b (6/12, 50%) e BVDV-2 (2/12, 16,7%). A reatividade dos isolados com um painel de anticorpos monoclonais criados contra proteínas do envelope (Erns, E1 e E2) demonstrou uma alta variabilidade antigênica entre os isolados. Assim, confirmou-se a circulação ativa da infecção por pestivírus bovino, com alta variabilidade genética e antigênica, em bovinos na fronteira oeste do RS, demonstrando a importância da contínua caracterização dos pestivírus circulantes em bovinos para manter atualizadas as medidas de diagnóstico e controle.

TERMOS DE INDEXAÇÃO: Pestivírus, soro, feto, bovinos, Brasil, BVDV, pestivírus, diarreia viral bovina, variabilidade, anticorpos monoclonais.

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INTRODUCTION

Bovine pestiviruses are associated with respiratory, digestive, hemorrhagic and reproductive infection and disease (Baker 1995). The infection has a worldwide distribution and is responsible for important economic losses and, therefore, is a major target of control programs (Houe 2003, Ridpath 2003). In Brazil, its presence was first described in the late 1960s and since then it has been systematically identified in several clinical and epidemiological presentations (Botton et al. 1998, Canal et al. 1998, Silveira et al. 2015, Flores et al. 2018).

Pestivirus of cattle comprise three viral species: bovine viral diarrhea virus 1 and 2 (BVDV-1 and -2) and the HoBi-like *pestivirus* (HoBiPeV). These viruses belong to the genus *Pestivirus* of the Flaviviridae family. BVDV-1, BVDV-2 and HoBiPeV were classified as *Pestivirus A*, *B* and *H*, respectively (Simmonds et al. 2017, Smith et al. 2017). These viruses are enveloped and have a positive polarity single-stranded RNA genome, 12.3kb in length, with a single open reading frame (ORF) containing two untranslated regions at the 5'- and 3'-ends (5' and 3'UTR, respectively) (Schweizer & Peterhans 2014). The 5'UTR region is highly conserved, and in association with N^{pro} and E2 regions, is used for phylogenetic analysis and classification of isolates into species and subgenotypes (Ridpath et al. 1994, Silveira et al. 2015). The BVDV-1 genotype is divided into 21 subgenotypes (BVDV-1a-u), BVDV-2 in four (BVDV-2a-d) and there are indicatives of at least four HoBiPeV subgenotypes (HoBiPev-3a-d) (Giangaspero et al. 2008, Dias et al. 2017, Yeşilbağ et al. 2017).

Glycoproteins Erns, E1 and E2 are present in the viral envelope and they are very variable (Schweizer & Peterhans 2014). The consequence of the genetic and antigenic variability, associated with the possibility of the emergence of new species and variants, creates the need for permanent monitoring of pestiviruses circulating in the field, as well as to update diagnostic techniques and vaccine composition (Ridpath 2003, Bauermann et al. 2013, Monteiro et al. 2018).

Several studies show that virus infection is widespread in the Brazilian cattle and seroprevalence levels varies according to the region of the country (Almeida et al. 2013, Monteiro et al. 2019). Bovine pestiviruses has already been isolated from animals with subclinical infection, herd with reproductive problems, abortions, animals with respiratory, digestive, hemorrhagic signs, mucosal disease (MD), persistently infected (PI) calves and fetal bovine serum (FBS) (Silveira et al. 2015, Flores et al. 2018). Phylogenetic analyses of isolates demonstrated that the three species are present and suggest a predominance of BVDV-1 (54.4%), followed by BVDV-2 (25.7%) and Hobi-like pestivirus (19.9%) samples (Flores et al. 2018). However, the comparison of bovine herd size and number of isolates indicates deficiency in laboratory diagnosis and the need to improve isolation and characterization of samples present in the field.

Fetal infection of pregnant cows is extremely important as it can produce gestational losses (embryonic loss, abortion, malformation, mummification, teratogeny, delivery a weak calf or birth of persistently infected animals (PI). PI calves usually have no apparent clinical signs, excrete the agent indefinitely, and serve as a major source of infection for other susceptible animals (Baker 1995). Fetal bovine serum (FBS) is a supplement for cell culture and used in production of veterinary and human vaccines. Commercial FBS is a pool of several fetuses collected from slaughterhouses and bovine pestiviruses were identified as contaminating (Xia et al. 2011, Monteiro et al. 2018). Brazil is a major producer and exporter of FBS and pestivirus contamination is a constant concern for industry and laboratories (Schirrmeier et al. 2004, Bauermann et al. 2013, Monteiro et al. 2018).

Thus, this study characterized genetically and antigenically twelve pestiviruses of cattle isolated in the western region of Rio Grande do Sul, Brazil, in order to investigate their genetic and antigenic variability.

MATERIALS AND METHODS

Cells and viruses. Procedures for amplification of pestivirus of bovine and reference viruses (BVDV-1 Singer, BVDV-2 VS-253, HoBiPeV - SV757/15) were carried out on pestivirus-*free* bovine kidney cells (MDBK - *Madin-Darby* bovine kidney). The cells were cultured in minimal essential medium (Dulbecco Minimum Essential Medium) (DMEM) supplemented with 10% equine serum, antibiotic (gentamicin 50µg/mL), antifungal (amphotericin B 1µg/mL) and maintained in at incubator with 5% CO_2 at 37°C.

Pestivirus of bovine samples. Twelve non-cytopathogenic bovine pestiviruses belonging to the stock of the Unipampa virology laboratory were used in this study. Ten viruses were isolated from 754 serum of fetuses samples collected from 2011 to 2013 in slaughterhouses in western Rio Grande do Sul, Brazil. Samples LV14/15 and LV84/11 were isolated from the blood of a calf with retarded growth and an animal with chronic weight loss, respectively. For viral isolation, 200 microliters of sample (fetal serum or blood) were individually inoculated for 60 minutes in 24-well plates with MDBK monolayer (Botton et al. 1998). After this, three blind passages were performed at 72 hours intervals. At the end of the third passage, the supernatant was stored and the cells subjected to indirect immunofluorescence assay (IFA) with a pool of monoclonal antibodies as a primary antibody. The primary antibodies demonstrated broad reactivity and ability to recognize the three different bovine pestiviruses (Kreutz et al. 2000, Flores et al. 2018). Positive samples were identified, storage and further cultured for analyses. All isolated viruses were originated from Uruguaiana, Itaqui, Barra do Quaraí, Alegrete, Itacurubi and Maçambará municipalities.

RNA extraction and RT-PCR. MDBK cells cultures were individually infected with the isolates in 24-well polyethylene plates. After 72 hours, the supernatants were collected and subjected to RNA extraction using TRIzol® Reagent (Life Technologies, Carlsbad/CA) according to the manufacturer's instructions. The cDNA synthesis was performed with the GoScript™ Reverse Transcription Random Primers kit (Promega, Madison/WI, USA) and used as a template for the amplification of the 5'UTR region by the polymerase chain reaction (PCR). For PCR, the set of primers HCV90-368 (Forw-CATGCCCATAGTAGGAC and Rev-CCATGTGCCATGTACAG) was used (RIDPATH; BOLIN; DUBOVI, 1994). The amplification conditions were: 94°C for 5 min, followed by 35 cycles of 94, 50 and 72°C for 30s each, and final extension at 72°C for 7 min.

Sequencing and phylogenetic analysis. The PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) and submitted to sequencing (ACTGene Análises Moleculares Ltda, Alvorada/RS, Brazil). Consensus sequence were obtained by evaluation of forward and reverse reading of each isolate and edited using the BioEdit Sequence Alignment Editor software

suite, version 7.0.5.3[6] (Hall 1999). These were submitted to the Basic Local Alignment Search Tool (BLAST)[7] for comparison with sequences available in GenBank and classification of the isolates for the three bovine pestivirus genotypes. Alignment of the 12 consensus sequences and 35 standard sequences was performed using the Clustal W (Thompson et al. 1997) software contained in BioEdit. Evolutionary analysis and phylogenetic reconstruction of the isolates and standard strains was performed using the Molecular Evolutionary Genetics Analysis (MEGA) version X (Tamura et al. 2011). The evolutionary history was inferred using the Neighbor-Joining method, and the evolutionary distances were computed using the Kimura 2-parameter method, with *bootstrap* values were calculated using 1000 replicates. Accession numbers of the reference sequences used in phylogenetic construction are in Fig.1.

Reactivity with a panel of monoclonal antibodies. The antigenic variability of the isolates was determined by the reactivity with 13 monoclonal antibodies (MAbs) directed to the glycoproteins Erns, E1 and E2 (Corapi et al. 1990, Kreutz et al. 2000). For this, MDBK cells infected with each isolate were trypsinized, resuspended and deposited on *multispot* slides, and after cell adhesion, cells

[6] Available at <http://www.mbio.ncsu.edu/bioedit/bioedit.html>

[7] Available at <http://www.ncbi.nlm.nih.gov/BLAST/>

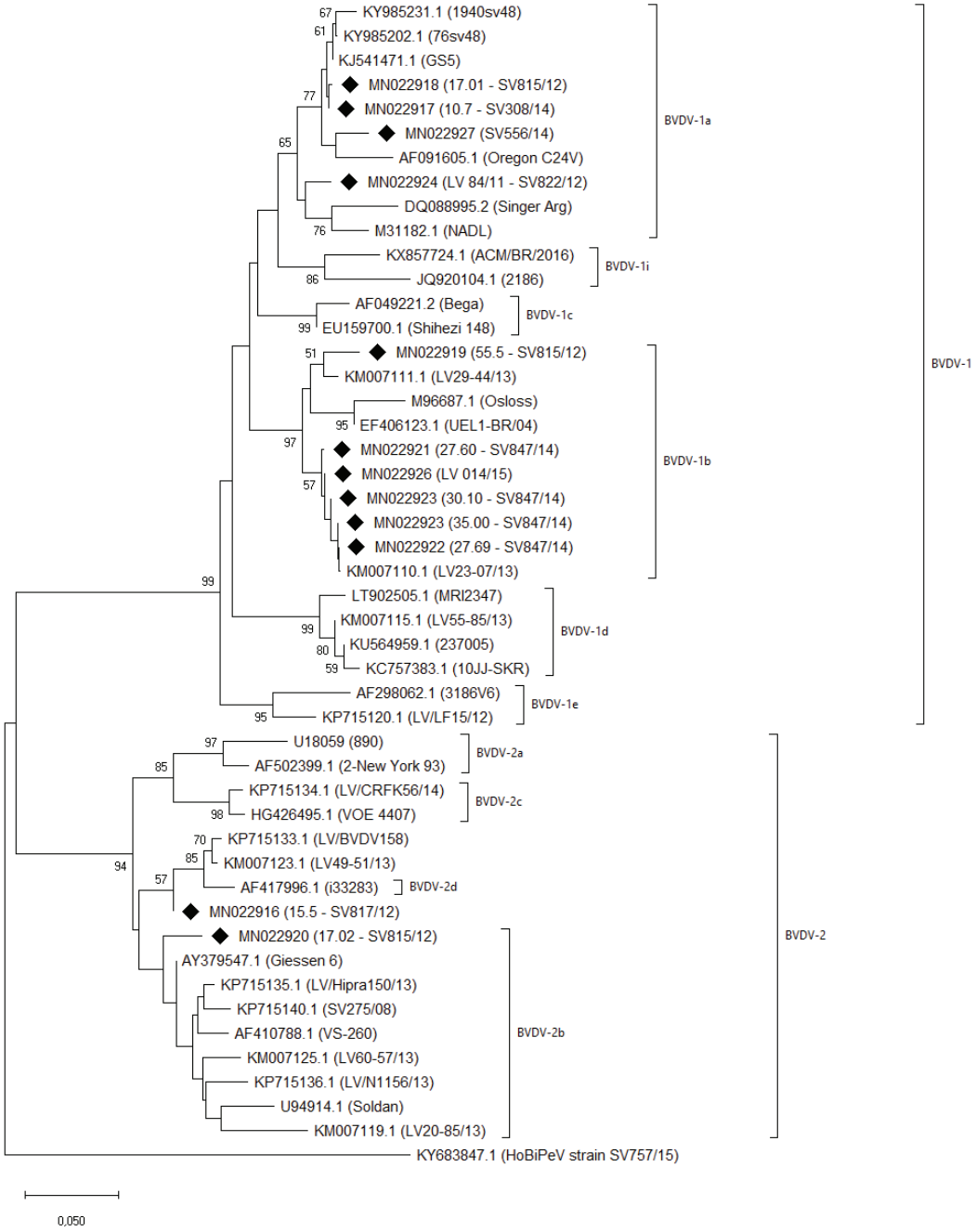


Fig.1. Phylogenetic analysis constructed by the Neighbor-Joining method, Kimura-2 parameter, based on the 5'UTR region of 12 nucleotide sequences of bovine pestivirus isolates (♦; 411 positions). Only *bootstrap* values (1000 replicates) above 50% are demonstrated.

were fixed with cold acetone. The ability of each MAb to recognize antigens of the isolates was investigated by the IFA technique (indirect immunofluorescence) (Botton et al. 1998). MAbs were used individually as primary antibody and an anti-mouse fluorescein-conjugated anti-IgG (Sigma-Aldrich, St. Louis/MO, USA) was used as secondary antibody. As a positive and negative control, standard strains (BVDV-1 Singer, BVDV-2 VS-253 and HoBiPeV SV757/15) and uninfected cells were used, respectively.

RESULTS

Individual analysis of 754 FBS allowed the isolation of 10 viruses, thus 1.3% of the samples tested were actively infected with bovine pestivirus. Two other samples from suspected cases were also included in the study. These viruses isolates provide an overview of the circulating samples in the region studied. All isolated samples were classified as non-cytopathic (NCP).

The genetic and antigenic characterization demonstrated that the twelve pestivirus isolates belong to BVDV-1 and -2 species and display a broad antigenic variability (Fig.1 and Table 1). Phylogenetic analysis identified ten samples as BVDV-1 (83.3%) and two viruses as BVDV-2 (16.7%). Among viruses classified as BVDV-1, four belong to the BVDV-1a genotype (4/12, 33.3%) and six are BVDV-1b (6/12, 50%). Among samples compatible with BVDV-2, isolate 17.02 was grouped as BVDV-2b, whereas 15.5 could not be classified in either BVDV-2 subgenotype (Fig.1).

The reactivity of the MAbs with the isolates confirmed a wide antigenic variability among the BVDVs (Table 1). No MAb used recognized 100% of the isolates. The level of recognition of the isolates by MAbs varied between 30.7 and 84.6%, reaffirming the wide variability among the isolates. The variability could be observed between the isolates and the standard strains, as well as among the different genotypes.

DISCUSSION

Pestivirus isolates from fetal serum and from animals with clinical signs were classified as BVDV-1 and BVDV-2 and confirmed the existence of genetic and antigenic variability among isolates. Among the analyzed samples, the presence of BVDV-1 (10/12) was predominant in relation to BVDV-2 (2/12). Despite of the limited number of viruses characterized, these findings are consistent with previous studies that characterized a more comprehensive sampling and demonstrate a predominance of BVDV-1 in Brazilian cattle herds (Weber et al. 2014, Silveira et al. 2015, Flores et al. 2018). The BVDV-1a (4/10) and BVDV-1b (6/10) subgenotypes were the two identified in the present study and reflect what has already been demonstrated in other regions of the country (Silveira et al. 2015, Flores et al. 2018). The representative circulation of bovine pestivirus genotypes in a given region can be determined when at least 50 virus samples are analyzed (Flores et al. 2018). Thus, the small number of samples studied herein can not be considered representative of the western region of Rio Grande do Sul, but they suggest the predominance of these two genotypes and demonstrate the circulation of these viruses in the cattle population.

In the present study, it was possible to determine that isolate 15.5 (SV817/12) belongs to the BVDV-2 species, without classification in subgenotype. The difficulty in determining the subgenotype also occurred with isolates of the BVDV-2 in other studies (Weber et al. 2014, Silveira et al. 2015, Monteiro et al. 2018). The analysis of the 5'UTR region alone may not be sufficient to determine the subgenotype for some viruses. In these situations, it is suggested to sequence the N^{pro} and E2 regions to obtain the definitive virus genotype classification (Pellerin et al. 1994, Ridpath et al. 1994, Silveira et al. 2015, Monteiro et al. 2018). No HoBiPeV isolates were identified in the present study. HoBiPeV are recognized as a new emerging bovine pestivirus species and represent the smallest group (<20%) among the three pestivirus species identified in Brazil (Silveira et al. 2015, Flores et al. 2018).

The evaluation of the envelope glycoproteins profile performed with MAbs confirmed the antigenic variability among the isolates. Erns, E1 and, especially E2 glycoproteins are responsible for the interactions of the virus with the cellular

Table 1. Reactivity of a panel of monoclonal antibodies (mAb) with bovine pestiviruses from western of Rio Grande do Sul State, Brazil

Isolates	Reactivity of monoclonal antibodies												
	E0	E1	E2								Undetermined		
	3.D.8	6.F.11	19.F.9	F.11.4.8	20.G.7	31.C.4	18.D.4	7.1.8	6.D.11	10.F.9	32.B.3	2.D.5	6.C.5
17.01 (SV815/12)	+	+	+	-	+	-	+	-	-	-	-	+	+
10.7 (SV308/14)	+	-	+	-	+	-	+	+	-	+	-	+	-
SV 556/14	-	+	-	-	+	+	-	+	+	-	-	-	+
LV 84/11 (SV822/12)	-	+	-	-	+	+	-	-	+	-	-	-	+
55.5 (SV815/12)	+	+	+	+	+	+	-	+	+	+	-	+	+
27.60 (SV847/14)	-	+	+	-	-	-	+	+	+	+	-	-	-
LV 014/15	+	+	+	-	-	-	+	-	+	-	-	+	-
30.10 (SV847/14)	+	-	+	-	-	+	+	+	+	+	-	+	-
35.00 (SV847/14)	+	+	+	-	-	+	+	+	+	-	-	+	-
27.69 (SV847/14)	+	+	+	+	+	+	+	+	+	-	-	+	-
15.5 (SV817/12)	+	+	+	-	+	+	-	+	+	-	-	-	-
17.02 (SV815/12)	+	+	-	-	-	-	-	-	+	-	-	-	+

receptors, and are associated with virulence and induction of the neutralizing antibodies (Schweizer & Peterhans 2014). The variability observed in these proteins reflects on the serological reactivity between different viral samples, being possible vaccine failures (Ridpath 2003, Otonel et al. 2014). Therefore, constant monitoring of the viruses circulating in the field, associated with the update of the vaccine strains, are some of the alternatives necessary to guarantee the control programs and vaccine efficacy (Ridpath 2003).

The use of a single monoclonal antibody in tests for identification and/or diagnosis of pestiviruses of bovine is not advisable since it may lead to false-negative results. For these cases, it is recommend to use a pool of monoclonal antibodies or specific antibodies for the conserved regions, such as the NS2/3 nonstructural protein, or polyclonal antibodies (Botton et al. 1998, Bauermann et al. 2013). Still, the genetic diversity also has consequences in the diagnosis by PCR. For this situation, it is recommend to use a set of primers with the capacity to detect the largest number of variants in small concentrations of genetic material (Giangaspero & Harasawa 2011, Monteiro et al. 2018).

The presence of more than one viral species infecting the same herd is a well-recognized possibility (Otonel et al. 2014). The explanation for this phenomenon is the constant mutations and recombination characteristic of pestiviruses or by the introduction into the herd of animals carrying different genotypes (Ridpath 2003, Fulton 2015). Genetic diversity is accompanied by the antigenic variability of the samples. In practical terms, genetic and antigenic diversity contribute to vaccine failures, when cross-serology among samples of distinct genotypes is not efficient (Fulton 2015). This situation reinforces that programs for the control and prevention of bovine pestivirus infection should follow the principle of identification and removal of persistently infected (PI) animals and using effective vaccines.

Isolation of bovine pestiviruses in FBS samples has been performed with some frequency. In previous study, that tested individual fetal serum the prevalence (0.7%) was similar (Botton et al. 1998). Monitoring of commercial batches of FBS has identified genome or virus infective, but without precision in determining the origin of infected herds (Xia et al. 2011, Monteiro et al. 2018). Despite the difficulty in obtaining, testing of individual FBS can be considered an excellent source of bovine pestiviruses, as it demonstrates active infection of pregnant animals and provides an overview of virus diversity circulating (Botton et al. 1998, Flores et al. 2018, Monteiro et al. 2018). The presence of fetal infection the virus infecting the fetus may also be associated with failure of fetal protection and consequent maintenance of the epidemiological cycle in herds. A cornerstone of any bovine pestiviruses control program is to prevent infection of pregnant cows (Ridpath 2003). In this way, monitoring of fetal samples may be helpful in evaluating the control program in certain regions. Diagnostic tests capable of detecting fetal infection of pregnant cows would be a major advance in disease control (Houe 2003, Ridpath 2003).

Economic losses produced by bovine pestiviruses are related to productive and, especially to reproductive failure (Houe 2003). In the present study, around 1.3% (10/754) of the fetal serum samples analyzed contained infectious virus. Although the consequences of these infections could not be determined, transplacentary BVDV infection may result in several outcomes, including fetal malformation, abortion or the birth of infected persistent animals. Another important aspect associated with fetal infection is the use of contaminated fetal bovine serum in the biological industry, including vaccines.

CONCLUSIONS

Pestiviruses circulate actively in cattle in the western region of the State of Rio Grande do Sul, Brazil. There are both genetic and antigenic variability among the pestiviruses isolated, being possible to detect the two major species of bovine pestiviruses, BVDV-1 and BVDV-2.

The numbers of samples evaluated is small, but corroborate with other studies that analyzed a larger number of samples of viruses.

Taken together, the results reinforce the need to continuous monitoring of the genetic and antigenic properties of circulating field samples.

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Conflict of interest statement.- The authors declare that they have no conflict of interest.

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Occurrence of anti-*Toxoplasma gondii* antibody and evaluation of risk infection factors in goats raised in Sergipe state, Brazil[1]

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ABSTRACT.- Rizzo H., Jesus T.K.S., Alcântara A.M., Carvalho J.S., Pinheiro Júnior J.W., Mota R.A & Silva T.R. 2020 **Occurrence of anti-*Toxoplasma gondii* antibody and evaluation of risk infection factors in goats raised in Sergipe state, Brazil.** *Pesquisa Veterinária Brasileira 40(5):374-380*. Departamento de Medicina Veterinária, Universidade Federal Rural de Pernambuco, Rua Manuel de Medeiros s/n, Dois Irmãos, Recife, PE 52171-900, Brazil, E-mail: hubervet@gmail.com

The objective was to determine, through indirect immunofluorescence reaction (RIFI, 1:64), the occurrence of IgG antibodies to *Toxoplasma gondii* and the risk factors associated with infection in goats in the state of Sergipe. To this study were used 675 samples of blood of animals from 41 farms of the three state mesoregions from 2013 to 2014. The occurrence of seropositive goats was 30.07%, with 90.24% of farms with seropositive animals. The distribution of titers obtained was 37.93%, 11.82%, 17.24%, 18.22%, and 17.77% for 64, 128, 512 and 1024 respectively. The risk factors observed were farms that did not have facilities (p=0.000, OR=2.30, CI 95%=1.41-3.74), with flooded soils (p=0.011, OR=2.94, CI 95%=1.27-6.79), which provided feed on the ground (p=0.032, OR=1.69, CI 95%=1.04-2.74), in uncovered cages (p=0.032, OR=1.69, CI 95%=1.04-2.74), pasture-based feed (p=0.003, OR=3.52, CI 95%=1.53-8.09), with access from cats to (p=0.031, OR=1.45, CI 95%=1.03-2.04) and introduced new breeders in the last five years (p=0.036, OR=1.58, CI 95%=1.02-2.74).

INDEX TERMS: *Toxoplasma gondii*, anti-*Toxoplasma gondii*, antibody, risk infection, goats, Sergipe, Brazil, caprine, infection.

RESUMO.- [**Ocorrência de anticorpo anti-*Toxoplasma gondii* e avaliação de fatores de risco de infecção em caprinos criados no estado de Sergipe, Brasil.**] O objetivo desse estudo foi determinar, através da reação de imunofluorescência indireta (RIFI, 1:64), a ocorrência de anticorpos IgG para o *Toxoplasma gondii* e os fatores de risco associados à infecção em rebanhos caprinos do estado de Sergipe, a partir de 675 amostras de sangue de animais de 41 propriedades das três mesorregiões do estado entre os anos de 2013 e 2014. A ocorrência de caprinos soropositivos foi de 30,07% com 90,24% de propriedades com animais soropositivos. A distribuição dos títulos obtidos foi de 37,93%, 11,82%, 17,24%, 18,22% e 17,77% para 64, 128, 512 e 1024 respectivamente. Os fatores de riscos observados foram propriedades que não possuíam instalações (p=0,000, OR=2,30, IC 95%=1,41-3,74), com terrenos alagados (p=0,011, OR=2,94, IC 95%=1,27-6,79), que disponibilizam a ração no chão (p=0,032, OR=1,69, IC 95%=1,04-2,74), em cochos descobertos (p=0,000, OR=1,97, IC 95%=1,32-2,94), alimentação à base de pastagem (p=0,003, OR=3,52, IC 95%=1,53-8,09), com acesso de gatos à fonte de água (p=0,031, OR=1,45, IC 95%=1,03-2,04) e que introduziram novos reprodutores nos últimos cinco anos (p=0,036, OR=1,58, IC 95%=1,02-2,74).

TERMOS DE INDEXAÇÃO: Anticorpo, anti-*Toxoplasma gondii*, fatores de risco, infecção, caprinos, Sergipe, Brasil, *Toxoplasma gondii.*

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INTRODUCTION

The most massive production of small ruminants found in Brazil is in the Northeast region (IBGE 2017). The goat farming in Sergipe is a complementary economic activity, characterized mainly by small herds without races and tested specializations in an extensive system (SEAGRI 2016).

One of the main diseases that affect goats is toxoplasmosis. It has clinical and economic importance as it is a congenital, abortive, and zoonotic disease (Medeiros et al. 2014, Silva et al. 2015). Toxoplasmosis is caused by *Toxoplasma gondii*, a parasite mandatory intracellular coccid whose primary host is felines, which eliminate oocysts in feces, contaminating soil, food, and water (Soares et al. 2010, Silva et al. 2015), leading to oral or aerosol infections, in addition to congenital (Silva et al. 2015) and venereal in goats (Wanderley et al. 2015). In humans, other forms of infection are the ingestion of raw milk, tissues, and organs of infected animals, direct contact with intrauterine secretions, and to a lesser extent by blood transfusion and organ transplants (Hill & Dubey 2002).

Goats are more susceptible to clinical toxoplasmosis when compared to other farm animals, causing economic losses in herds around the world due to reproductive disorders, such as abortions of mummified fetuses and births of weak puppies, which can be repeated in subsequent pregnancies (Nunes et al. 2013, Silva et al. 2015). The clinical signs are variable and unspecific for a definitive diagnosis of toxoplasmosis (Spósito Filha & Oliveira 2009). It is necessary to use complementary tests through direct methods: polymerase chain reaction (PCR), hybridization, isolation, immunohistochemistry and histological; and indirect techniques: serology, through the Sabin-Feldman test, indirect hemagglutination, complement fixation, immunoenzymatic assay (ELISA), and indirect immunofluorescence reaction (RIFI). The last one is considered the "gold standard test" due to its sensitivity in both the acute and chronic phases of the infection (Hill & Dubey 2002).

Infection with *T. gondii* has a "cosmopolitan characteristic", and in Brazil, it has been diagnosed by RIFI since 1985 in studies carried out in Minas Gerais. Since then, several kinds of research have been developed (Fialho et al. 2009). In the Northeast region, despite having the most extensive national herd, there are few surveys of goats seropositive for *T. gondii*. They are carried out in the states of Bahia (Uzêda et al. 2004), Alagoas (Anderlini et al. 2011), Pernambuco (Silva et al. 2003, Bispo et al. 2011, Pereira et al. 2012, Lúcio et al. 2016), Paraíba (Faria et al. 2007), Rio Grande do Norte (Lima et al. 2008), Ceará (Cavalcante et al. 2008), Maranhão (Soares et al. 2010, Moraes et al. 2011) and Piauí (Santos et al. 2018), which had an occurrence ranging from 4.34% to 47.56%.

Concerning the main risk factors for the spread of the disease in goat herds in the Northeast, the following can be mentioned: sex, animals of advanced age, extensive or semi-intensive breeding, properties located in hot regions, proximity to urban areas and presence of cats in herds (Anderlini et al. 2011, Bispo et al. 2011, Abu-Dalbouh et al. 2012, Garcia et al. 2012, Santos et al. 2018).

Considering the lack of epidemiological data on the presence of anti-*T. gondii* IgG antibodies in goats in the state, the present study was conducted to determine the occurrence and risk factors for parasite infection in herds in the three mesoregions of Sergipe.

MATERIALS AND METHODS

The study was carried out in the Sergipe state (9°30'49" and 11°34'05" latitude and 36°23'40" and 38°15'00" longitude), Northeast region, Brazil, comprising 75 municipalities with a total area of 21,918,493km2 divided into three edaphoclimatic mesoregions (Agreste, Leste and Sertão). It has a semi-arid climate in the west and part of the Sertão and tropical in the other regions with higher humidity on the coast (Santos et al. 2014, SEAGRI 2016).

In selecting the municipalities and properties that participated in the study, the division of the state, ease of access, convenience, and availability of producers were taken into account. The goats were randomly selected from males and females, apparently healthy, of different zootechnical patterns, and aged over six months. The sample calculation was carried out according to Thrusfield (1995), using the following parameters: population of 19,042 goats (IBGE 2017), 95% confidence interval (CI), and a percentage of sample error margin of 5%, with a prevalence of ±27.07%, (Pita Gondim et al. 1999, Silva et al 2003, Uzêda et al. 2004, Faria et al. 2007, Cavalcante et al. 2008, Lima et al. 2008, Soares et al. 2010, Anderlini et al. 2011, Bispo et al. 2011, Moraes et al. 2011, Pereira et al. 2012, Silva et al. 2015, Lúcio et al. 2016), resulting in a sample of 369 animals. The Win Episcope 2.0 software was used to calculate the number of animals sampled from the above parameters.

Blood samples were collected from 2013 to 2014, totaling 675 goats (107 males and 568 females) from 41 farms in fifteen municipalities, where 4 to 21 samples per farm were obtained. The total number of goats per mesoregion was 235, 178, and 262 for the Agreste, Leste, and Sertão regions, respectively (Fig.1).

During visits to the properties, epidemiological questionnaires were applied to assess the risk factors associated with *Toxoplasma*

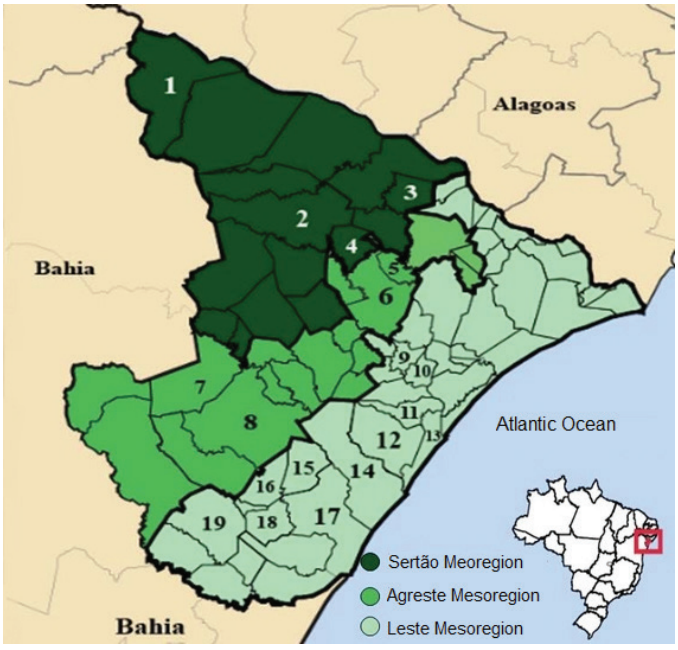


Fig.1. Map of Sergipe divided into its three mesoregions, numerically indicating the municipalities of goat herds submitted to RIFI for *Toxoplasma gondii*. Sertão Mesoregion: 1) Canindé de São Francisco, 2) Nossa Senhora da Glória, 3) Pinhão, and 4) Pedra Mole. Agreste Mesoregion: 5) Macambira, 6) Itabaiana, 7) Lagarto, and 8) Poço Verde. Leste Mesoregion: 9) Neópolis, 10) Nossa Senhora do Socorro, 11) Aracaju, 12) São Cristóvão, 13) Itaporanga D'Ajuda, 14) Salgado, and 15) Estância.

gondii infection. The questionnaires consisted of 34 closed questions, encompassing the characteristics of the herd based on history, breeding purpose, management health, nutritional and reproductive, facilities, and data on the producer.

Blood samples were collected from venipuncture of the jugular with sterile 40x12 needles in a vacuum tube (Vacutainer®), without anticoagulant, and transported under refrigeration. They were centrifuged at the Clinical Pathology Laboratory of "Hospital Veterinário Dr. Vicente Borelli", from "Faculdade Pio Décimo", Aracaju, at 1600g for 10 minutes to obtain serum, stored in Eppendorf tubes and frozen at -20ºC until analysis.

Serological diagnosis for detection of anti-*T. gondii* antibodies by RIFI was carried out at the Bacteriosis Laboratory of the Department of Veterinary Medicine of the Federal Rural University of Pernambuco using the cutoff point 64 (Camargo 1964). Only reactions where the presence of fluorescence was total, around the surface of the tachyzoites (Paré et al.1995), seen through a fluorescence microscope, with a 40x objective, were considered positive.

For the execution of the statistical calculation using the EpiInfo 3.5.1 program, a descriptive statistical analysis was performed, determining the absolute and relative frequencies. For the analysis of risk factors associated with *T. gondii* infection, univariate analysis of the variables of interest was performed using Pearson's chi-square test, and when necessary, Fisher's exact test. Subsequently, logistic regression analysis was performed considering the result of the immunofluorescence antibody test (IFAT, reagent or non-reagent) as a dependent variable for *T. gondii* infection. The independent or explanatory variables considered in the model were those that presented statistical significance <0.20. This probability was stipulated so that possible risk factors were not excluded from the analysis (Hosmer & Lemeshow 1989).

The research procedures were approved by the Bioethics Committee of the "Faculdade Pio Décimo", under protocol No. 06/2011.

RESULTS

The occurrence of goats seropositive to *Toxoplasma gondii* in the 41 herds evaluated in the state of Sergipe was 30.07% (203/675) with a high index of properties with positive animals, 90.24% (37/41). Concerning the mesoregions, no statistically significant difference was observed (p=0.094) with occurrences of 35.39%, 25.53%, and 30.53% in the Leste, Agreste, and Sertão respectively (Table 1).

The titers of seropositive goats varied from 64 to 1024. Of these, the titration 64 (37.93%) showed a statistically significant difference (p<0.05) from the others that show similar percentages of 11.82%, 17.24%, 18.22%, and 17.77% for 128, 256, 512 and 1024 respectively (Table 2).

Table 1. Detection of anti-*Toxoplasma gondii* antibodies determined by indirect immunofluorescence reaction (1:64) in goat serum, according to property, animals and mesoregion in the Sergipe state (2013-2014)

Municipality	Positives/Properties (%)	Caprines (%)		
		Male	Female	Positives/Total
Aracaju	3/3 (100)	10/16 (62.5)	8/34 (23.52)	18/50 (36)
Estância	1/1 (100)	2/3 (66.66)	12/17 (70.58)	14/20 (70)
Itaporanga D'Ajuda	1/1 (100)	0/4 (0)	9/16 (56.25)	9/20 (45)
Nossa Senhora do Socorro	1/1 (100)	0/3 (0)	3/15 (20)	3/18 (16.66)
Salgado	2/2 (100)	2/3 (66.66)	5/27 (18.51)	7/30 (23.33)
São Cristóvão	1/1 (100)	1/3 (33.33)	3/17 (17.64)	4/20 (20)
Neópolis	1/1 (100)	0/1 (0)	8/19 (42.1)	8/20 (40)
Total Leste mesoregion	10/10 (100)	15/33 (45.45)	48/145 (33.1)	63/178 (35.39)
Itabaiana	1/1 (100)	1/2 (50)	13/18 (72.22)	14/20 (70)
Lagarto	1/1 (100)	1/5 (20)	2/14 (14.28)	3/19 (15.78)
Macambira	2/3(66.66)	1/9(11.11)	10/33 (30.3)	11/42 (26.19)
Poço Verde	9/11 (81.81)	4/19 (21.05)	28/135 (20.74)	32/154 (20.77)
Total Agreste mesoregion	13/16 (81.25)	7/35 (20)	53/200 (26.5)	60/235 (25.53)
Canindé de São Francisco	6/6 (100)	5/14 (35.71)	40/105 (38.09)	45/119 (37.81)
Nossa Senhora da Glória	6/6 (100)	3/18 (16.66)	19/75 (25.33)	22/93 (23.65)
Pedra Mole	0/1 (0)	0/3 (0)	0/8 (0)	0/11 (0)
Pinhão	2/2 (100)	2/4 (50)	11/35 (31.42)	13/39 (33.33)
Total Sertão mesoregion	14/15 (93.33)	10/39 (25.64)	70/223 (31.39)	80/262 (30.53)
TOTAL	37/41 (90.24)	32/107 (29.9)	171/568 (30.1)	203/675 (30.07)

Table 2. Anti- *Toxoplasma gondii* antibody titers in 203 positive goats by indirect immunofluorescence reaction (RIFI) in the mesoregion of the Sergipe state (2013-2014)

Mesoregions	Titration (%)				
	64	128	256	512	1024
Agreste	19 (9.35)	6 (2.95)	12 (5.91)	11 (5.41)	12 (5.91)
Leste	24 (11.82)	6 (2.95)	17 (8.37)	9 (4.43)	7 (3.44)
Sertão	34 (16.74)	12 (5.91)	6 (2.95)	17 (8.37)	11 (5.41)
Total	77 (37.93)[a]	24 (11.82)[b]	35 (17.24)[b]	37 (18.22)[b]	30 (17.77)[b]

[a,b] Different lowercase letters on the same line indicate a statistically significant difference (Chi-square P<0.05).

In the univariate analysis, twelve variables showed significance, p<0.05 (Table 3). However, when submitted to multivariate logistic regression analysis, the ones confirmed as risk factors for *T. gondii* infection were properties that: did not have facilities (p=0.000, OR=2.30, CI 95%=1.41-3.74); with flooded land (p=0.011, OR=2.94, CI 95%=1.27-6.79); which provided the feed on the ground (p=0.032, OR=1.69, CI 95%=1.04-2.74); in uncovered hods (p=0.000, OR=1.97, CI 95%=1.32-2.94); pasture-based feed (p=0.003, OR=3.52, CI 95%=1.53-8.09); with access by cats to a water source (p=0.031, OR=1.45, CI 95%=1.03-2.04) and that have introduced new breeders in the last five years (p=0.036, OR=1.58, CI 95%=1.02-2.74).

Table 3. Goats seropositive for *Toxoplasma gondii*, through RIFI (1:64), according to the studied variable, from rural properties in the Sergipe state (2013-2014)

Variable	RIFI % (soropositives/total)	OR (CI 95%)	P-value
Mesoregion			
Leste	36.51 (65/178)	-	
Agreste	25.53 (60/235)	0.63 (0.40-0.98)	0.094
Sertão	30.53 (80/262)	1.28 (0.85-1.94)	
Sex			
Female	30.21 (171/566)	1.04 (0.65-1.69)	0.478
Male	29.35 (32/109)		
Breed			
Anglo-Nubiana	28.33 (17/60)		
Boer	14.81 (8/54)		
Pardo Alpina	31.25 (5/16)	-	0.012
Saanen	26.38 (57/216)		
No defined breed	34.57 (111/321)		
Toggenburg	62.5 (5/8)		
Age of animals (years)			
1 ≤ 2	30.56 (114/373)	-	
> 2	30.51 (83/272)	1.00 (0.70-1.42)	0.468
≤ 1	20 (6/30)	0.57 (0.18-1.50)	
Total goats			
Up to 50	33.87 (84/248)	-	0.061
Between 51 and 100	37.28 (22/59)	1.16 (0.61-2.17)	
Above 100	26.35 (97/368)	0.60 (0.33-1.13)	
Breeding purpose			
Beef	32.55 (97/298)	1.23 (0.87-1.73)	0.122
Milk	28.11 (106/377)		
Breeding regime			
Extensive	35.08 (40/114)	-	
Intensive	40.74 (44/108)	1.27 (0.71-2.27)	0.006
Semi-extensive	26.32 (119/452)	0.52 (0.33-0.83)	
Reproduction biotechnologies			
No	35.56 (175/492)	3.05 (1.97-4.81)	0.000
Yes	15.3 (28/183)		
Property size (Ha)			
< 30	25.88 (95/367)	-	
Between 30 and 100	33.46 (83/248)	1.44 (1.00-2.08)	0.016
> 100	41.66 (25/60)	1.42 (0.76-2.62)	
Type of land			
Rough	27.77 (30/108)	-	
Flooded	60 (21/35)	3.75 (1.76-8.18)	0.000
Flat	28.57 (152/532)	0.27 (0.12-0.57)	

Variable	RIFI % (soropositives/total)	OR (CI 95%)	P-value
Facilities			
Masonry sheepfold	22.36 (36/161)	-	
Clay floor sheepfold	27.35 (29/106)		0.000
Slatted floor sheepfold	27.43 (62/226)		
No facilities	41.75 (76/182)		
Disinfect facilities			
No	40 (64/160)	1.80 (1.21-2.65)	0.001
Yes	26.99 (139/515)		
Feed provision			
Covered hod	21.68 (80/369)	-	
Uncovered hod	38.82 (73/188)	2.29 (1.53-3.43)	0.000
On the ground	42.37 (50/118)	1.16 (0.70-1.90)	
Feeding			
Concentrated	9.72 (7/72)	-	
Access to pasture	36.36 (160/440)	5.31 (2.35-14.02)	0.000
High volume in the hod	22.08 (36/163)	0.50 (0.32-0.76)	
Food storage			
Open location	39.62 (63/159)	1.76 (1.19-2.59)	0.002
Closed location	27.13 (140/516)		
Water supply			
Direct from source (weir/dam)	38.93 (44/113)	-	
Containers inside the facilities	28.47 (131/460)	0.62 (0.40-0.99)	0.077
Containers outside the facilities	27.45 (28/102)	0.95 (0.57-1.57)	
Presence of cats			
No	29.13 (44/151)	0.94 (0.61-1.42)	0.429
Yes	30.34 (159/524)		
Cats access to water			
No	27.16 (119/438)	0.68 (0.48-0.98)	0.019
Yes	35.16 (83/236)		
Cats access to food storage			
No	30.27 (145/479)	1.03 (0.70-1.51)	0.469
Yes	29.59 (58/196)		
Performs quarantine			
No	27.60 (106/384)	0.76 (0.54-1.07)	0.064
Yes	33.33 (97/291)		
Contact between healthy and sick animals			
No	27.2 (71/261)	0.79 (0.55-1.13)	0.113
Yes	31.88 (132/414)		
Matrix acquisition in the last five years			
No	27.14 (76/280)	0.78 (0.55-1.11)	0.094
Yes	32.15 (127/395)		
Acquisition of breeders in the last five years			
No	26.37 (96/364)	0.67 (0.48-0.94)	0.021
Yes	34.51 (107/310)		

DISCUSSION

The rate of 30.07% of goats seroreagent to *Toxoplasma gondii* in the state of Sergipe was similar to that described by Pereira et al. (2012) 31.73% (53/167) in Pernambuco, but lower than the 47.56% (78/164) reported by Bispo et al. (2011) in the same state and 38.98% (177/454) described by Anderlini et al. (2011) in Alagoas. Lower rates in the Northeast were observed in São Luís in Maranhão (Moraes et al. 2011),

Bahia (Uzêda et al. 2004), Mossoró in Rio Grande do Norte (Lima et al. 2008), southern Piauí (Santos et al. 2018) and Paraíba (Faria et al. 2007) presenting 4.34% (2/46), 16.35% (61/373), 17.06% (65/381), 22.07% (32/145) and 24.50% (75/306), respectively.

Regarding the outbreaks of the 41 properties studied, 37 (90.24%) presented at least one positive animal, demonstrating the dissemination of the agent in Sergipe's herds, as observed in Pernambuco's herds with 100% (10/10) and 66.66% (4/6) and in Rio Grande do Norte with 92.85% (13/14) of outbreaks (Silva et al. 2003, Cavalcante et al. 2008, Pereira et al. 2012).

There was no significant difference in the mesoregions, even in the Leste, with a humid and hot climate in Agreste and Sertão; a condition that favors the maintenance and sporulation of the oocyst in the environment. Similar results were obtained by Silva et al. (2003) in Pernambuco when comparing Zona da Mata and Agreste mesoregions (47.88% x 52.11%). Anderlini et al. (2011), when analyzing the same variables (Agreste, Leste, and Sertão) in Alagoas, observed that the Leste mesoregion showed a significant result with 66.15% (43/65) of seropositive animals. Also, this demonstrated the favorable climatic condition for the parasite's biological cycle since the values obtained in the Sertão mesoregion were only 18.18% (22/121) and 41.49% (112/268) in the Agreste.

The highest occurrence of goats showing titers of 64 in this study is similar to the results of Uzêda et al. (2004) and Pereira et al. (2012) with low titers. However, with a cutoff of 16, the highest titers presented by both were, respectively, 14.75% (9/61) for titers over 256, and 0.59% (1/167) for 4,096 titers. The higher occurrence of low titers suggests that these animals were infected (Maley et al. 1997).

As for the availability of food, both breeding systems had significance in the values obtained. They are properties belonging to small producers, subsistence breedings, whose sanitary measures and management practices are inadequate, which favors contact with contaminated sources of infection.

Anderlini et al. (2011) reported that in intensive regimes, there is a greater chance of high HIV-positive rates due to the higher concentration of animals, exposing them even more to contaminated food. The acquisition of new animals, the lack of installation, and the animal's access to the pasture had significant results, corroborating the deficiency of management on the property.

The type of flooded terrain showed a higher rate of animals reactive to the infectious agent when compared to flat and rugged terrain, probably since they present better conditions for the sporulation of oocysts and their greater permanence in the environment. The cat's access to water is fundamental in the spread of the disease since it occurs through the ingestion of oocysts of the parasite released in the soil. Due to its potential for expansion higher than the evacuation site, it can contaminate unprotected water sources (springs, artesian wells, distribution network) and pastures, especially in rainy periods, making the agent able to conclude their biological cycle in the organism of the intermediate host (goats) (Dubey 2010, Santos et al. 2018).

CONCLUSION

The occurrence of *Toxoplasma gondii* in goats in Sergipe is correlated mainly with deficient sanitary management. The results show characteristics that favor the maintenance of the agent in the environment.

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Conflict of interest statement.- The authors declare that they have no conflict of interest.

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ELISA of amyloid A in paired bronchoalveolar lavage fluid and serum samples of healthy horses[1]

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ABSTRACT.- Di Filippo P.A., Ribeiro L.M.F., Meireles M.A.D., Lannes S.T., Mello L.M., Gobbi F.P., Toledo L.F.A. & Lessa D.A.B. 2020. **ELISA of amyloid A in paired bronchoalveolar lavage fluid and serum samples of healthy horses.** *Pesquisa Veterinária Brasileira 40(5):381-384.* Laboratório de Clínicas e Cirurgia Veterinária, Centro de Ciências e Tecnologias Agropecuárias, Universidade Estadual do Norte Fluminense "Darcy Ribeiro", Av. Alberto Lamego 2000, Campos dos Goytacazes, RJ 28013-602, Brazil. E-mail: pdf@uenf.br

Pulmonary disorders are common in horses, and treatment efficiency depends on an adequate diagnosis. Amyloid A is the most sensitive indicator of pathology in horses. The objective of this study was to establish the concentration of amyloid A of bronchoalveolar lavage fluid (BALF) in healthy horses. Health condition of horses was considered normal based on physical examination, complete blood count, biochemical parameters, and BALF cytology. Blood and BALF were collected from thirty adult female horses. Amyloid A concentrations in serum and BALF were measured using commercial ELISA tests. Amyloid A was detected in serum (mean ± SD = 3.71±2.51) and BALF (mean ± SD = 0.000745±0.000785) of all horses. In conclusion, SAA can also be measured in bronchoalveolar fluid, affording early detection of respiratory infections or inflammatory conditions.

INDEX TERMS: ELISA, amyloid A, bronchoalveolar lavage, serum, healthy horses, acute phase protein, biomarker, lung, inflammation, horses.

RESUMO.- [Detecção de amilóide A no lavado broncoalveolar de equinos hígidos]. Distúrbios pulmonares são comuns nos cavalos e a eficiência do tratamento depende de um diagnóstico adequado e precoce. A amilóide A é um biomarcador sensível na deteccção de patologias inflamatórias e infecciososa em cavalos. O objetivo deste estudo foi estabelecer a concentração de amilóide A no líquido broncoalveolar (LBA) em cavalos saudáveis. Os cavalos foram considerados saudaveis baseado nos achados de normalidade do exame físico, hemograma, parâmetros bioquímicos e citologia do LBA. Sangue e LBA foram coletados de 30 fêmeas equinas adultas. Os níveis de Amilóide A no soro e no LBA foram mensurados por meio do teste de ELISA. A amilóide A foi detectada no soro (média ± DP = 3,71±2,51) e no LBA (média ± DP = 0,000745±0,000785) de todos os animais. Conclui-se que a amilóide A também pode ser mensurada no LBA, auxiliando no diagnóstico precoce de processos inflamatórios e infecciosos pulmonares.

TERMOS DE INDEXAÇÃO: Amilóide A, lavado broncoalveolar, equinos hígidos, proteínas de fase aguda, biomarcadores, pulmões, inflamação, cavalos.

INTRODUCTION

Inflammatory airway disease (IAD) is one of the most important pulmonary disorders in athletic horses. This syndrome is characterized by cough, decreased performance, and delayed recovery after exercise with normal respiratory effort at rest (Couëtil et al. 2007). Inflammation is a complex process, and early diagnosis is essential to devise and implement an effective treatment plan (Crisman et al. 2008). Markers of inflammatory disease have recently attracted attention in the scientific community. In equine medicine, for example, the usefulness of clinically applied inflammatory markers such as serum amyloid A protein (SAA) has been reported

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in previous studies (Hultén & Demmers 2002, Jacobsen & Andersen 2007).

Equine SAA increases rapidly and considerably (more than 100 times). However, this increase does not afford the exact diagnosis; rather, it only broadly signals the presence of a pathology (Hultén et al. 1999, Cywinska et al. 2013). High serum SAA concentration was found in horses with inflammatory airway disease (Viner et al. 2017), heaves (Lavoie-Lamoureux et al. 2012), infectious respiratory diseases (equine influenza virus, equine herpesvirus-4 and *Streptococcus equi* subspecies *equi),* and bacterial pneumonia (Hobo et al. 2007).

The liver is the main site of SAA, but several extrahepatic isoforms of SAA have been demonstrated in horses (McDonald et al. 2001, Jacobsen et al. 2006a, Christoffersen et al. 2010). The extrahepatic production of SAA works as a local and immediate defense against tissue injury from inflammatory challenges until a systemic/hepatic response is elicited (Uhlar and Whitehead 1999). Studies describing the detection of amyloid A in bronchoalveolar lavage fluid are scarce. Therefore, the aim of this study was to establish the pattern of amyloid A concentrations in bronchoalveolar lavage fluid (BALF) obtained from healthy horses using ELISA tests.

MATERIALS AND METHODS

This study was approved by the Ethics Committee on Animal Experiments of "Universidade Estadual do Norte Fluminense Darcy Ribeiro" (CEUA-UENF), under protocol number 901139.

Thirty crossbreed female horses whose ages ranged from 5-7 years and body weight between 400 and 425kg were studied. All horses were kept in semi-confinement, in 10-m^2 stall boxes with cement floor and no bedding. Feeding included 3 to 4kg/day of commercial horse concentrate per animal, containing 12% total protein, 4kg of Coast-Cross grass hay/animal per day, mineral salt, and water *ad libitum*. The animals were dewormed every four months and vaccinated against influenza, tetanus, rabies, equine adenitis, and leptospirosis. The horses had no history of respiratory disease or medical treatment during the 30 days preceding data collection.

The animals included were considered healthy after a routine physical evaluation, pulmonary auscultation and percussion, endoscopy examination, complete blood count, and cytological analysis of bronchoalveolar lavage fluid according to the values described by Viscardi et al. (2015), and Couëtil et al. (2016).

Bronchoalveolar lavage was performed in the standing horse using a special silicone BAL catheter (BIVONA®), under mild sedation (detomidine, 0.5mg/kg bwt i.v). The catheter was advanced via the trachea until being wedged in a distal bronchus. The cuff was then filled with 8 mL air and 250 mL of warm (37°C) sterile 0.9% saline (divided into 3 boluses) were sequentially instilled and immediately aspired. Samples were considered adequate if at least 40% of the infused liquid was recovered and when surfactant and turbidity were observed. After collection, the aliquots of BALF were pooled and macroscopically examined to evaluate the color, transparency, and the presence of flocculent debris. Flocculent samples were filtrated through two layers of gauze to remove excess mucus strands and other debris.

The samples were maintained at -4°C in sterile test tubes for cytological processing and at -20°C for SAA determination. Within 1h of collection the samples used in the cytological analysis were cytocentrifuged (Cytopro 7620, Wescor®) at 110*g* for 5 min and stained with May-Grunwald, Giemsa (Merck®). Differential cell counts were performed by counting 500 cells, excluding epithelial cells.

Venous blood samples (7mL) were collected form the jugular vein with a Vacutainer (BD) into plain tubes. Samples were centrifuged (1500*g*, 10 min) and the serum separated and stored at -20°C for subsequent SAA analysis.

The amyloid A levels in paired BALF and serum samples were measured using the enzyme linked immunosorbent assay (ELISA, PHASE™ SAA Assay, Tridelta Ltd., Ireland) previously validated for use in equine studies (Coutinho da Silva et al. 2014, Leclere et al. 2015, Turło et al. 2015a, 2015b). Samples were evaluated in duplicate and only serum samples were diluted (1:2000), and the results appropriately recalculated. Data are expressed as mg/mL.

The data obtained were submitted to descriptive analysis, and means, maximum and minimum values were determined using the SAS statistical program.

RESULTS AND DISCUSSION

The results are summarized in Table 1 and 2. BALF cytology profile of the healthy horses was characterized by the prevalence of alveolar macrophages and lymphocytes, followed by neutrophils, mast cells, eosinophils and a small percentage of epithelial cells. These results are similar to the findings described by Viscardi et al. (2015), Wysocka & Kluciński (2015) and in a recent Consensus Statements of the American College of Veterinary Internal Medicine (Couëtil et al. 2016).

The SAA concentrations in serum of healthy horses were similar to the values described by other authors using the same methodology (Coutinho da Silva et al. 2014, Turło et al. 2015a, 2015b). Increased in SAA concentrations were found in horses with infectious and noninfectious respiratory diseases (Viner et al. 2017). SAA values from horses with *Streptococcus equi* subsp. *equi* were significantly higher, when compared to horses with viral infections (EIV/EHV-4). However, due to the high similarity in SAA values between these two groups it was not possible to differentiate them based solely on SAA concentrations. For Viner et al. (2017), SAA may assist in the differentiation between respiratory infections and non-infectious inflammatory respiratory diseases presenting similar clinical signs.

Increased in SAA concentrations have been observed in horses infected with influenza virus (Hultén et al. 1999), *Streptococcus zooepidemicus (*Hobo et al. 2007), and in foals with *Rhodococcus equi* pneumonia (Hultén & Demmers 2002). In a study with horses with influenza virus infection, Hultén et al. (1999) observed that SAA reacts more rapidly than fibrinogen. The authors concluded that measurement of SAA is useful as a method of anticipating the clinical condition in

Table 1. Percentage of cells recovered from bronchoalveolar lavage fluid in healthy horses

Percentage of cells					
Macrophages	Lymphocytes	Neutrophils	Eosinophils	Mast cell	Epithelial cells
43.80±17.01	36.4±21.54	4.6±3.80	1.8±0.84	1.60±0.84	0.70±1.90

Table 2. Amyloid A concentration in healthy horses

Amyloid A concentration (mg/mL)					
Serum			BALF		
Median	Minimum	Maximum	Median	Minimum	Maximum
3.71	0.21	9.43	0.000745	0.000054	0.002518

BALF = bronchoalveolar lavage fluid.

equine influenza virus infection. On the other hand, a study developed with the objective of evaluating the usefulness of weekly tests of serum amyloid A (SAA) and plasma fibrinogen concentrations in foals to achieve early diagnosis of *R. equi* pneumonia prior to the onset of clinical signs, demonstrated that while SAA concentrations may rise during clinically manifest *R. equi* pneumonia, SAA does not represent a reliable early marker of Rhodococcosis when plasma concentrations are tested once a week. At the same time, this study raises the possibility that plasma fibrinogen monitoring starting at 1 week of age and repeated on a weekly basis, could serve as a screening test allowing clinicians to identify foals as suspected of *R. equi* infection (Passamonti et al. 2015). The SAA response is dependent on the nature and intensity of the inflammatory stimulus (Hultén & Demmers 2002, Jacobsen & Andersen 2007, Crisman et al. 2008), justifying the diferente results described above. According to Jacobsen & Andersen (2007), after having excluded the existence of coexisting noxious stimuli, plasma SAA concentrations may also serve as a good indicator of disease status, reflecting clinical improvement or deterioration as well as the quality of the response to treatment.

Horses with recurrent airway obstruction (RAO, or heaves) also had increased concentrations of serum amyloid A (Lavoie-Lamoureux et al. 2012). Also, SAA concentrations were 3.5 times higher in horses with inflammatory airway disease (IAD) when compared to controls horses (Bullone et al. 2015). In other words, it has been suggested that SAA increases proportionately to the extent of tissue damage. However, another study revealed that there was no difference in SAA concentration between exercise-intolerant horses with and without IAD (Leclere et al. 2015). The results of that study were associated with the small number of samples used, not with the ineffectiveness of SAA as a blood marker of airway inflammation. Importantly, SAA is considered a "major" acute phase protein, with a very rapid (24-48 hours) and pronounced (10-fold or over) increase with inflammation (Crisman et al. 2008).

Although the main source of acute phase proteins is the liver, extrahepatic SAA isoforms may also be produced (Upragarin et al. 2005, Christoffersen et al. 2010). In horses, SAA protein has been detected in colostrum (McDonald et al. 2001), synovial fluid (Jacobsen et al. 2006a), and in the endometrium of mares with uterine *Escherichia coli* infection (Christoffersen et al. 2010). Yet, the present study is the first report on lung SAA in healthy horses.

The local functions of SAA protein have not yet been fully understood, but it has been suggested that SAA is part of the innate host response against the invasion of microorganisms or assist in maintenance of tissue functions in organs that communicate with the environment, such as the lungs (Urieli-Shoval et al. 1998). The production of this protein in the mammary gland may have protective effects in the neonatal intestine (Duggan et al. 2008). In joints, SAA may contribute to inflammation and cartilage destruction, acting as a chemoattractant for leukocytes, inducing cyclooxygenase metabolite formation, and regulating metalloproteinase activity (O'Hara et al. 2004). Thus, the syntheses of SAA in joints suggests an important pathophysiological role in inflammatory arthritis in horses (Jacobsen et al. 2006b). In addition, the local determination of SAA levels improves diagnosis accuracy, because it provides information on the inflammatory/infectious status of the particular organ of interest (Jacobsen & Andersen 2007). Early detection of respiratory disease in horses is crucial, and SAA should be assessed in that context. Depending on the nature of the inflammatory stimulus, SAA values have been reported to start increasing by 4 to 12h following acute inflammation, reach peak values within 48h and return to baseline concentrations between 3 days and 3 weeks (Hultén & Demmers 2002, Jacobsen & Andersen 2007, Crisman et al. 2008). However, in healthy animals it is common to obtain low or undetectable SAA values (Crisman et al. 2008), such as those observed in BALF of the animals in this study (Table 2).

CONCLUSIONS

The determination of serum amyloid A protein (SAA) in bronchoalveolar lavage fluid (BALF) is possible, therefore, healthy horses have a low SAA concentration in BALF.

The knowledge of SAA values in healthy horses may help in the interpretation of samples from diseased animals facilitating early detection of airway disease in horses.

Conflict of interest statement.- The authors declare that this paper has no conflict of interest and none of the authors has any other financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Performance of the Dot-blot test method for detecting antibodies to *Sarcocystis* spp. in cattle[1]

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ABSTRACT.- Ferreira, M.S.T., Fernandes F.D., Alves, M.E.M, Braunig P., Sangioni, L.A. & Vogel F.S.F. 2020. **Performance of the Dot-blot test method for detecting antibodies to *Sarcocystis* spp. in cattle.** *Pesquisa Veterinária Brasileira 40(5):385-388*. Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria, Avenida Roraima 1000, Santa Maria, RS 97105-900, Brazil. E-mail: fagnermedvet@gmail.com

Serological techniques can detect antibodies against *Sarcocystis* spp., *Neospora caninum* and *Toxoplasma gondii* antigens in single or mixed infections. Immunofluorescent antibody tests (IFAT) is considered the gold standard technique for Sarcocystosis diagnostic in cattle serum and a positive IFAT result reflects *Sarcocystis* spp. infection. Therefore, the aims of the present study were to compare IFAT and Dot-blot for sarcocystosis diagnostic in experimentally infected mice and to investigate serological cross-reactions with *N. caninum* and *T. gondii* in these methods. Mice (*Mus musculus*) were inoculated intraperitoneally with bradizoites of *Sarcocystis* spp. or tachyzoites of *N. caninum* or *T. gondii.* Serum samples were obtained and analyzed by IFAT and Dot-blot for the three protozoa. Serum from *N. caninum* and *T. gondii* experimentally infected mice were tested by IFAT and reacted only to *N. caninum* or *T. gondii* antigens, respectively. Specific antibodies against *Sarcocystis* spp. were present in all animals experimentally infected with this protozoan, with IFAT titers from 10 to 800. Serum samples from mice experimentally infected with *Sarcocystis* spp., *N. caninum* and *T. gondii* and tested by Dot-blot demonstrated no cross reaction between protozoa. A Dot-blot using *Sarcocystis* spp. antigen appears to be a good alternative to IFAT in the serological diagnosis of Sarcocystosis.

INDEX TERMS: Dot-blot test, antibodies, *Sarcocystis* spp., cattle, serological diagnosis, IFAT, protozoan.

RESUMO.- [**Desempenho do teste Dot-blot para detecção de anticorpos para *Sarcocystis* spp. em bovinos.**] As técnicas sorológicas podem detectar anticorpos contra os antígenos de *Sarcocystis* spp., *Neospora caninum* e *Toxoplasma gondii* em infecções únicas ou mistas. O teste de anticorpos imunofluorescentes (IFAT) é considerado a técnica padrão-ouro para o diagnóstico de sarcocistose no soro de bovinos e um resultado positivo de IFAT reflete *Sarcocystis* spp. infecção. Portanto, os objetivos do presente estudo foram comparar IFAT e Dot-blot para diagnóstico de sarcocistose em camundongos infectados experimentalmente e investigar reações cruzadas sorológicas com *N. caninum* e *T. gondii* nesses métodos. Os camundongos (*Mus musculus*) foram inoculados intraperitonealmente com bradizoítos de *Sarcocystis* spp. ou taquizoítos de *N. caninum* ou *T. gondii*. As amostras de soro foram obtidas e analisadas por IFAT e Dot-blot para os três protozoários. O soro de *N. caninum* e *T. gondii* infectados experimentalmente foram testados por IFAT e reagiram apenas aos antígenos de *N. caninum* ou *T. gondii*, respectivamente. Anticorpos específicos contra *Sarcocystis* spp. estavam presentes em todos os animais experimentalmente infectados com este protozoário, com títulos de IFAT de 10 a 800. Amostras de soro de camundongos infectados experimentalmente com *Sarcocystis* spp., *N. caninum* e *T. gondii* e testadas por Dot-blot não demonstraram reação cruzada entre protozoários. Um Dot-blot usando *Sarcocystis* spp. O antígeno parece ser uma boa alternativa ao IFAT no diagnóstico sorológico da sarcocistose.

TERMOS DE INDEXAÇÃO: Dot-blot, anticorpos, *Sarcocystis* spp., bovinos, diagnóstico sorológico, IFAT, protozoário.

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INTRODUCTION

Sarcocystis spp., *Neospora caninum* and *Toxoplasma gondii* are coccidians belonging to Sarcocystidae family. They have worldwide distribution and may cause infections in ruminants inducing productive and economic losses (Tenter 1995, Dubey 2003, 2009). Although the prevalence of *Sarcocystis* spp. infection in cattle herds is approximately 90% to 100% in many countries, Sarcocystosis in cattle are frequently asymptomatic making difficult the diagnosis (Moré et al. 2011, Ruas et al. 2001, Akhlaghi et al. 2016).

Diagnosis of acute Sarcocystosis is difficult because, the clinical signs are uncommon, unspecific and the parasitemia is frequently low and consequently undetectable (Ndiritu et al. 1996, Dubey et al. 2016). Diagnosis of chronic Sarcocystosis is usually made by conventional laboratory methods as microscopy, immunoassays and tissue digestion (Fayer & Dubey 1986, Gajadhar et al. 1987, Cawthorn & Speer 1990). Several serological methods as immunofluorescent antibody tests (IFAT), enzyme linked immunosorbent assays (ELISA), and immunoblots (as Western blot and Dot-blot) have been developed for diagnosis the bovine Sarcocystosis (Moré et al. 2008). Serological techniques detect antibodies against the protozoans *Sarcocystis* spp., *N. caninum* and *T. gondii* antigens in single or mixed infections (Uggla et al. 1987, Dubey et al. 1996). However a specific and sensitive serological test which detects acute and chronic infection and suitable for screening a large number of animals is lacking for *Sarcocystis* spp.

The aims of the present study were a) to compare two serological tests (IFAT and Dot-blot) for Sarcocystosis diagnostic, and b) to check the cross-reactions of *N. caninum* and *T. gondii* using IFAT and Dot-blot in serum of experimentally infected mice.

MATERIALS AND METHODS

Animal inoculation and serum samples. Seven mice (*Mus musculus*), male, approximately 6 weeks of age with initial weight of 30g, without previous contact with parasites were used to obtain hyperimmune serum against *Sarcocystis* spp., *Neospora caninum* and *Toxoplasma gondii*. Two animals were inoculated intraperitoneally with $2x10^5$ bradyzoites of *Sarcocystis* spp. obtained from cysts of a naturally infected bovine heart and purified by previously described by García-Lunar et al. (2015). Similarly, two animals were inoculated intraperitoneally with $2x10^5$ tachyzoites of *N. caninum* and two animals with *T. gondii*. Parasites were obtained from culture in VERO cells as described in the item 2.2. After 20 days of infection, blood was collected from cardiac puncture and serum was obtained. One naive animal was used as a negative experimental control. Samples were identified and stored at -20°C until processing.

Antigens preparation of sorological tests. Tachyzoites from the NC-1 strain of *N. caninum* (Dubey et al. 1988) and RH-strain of *T. gondii* (Sabin, 1941) were cultured in VERO cells (African green monkey kidney cells) in RPMI 1640 culture medium (Invitrogen, Brazil), supplemented with 10% fetal bovine serum (Nutricell, Brazil) with 5% CO_2 at 37°C. Tachyzoites from *N. caninum* were obtained after cellular suspension and disruption, and solution was decanted during 30 minutes, at 4°C, in sterile tube to diminish cell debris. Tachyzoites from *T. gondii* were obtained from frozen aliquots and supplemented with RPMI and fetal serum. Supernatant was recovered from each suspension and centrifuged at 1.500 x g for 10 minutes. Tachyzoites were counted using Neubauer's chamber and re-diluted with RPMI to a final concentration of $2x10^5$ tachyzoites/mL. The pellets were stored at −80°C until use for total protein extraction or IFAT (Pinheiro et al. 2005).

Sarcocystis spp. bradyzoites and/or merozoites were obtained from microscopic analysis of myocardium samples from naturally infected cattle and then purified as described by García-Lunar et al. (2015) and then used as antigen (Moré et al. 2011). Briefly, 100g of minced myocardium were mixed with 400ml of digestion solution (2.5% pepsin, 1% HCl) and were placed in a magnetic stirrer for 20 minutes at 37°C. The homogenate was filtered using 300µm gauze into a 50ml tubes and centrifuged at 500 x g for 5 minutes. The supernatant was discarded and the pellet was washed in 30ml of PBS, 13.5ml of isotonic Percoll® (GE Healthcare) and 1.5ml saline solution (1.5M NaCl) and then centrifuged (4.000 x g for 10 min) (Pertoft et al. 1980). The supernatants and the upper layer above the pellet were discarded and the pellet was washed three times with PBS to a final concentration of $2x10^5$ bradyzoites/mL. Pellets with bradyzoites were frozen at -80°C until use for total protein extraction or for their use in IFAT (Moré et al. 2008, Fernandez-García et al. 2009).

IFAT. Slide preparation and IFAT for *N. caninum* and *T. gondii* were performed as described by Dubey et al. (1988). Bradyzoites of *Sarcocystis* spp. were fixed in slides as described by García-Lunar et al. (2015).

In order to investigate antibodies against *N. caninum* and *T. gondii*, serum samples were used at the dilution of 1/64 (Devens et al. 2014) in phosphate-buffered saline solution (PBS; phosphate 0.1M, NaCl 0.33M, pH 7.2). Serum samples were analyzed for antibodies against *Sarcocystis* spp. in serial dilutions starting at 1/10, dilution in PBS (Tenter 1988). A commercial goat anti-mouse IgG fluorescein isothiocyanate conjugate (Sigma Bio Sciences, St Louis, USA) was used as secondary antibody. Mouse positive and negative sera controls were included on each slide to each parasite. After incubations, slides were observed at 400x magnification under fluorescent microscope (Leica CTR 4000/EBQ 100, Leica Microsystems, Germany) and complete fluorescence of tachyzoites and bradyzoiytes was considered positive. IFAT were used as a gold standard serologic test, since it presents good sensitivity and specificity to diagnose *Sarcocystis* spp., *N. caninum* and *T. gondii* infections (Moré et al. 2008).

Total protein extraction. Total protein extraction was performed using the Radio-Immunoprecipitation Assay Buffer (RIPA Buffer - Sigma Bio Sciences, St Louis, USA) buffer following the manufacturer's recommendations. RIPA Buffer were added (300µL) were added to the pellets containing *N. caninum*, *T. gondii* tachyzoites, *Sarcocystis* spp. bradyzoites, VERO cells and bovine myocardium. The lysate was centrifuged at 8.000 x g for 10 minutes at 4°C to sediment the cellular debris. The supernatant containing the protein solution was carefully transferred to a microtube where 3µl of protease inhibitor cocktail (Sigma Bio Sciences, St Louis, USA) was added and stored at -20°C until the time of use.

Dot-blot. Nitrocellulose membrane with 0.45µm porosity was used and approximately 12µg/mL of each parasites purified antigenic protein was distributed. After drying for 20 minutes at room temperature, membranes were washed three times for five minutes under shaking with PBS-T wash solution (0.05% Tween 20 and PBS) and then blocked with blocking solution (PBS-T with 5% non-fat dry milk) for 16 hours at 4°C. Mice serum samples and negative control serum, were diluted (1/50) in PBS-T and then distributed on the membrane and incubated at 37°C for one hour under slight shaking as previously described (Tenter 1988, Pinheiro et al. 2005). After

incubation, the membrane was washed three times for five minutes and then incubated with horseradish peroxidase (HRP)-conjugated produced in goat anti-mouse immunoglobulin G (1/2000, Sigma Bio Sciences, St Louis, USA) under shaking for 1 hour at 37°C. The enzymatic reaction was revealed with a developing solution (9mL of 50mM Tris-HCl, 1mL of 0.3% Nickel Sulphate, 30µL of Hydrogen Peroxide, 0.006g of DAB) (Sigma Bio Sciences, St Louis, USA) for 20 minutes at room temperature (Pinheiro et al. 2005).

RESULTS AND DISCUSSION

Neospora caninum or *Toxoplasma gondii* experimentally infected mice tested by IFAT for the 3 protozoans (*N. caninum*, *T. gondii* and *Sarcocystis* spp.) reacted only to *N. caninum* or *T. gondii* antibodies, respectively. Specific antibodies against *Sarcocystis* spp. were found in two animals experimentally infected only by this protozoan (one animal showed titer of 50 and other animal presented titer of 800 at IFAT). As previously reported, IFAT is considered the gold standard technique for Sarcocystosis serologic diagnostic in cattle and a positive IFAT result reflects *Sarcocystis* spp. infection (Garcia et al. 2008, Moré et al. 2008). Therefore, in the present study was established that IFAT would be the standard technique to be compared with Dot-blot test. Moré et al. (2008) have described IFAT at 1:25 dilution to be a suitable method for diagnosis of Sarcocystosis in cattle.

Serum samples from mice experimentally infected with *Sarcocystis* spp. and tested by Dot-blot demonstrated the presence of antibodies against this protozoan (at 1:50 dilution) but no cross-reactions were observed in the same sample and same dilution against *N. caninum* or *T. gondii*. Only antibodies against *N. caninum* and no serological cross-reactions with *T. gondii* and *Sarcocystis* spp. were detected in serum samples from mice inoculated with *N. caninum*. As well as, no cross-reactions against *N. caninum* and *Sarcocystis* spp. was observed in serum samples from mice infected by *T. gondii*, showing only specific antibodies against *T. gondii*. These results showed the Dot-blot sensitivity for coccidian immunological diagnostic in mice and suggest this method as a potential serological test for bovine *Sarcocystis* spp. detection.

Although there are no studies that indicate the best experimental model for infection with *Sarcocystis* species that infect cattle, in this study was possible to produce antibodies against *Sarcocystis* spp. in *Mus musculus* mice as demonstrated by the two serological diagnostic tests performed. Animals inoculated with *Sarcocystis* spp. revealed antibodies against this protozoan at Dot-blot technique and showed a high titer of antibodies at IFAT (1:800). Dot-blot proves to be a sensitive test for IgG antibodies against *Sarcocystis* spp. in this study and similar result was observed by Tenter (1988) in mice infected with *Sarcocystis* and in agreement with results described by Ndiritu et al. (1996) in experimentally infected bovine. Tenter (1988) compared Dot-blot with the conventional diagnostic methods ELISA and IFAT for serological detection of *Sarcocystis muris* in immunized and experimentally infected rodents and demonstrated that Dot-blot was sensitive for detection of IgG in immunized mice showing sensitivity equivalent to ELISA and IFAT using serum from infected animals.

The results of the present study suggest that Dot-blot using *Sarcocystis* spp. antigens present good sensitivity and specificity compared to IFAT and this method should be evaluated using bovine serum samples from naturally infected animals as a possible screening test to detect antibodies against *Sarcocystis* spp. that could be applied into the field and consequently Dot-blot may be used as an alternative to IFAT, the gold standard technique to bovine Sarcocystosis.

IFAT is a technique that requires expensive equipment as fluorescence microscope is laborious, and difficult to interpret requiring trained technicians (Pappas 1988, Saville et al. 2004). Therefore, diverse serological diagnostic methods have been developed, to make the diagnosis faster and more precise (Moré et al. 2008, Dubey et al. 2015). In addition, the clinical signs of bovine Sarcocystosis when present are non-specific and not always causing visible macroscopic lesions during meat inspection, therefore these serological methods help in infection diagnosis in order to establish disease control measures (Blagojevick & Antic 2014). Serological tests also allow the diagnosis of *N. caninum* and *T. gondii,* in individual or mixed infections (Dubey et al. 1996, Moré et al. 2008). Therefore, Dot-blot may be considered as an alternative specific and sensitive method which makes possible to evaluate a large number of samples into the field in order to facilitate the diagnosis of *Sarcocystis* infection in cattle herds (Holmdahl et al. 1993, Ndiritu et al. 1996, Guclu et al. 2004). Further studies should be employed allowing the standardization of this method for diagnostic of *Sarcocystis* spp. in bovine serum samples from naturally infected animals.

CONCLUSION

Dot-blot showed same specificity and sensibility as IFAT for immunological diagnostic of *Sarcocystis* spp. in experimentally infected mice and this immunoblot test did not demonstrate serological cross-reactions with *Neospora caninum* and *Toxoplasma gondii*.

Compliance with ethical standards.- All experimental practices involving animals were approved by the Ethics Committee for Animal Experimentation at "Universidade Federal de Santa Maria" (UFSM) (Protocol number 87352511).

Conflict of interest statement.- The authors declare that they have no conflict of interest.

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Epidemiological and pathological aspects of cardiomyopathies in cats in southern Brazil[1]

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ABSTRACT.- Argenta F.F., Mello L.S., Cony F.G., Pavarini S.P., Driemeier D. & Sonne L. 2020. **Epidemiological and pathological aspects of cardiomyopathies in cats in southern Brazil**. *Pesquisa Veterinária Brasileira 40(5)389-398*. Setor de Patologia Veterinária, Departamento de Patologia Clínica Veterinária, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, Porto Alegre, RS 91540-000, Brazil. E-mail: lusonne@yahoo.com.br

Cardiomyopathies are considered one of the most important causes of heart failure in cats and are subdivided into three main morphological types: hypertrophic (HCM), dilated (DCM), and restrictive (RCM). This study aimed to determine the frequency and types of cardiomyopathies in cats diagnosed in southern Brazil, with an emphasis on their epidemiological and pathological aspects. Necropsy reports filed in a veterinary pathology laboratory were reviewed, and cats diagnosed with cardiomyopathy were selected for the study. Animal identification data, history and clinical signs, and gross lesions, were reviewed and compiled. During the study period, 1.594 cat necropsies were performed, of which 72 (4.5%) comprised a diagnosis of cardiomyopathy. HCM was the most frequent followed by CMR and CMD, representing 77.8%, 12.5% and 9.7%, respectively. Age ranged from three months to 18 years, with a median age of seven years. In relation to sex, 62.5% were males and 37.5% females. In 76.4% of the cases, it affected cats without a breed defined. Restrictive mixed dyspnea and hydrothorax were the main signs or findings of the clinical examination. Sudden death and acute paresis of the pelvic limbs due to aortic thromboembolism have also been described. In HCM, myocardial thickening was observed, with a reduction in the ventricular chamber. Hypertrophy, disarray, and fibrosis of the myofibers were the main histological findings. In RCM, whitish and thickened endocardium was seen in most cases. DCM was characterized by dilated cardiac chambers, and microscopic examination revealed no significant findings. The main extra cardiac lesions revealed pulmonary edema and congestion, hydrothorax and chronic passive congestion of the liver. Cardiomyopathies are important causes of death in cats and should be included in the differential diagnosis of patients with cardio respiratory clinical signs and in cases related to sudden death and acute paresis of the pelvic limbs.

INDEX TERMS: Epidemiology, pathology, cardiomyopathy, cats, Brazil, felines, hypertrophic cardiomyopathy, dilated cardiomyopathy, restrictive cardiomyopathy.

RESUMO.- [**Aspectos epidemiológicos e patológicos das cardiomiopatias em gatos no Sul do Brasil.**] As cardiomiopatias são consideradas umas das mais importantes causas de insuficiência cardíaca em gatos e são subdivididas morfologicamente em três principais tipos: cardiomiopatia hipertrófica (CMH), dilatada (CMD) e restritiva (CMR). Este trabalho teve como objetivo determinar a frequência e os tipos de cardiomiopatias em gatos diagnosticados no Sul do Brasil, abordando seus aspectos epidemiológicos e patológicos. Foram revisados os laudos de necropsias de gatos e selecionados para o estudo de diagnóstico de cardiomiopatia. Os dados referentes à identificação do animal, o histórico/

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sinais clínicos e lesões macroscópicas foram revisados e compilados. No período estudado, foram realizadas 1.594 necropsias de gatos, destas, 72 (4,5%) compreenderam diagnóstico de cardiomiopatia. A CMH foi a mais frequente seguida pela CMR e CMD, representando 77,8%, 12,5% e 9,7%, respectivamente. A idade variou de três meses a 18 anos, com a idade mediana de sete anos. Em relação ao sexo, 62,5% eram machos e 37,5% fêmeas. Em 76,4% dos casos, afetou gatos sem raça definida. Dispneia mista restritiva e hidrotórax foram os principais sinais ou achados do exame clínico. Morte súbita e paresia aguda de membros pélvicos em razão do tromboembolismo aórtico também foram descritos. Na CMH, observou-se espessamento do miocárdio, com redução da câmara ventricular. Hipertrofia, desarranjo e fibrose das miofibras foram os principais achados histológicos. Na CMR, visualizou-se endocárdio esbranquiçado e espessado na maioria dos casos. A CMD caracterizou-se pela dilatação das câmaras cardíacas, e sem lesão histológica significante. As principais lesões extracardíacas encontradas foram edema e congestão pulmonares, hidrotórax e congestão passiva crônica do fígado. As cardiomiopatias são causas importantes de morte em gatos, devem ser incluídas no diagnóstico diferencial de pacientes com sinais clínicos cardiorrespiratórios e também em casos relacionados a morte súbita e paresia aguda dos membros pélvicos.

TERMOS DE INDEXAÇÃO: Epidemiologia, patologia, gatos, Brasil, felinos, cardiomiopatia hipertrófica, cardiomiopatia dilatada, cardiomiopatia restritiva.

INTRODUCTION

Cardiomyopathies are important progressive heart diseases, especially in felines and canines, which resemble the presentation of the diseases in humans (Miller & Gal 2017). Cardiomyopathy is defined as a diverse group of heart muscle diseases, in which the myocardium is structurally and functionally altered (Elliott et al. 2008). Cardiomyopathies are considered one of the most important causes of heart failure in cats and are classified as primary when the origin or suspicion is genetic and secondary when there is a known cause that is not genetic (Robinson & Robinson 2016). Cardiomyopathies are further subdivided into three main morphological types: hypertrophic (HCM), dilated (DCM), and restrictive (RCM) (Robinson & Robinson 2016). In HCM, myocardial hypertrophy of an unknown etiology is identified (Fox 2003), but an autosomal recessive genetic character is suspected in cats and humans (Meurs et al. 2005, 2007). CMD is characterized by the dilation of the four cardiac chambers (Robinson & Robinson 2016) and RCM by the restriction of ventricular filling and distention due to the large amount of fibrous connective tissue (Fox 2004, Kimura et al. 2016).

In cats, cardiomyopathies are the most frequent cardiovascular diseases and are important causes of deaths (Ferasin 2009a). In Brazil, there are few published data sources related to cardiomyopathies in this species. Therefore, this study aimed to determine the frequency and types of cardiomyopathies in cats diagnosed in southern Brazil, with an emphasis on their epidemiological and pathological aspects.

MATERIALS AND METHODS

Necropsy reports filed in a veterinary pathology laboratory from January 2008 to December 2018 were reviewed, and cats diagnosed with cardiomyopathy were selected for the study. All cats in the study were from Rio Grande do Sul, Brazil, especially from the metropolitan region of Porto Alegre. The classification of cardiomyopathies was based on the morphological changes described by Robinson & Robinson (2016). Cases with a medical record or/and pathological features consistent with chronic renal failure, endocrine disorders, hypertension, myocarditis and neoplasms with cardiac involvement were excluded from the study. Animal identification data, such as breed, sex and age, history and clinical signs, and gross lesions were reviewed and compiled. From the selected cases, archived paraffin blocks were searched for the preparation of histological slides, for subsequent staining by hematoxylin and eosin (HE) techniques and visualization by optical microscopy. For the evaluation of the degree of fibrosis, histological sections of the heart were submitted to Masson's trichrome (MT) stain.

RESULTS

From January 2008 to December 2018, 1,594 cat necropsies were reported, of which 72 were cardiomyopathic cases, representing 4.5% of the cases (respecting the exclusion criteria). Regarding the morphological classification, 77.8% were cases of HCM, 12.5% restrictive and 9.7% dilated. Age ranged from three months to 18 years, with a median age of seven years (mean age of 7.6 years). In relation to sex, 62.5% were males and 37.5% females. In 76.4% of the cases, it affected cats mixed breed, and 23.6%, cats with a defined breed, mainly Persian and Siamese. Clinical signs and/or physical examination findings were described in 57 cases and included: restrictive mixed dyspnea (45.6%), hydrothorax (33.3%), vomiting (29.8%), lethargy (28.1%), anorexia (21.1%) and cyanosis (18.5%). Acute paresis of the pelvic limbs was identified in 14%, and sudden death reported in 26.3% of cases. No significant changes were identified in the epidemiological data and clinical presentation regarding the morphological classifications of cardiomyopathies.

The hearts of cats with HCM were grossly enlarged, due to a marked thickening of the myocardium, predominantly of the left ventricle and interventricular septum, with a reduction in the left ventricular chamber (Fig.1A). Multifocal to coalescent whitish areas in the myocardium were visualized in 66.1% of the cases. Cardiac lesions secondary to myocardial thickening, such as right ventricular dilatation, left atrial dilatation and atrial thrombosis, were described in 33.9%, 28.6% and 8.9% of cases, respectively. Histologically, in 50% of HCM cases, lesions were identified in the left ventricle and interventricular septum; 41.1% only in the left ventricle, and 8.9% left and right ventricle, in addition to the interventricular septum. These lesions were mainly characterized by varying degrees of myofiber hypertrophy, multifocal disarray that is characterized by the interweaving of myofibers, and proliferation of interstitial myocardial multifocal fibrous connective tissue (fibrosis) (Fig.1B and 1C), evidenced mainly by the histochemical technique of MT (Fig.1D). Table 1 details the histological lesions in cats with HCM.

In RCM, the hearts were grossly enlarged, whitish and frequently globose. In 77.8% of cases, diffusely whitish and thickened endocardium was observed (Fig.2A). In the

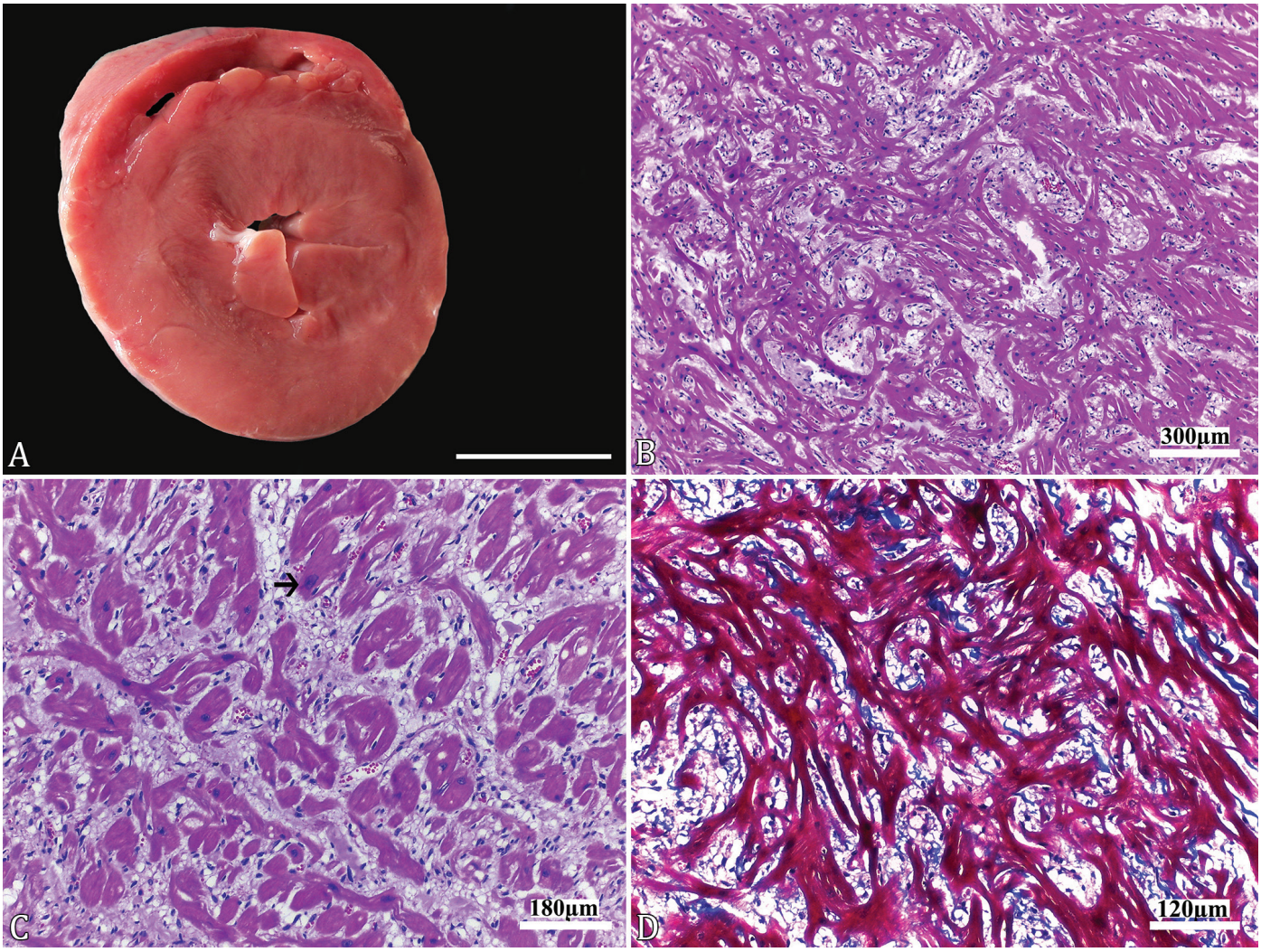


Fig.1. Pathological aspects of hypertrophic cardiomyopathy in cats. (**A**) Cross-sectional view of the heart with thickening of the myocardium, predominantly of the left ventricle and interventricular septum, with consequent reduction of the left ventricular chamber. Bar = 2.0cm. (**B**) Left ventricular myocardium with hypertrophy and multifocal disarray characterized by interweaving of myofibers, associated with proliferation of the interstitial multifocal fibrous connective tissue (fibrosis). HE, bar = 300µm. (**C**) Left ventricular myocardium with hypertrophy, disarray and moderate interstitial multifocal fibrosis. Multifocal karyomegaly is also shown (arrow). HE, bar = 180µm. (**D**) Left ventricular myocardium with hypertrophy, disarray and blue-stained interstitial multifocal fibrosis. MT, bar = 120µm.

Table 1. Histological lesions observed in the heart of cats with hypertrophic cardiomyopathy

Histological lesion	Histological grade			
	Discrete	Moderate	Accentuated	No change
	n	n	n	n
Myofiber hypertrophy	18	23	10	5
Myofiber disarray	21	17	9	9
Myocardial fibrosis	24	13	5	14
Cardiomyocyte vacuolization	22	6	0	28
Vessel wall thickening	19	5	0	32
Cardiomyocyte cariomegaly	14	4	1	37
Cardiomyocyte necrosis	6	4	1	45
Inflammatory infiltrate in the myocardium	6	3	0	47
Myocardial adipocyte infiltrate	4	4	0	48
Multifocal mineralization	0	1	0	55

n = Number of cases.

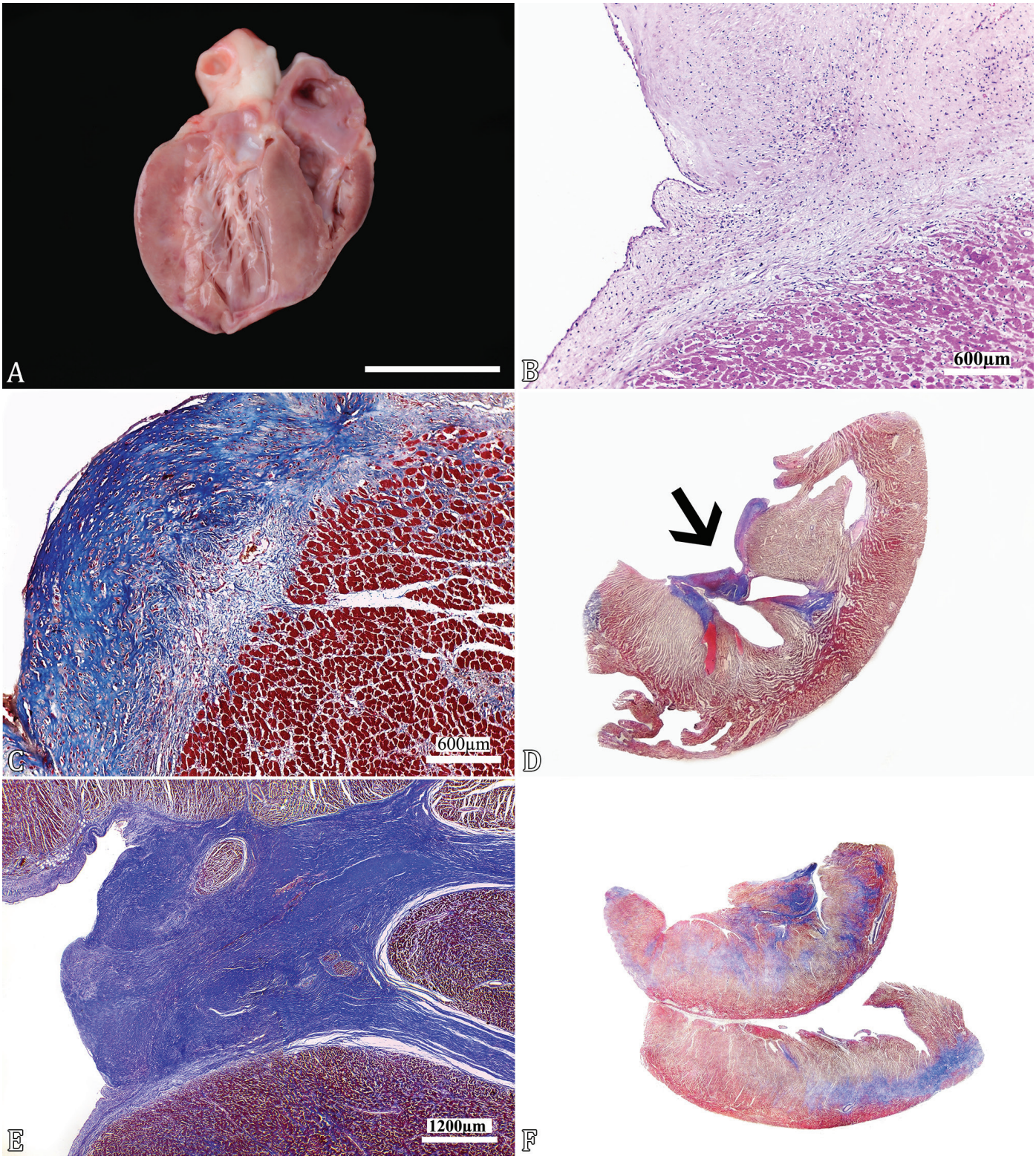


Fig.2. Pathological aspects of restrictive cardiomyopathy in cats. (**A**) Longitudinal section of the heart with whitish endocardium. Moderate left ventricular and interventricular septal hypertrophy associated with multifocal whitish areas in the myocardium. Bar = 4.5cm. (**B**) Thickened endocardial region resulting from intense fibrosis. HE, bar = 600µm. (**C**) Intense fibrosis in the endocardial region. MT, bar = 600µm. (**D**) Sub macroscopic aspect of restrictive cardiomyopathy in cats, with the formation of irregular trabeculae of fibrous connective tissue interconnecting the interventricular septum with the free wall of the left ventricle. MT. (**E**) Formation of trabeculae of fibrous connective tissue. MT, bar = 1200µm. (**F**) Sub macroscopic aspect of restrictive cardiomyopathy in cats with predominance of fibrosis in the myocardial region. MT.

remaining 22.2%, whitish irregular bands interconnecting the left ventricular free wall to the interventricular septum were visualized. Left ventricular and interventricular septal myocardial hypertrophy, right ventricular and left atrial dilatation, and atrial thrombosis were described in 66.7%, 55.6% and 11.1%, respectively. In 88.9% of the cases of RCM, lesions were microscopically identified in the left ventricle and interventricular septum, and in the remaining 11.1%, there were cases with histological lesions in the left atrium and ventricle, in addition to the septum. Intense multifocal to coalescent fibrosis in the endocardial region (Fig.2B), evidenced mainly by MT staining (Fig.2C), was identified in 66.7% of the cases. In cases with predominance of endocardial fibrosis, 22.2% had irregular bands of fibrosis interconnecting the left ventricular free wall to the interventricular septum (Fig.2D and 2E). Fibrosis presented the myocardial form in 33.3% of cases (Fig.2F). Other histological findings included: moderate hypertrophy and disarray of myofibers, and cardiomyocyte karyomegaly (55.6% each), mild inflammatory infiltrate of lymphocytes and plasma cells in the epicardium and myocardium (22.2%), discrete multifocal mineralization (22.2%) and focal endocardial cartilaginous metaplasia (11.1%).

Cats with DCM had enlarged and globose hearts due to dilation of the cardiac chambers, predominantly of the ventricles. The ventricular walls were thin and flaccid, with flattening of the papillary muscles (Fig.3). Histological lesions were observed in 14.3% of the cases, characterized by the discrete multifocal perivascular fibrosis in the left ventricular myocardium and a slight thinning of the cardiac fibers, which were multifocally wavy in the ventricles and interventricular septum.

The main extra cardiac lesions found in cats with cardiomyopathies were: varying degrees of pulmonary edema and congestion (94.4% and 69.4%, respectively) (Fig.4A), hydrothorax (50%), chronic passive hepatic congestion (44.4%) (Fig.4B) and cyanosis (26.4%) (Fig.4C and 4D). Thrombus located at the bifurcation of the aorta in internal iliac arteries was reported in 11.1% of cases. These cats had diminished, firm pelvic limb muscles with whitish multifocal areas. In 4.2% of cases observed, the extremities of the pelvic limbs, skin, and hair were dry, with multifocal areas of cutaneous continuity solution (gangrene) (Fig.4F). Table 2 details the extra cardiac lesions of cats with cardiomyopathy.

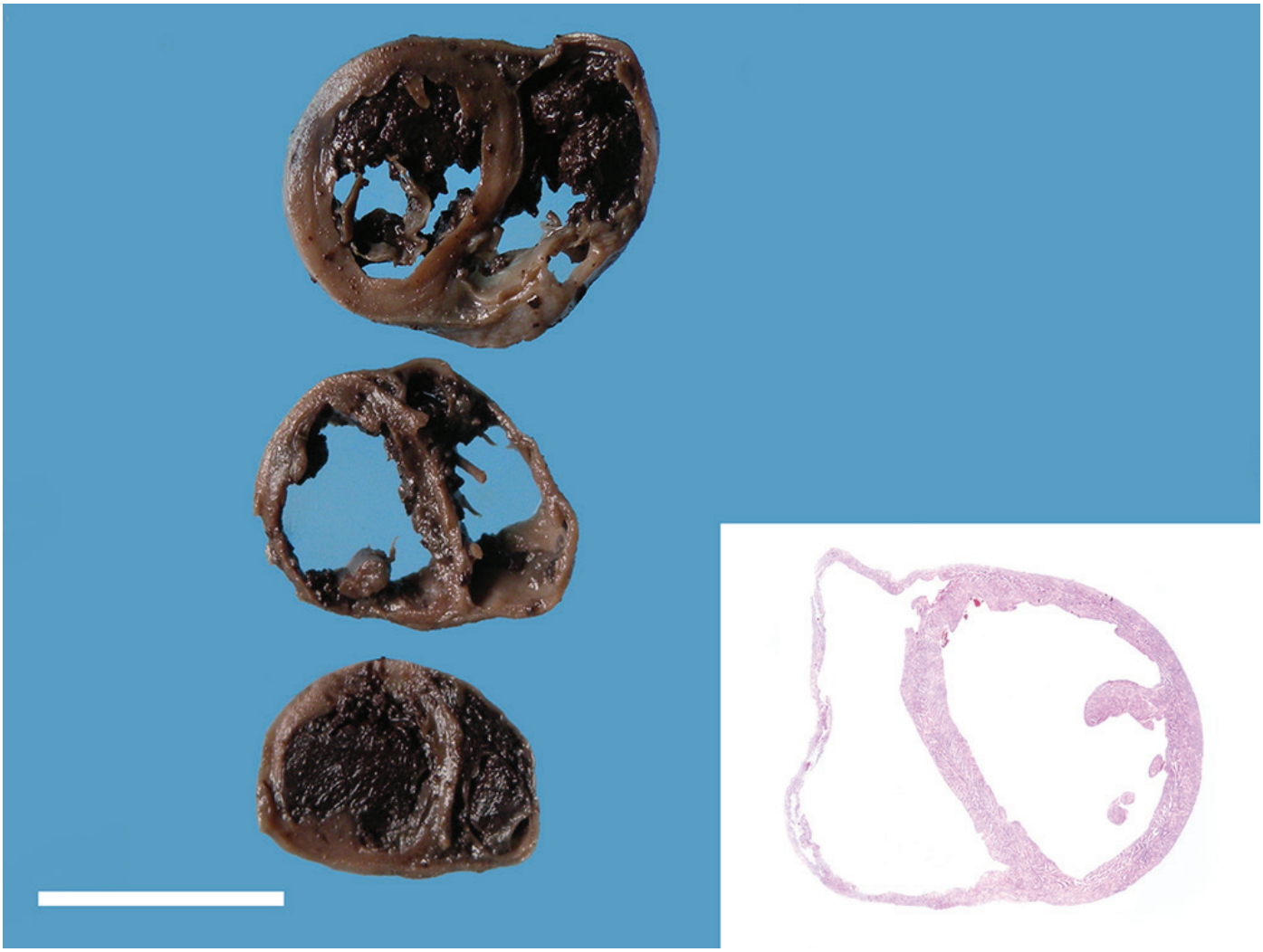


Fig.3. Gross and sub macroscopic aspects of dilated cardiomyopathy in cats. Ventricular dilatation, with thin and flaccid ventricular walls (10% formalin-fixed sample) Insert: sub macroscopic aspect, with intense ventricular dilation. HE, bar = 2.0cm.



Fig.4. Extra cardiac lesions in cats with cardiomyopathies. (**A**) Bright pleural surface lungs with red multifocal areas in a cat diagnosed with hypertrophic cardiomyopathy. (**B**) Thoracic cavity with large amount of free serous and yellowish fluid in the cavity associated with congestion and marked lobular pattern of the liver in a cat with hypertrophic cardiomyopathy. (**C**) Plantar surface of purplish-colored thoracic limbs in a cat with hypertrophic cardiomyopathy. (**D**) Purplish oral mucosa and tongue in a case of hypertrophic cardiomyopathy. (**E**) Thrombus located at the aorta artery bifurcation in the internal iliac arteries in a case of hypertrophic cardiomyopathy. (**F**) Extremities of pelvic limbs, skin and dry-looking hair with multifocal areas of cutaneous continuity solution in the same case as above. Insert: pelvic limb muscles with whitish multifocal areas.

Table 2. Extra cardiac pathological lesions of cats with cardiomyopathy

Lesion	Total (n = 72)	HCM (n = 56)	RCM (n = 9)	DCM (n = 7)
	n (%)	n (%)	n (%)	n (%)
Pulmonary edema	68 (94.4)	53 (94.6)	8 (88.9)	7 (100)
Pulmonary congestion	50 (69.4)	35 (62.5)	8 (88.9)	7 (100)
Hydrothorax	36 (50)	27 (48.2)	5 (55.6)	4 (57.1)
Chronic passive congestion of the liver	32 (44.4)	26 (46.4)	3 (33.3)	3 (42.9)
Cyanosis	19 (26.4)	16 (28.6)	1 (11.1)	2 (28.6)
Ascites	15 (20.8)	9 (16.1)	4 (44.4)	2 (28.6)
Hydropericardium	14 (19.4)	10 (17.9)	3 (33.3)	1 (14.9)
Pulmonary atelectasis	12 (16.7)	10 (17.9)	2 (22.2)	0 (0)
Diffuse subcutaneous edema	8 (11.1)	7 (12.5)	1 (11.1)	0 (0)
Aortic thromboembolism	8 (11.1)	7 (12.5)	1 (11.1)	0 (0)
Bilateral nasal serous secretion	6 (8.3)	5 (8.9)	1 (11.1)	0 (0)
Gangrene of the pelvic limbs	3 (4.2)	3 (5.4)	0 (0)	0 (0)

n = Number of cases, HCM = hypertrophic cardiomyopathy, RCM = restrictive cardiomyopathy = DCM = cardiomyopathy dilated, % = percentage.

DISCUSSION

In the present study, the frequency of cardiomyopathies observed in cats was 4.5%. In the literature, heart disease in cats was found to have a frequency of 4.2 to 5.7% (O'neill et al. 2014, Rodrigues et al. 2017). In studies of the causes of death and reasons for euthanasia, cardiomyopathies accounted for approximately 1% of total feline necropsies (Togni et al. 2018, Withoeft et al. 2019). In cats, cardiomyopathies are the most frequent cardiovascular diseases and are important causes of death in the species (Ferasin 2009a). HCM is most commonly diagnosed, clinically and by *post mortem* examination (Atkins et al. 1992, Rush 1998, Riesen et al. 2007, Togni et al. 2018), followed by RCM and DCM (Ferasin et al. 2003, Locatelli et al. 2018). This corroborates the findings of the present study. In the past, DCM was the second most diagnosed form in cats; however, its decreased frequency over the years may be related to the supplementation of taurine in diets after the discovery of the association of this amino acid deficiency with this condition in cats (Pion et al. 1987, Ferasin et al. 2003).

Cats with cardiomyopathies present a wide variation in age range (Bright et al. 1992, Ferasin 2009a). Researchers describe that middle-aged cats are the most affected, with an average age ranging from four to 10 years (Atkins et al. 1992, Bright et al. 1992, Rush et al. 2002, Ferasin et al. 2003, Riesen et al. 2007, Ferasin 2009a, Payne et al. 2010, Biasato et al. 2015, Kimura et al. 2016, Spalla et al. 2016, Miller & Gal 2017, Locatelli et al. 2018). However, there are reports of two-month-old cats with HCM (Fujii et al. 2001), and in the present study it was possible to identify three-month-old cats diagnosed with cardiomyopathy. Cats of mixed breed were the most affected in the present study, as described in the literature (Ferasin et al. 2003, Spalla et al. 2016, Locatelli et al. 2018); however, these data may be related to the fact that these animals represented the majority of the feline population referred to in the pathology laboratory. In the present study, males represented more than 60% of cases, as identified by numerous researchers (Atkins et al. 1992, Bright et al. 1992, Riesen et al. 2007, Fox et al. 2014, Payne et al. 2015, Spalla et al. 2016, Locatelli et al. 2018); however, in the study by Ferasin et al. (2003), no significant sexual predisposition was identified. In human medicine, HCM patients have a MYBPC3 mutation, that is an abnormal phenotype that develops earlier in men than in women (Christiaans et al. 2010), and this has also been reported in Maine Coon cats (Kittleson et al. 1999).

The clinical signs of felines with cardiomyopathies are varied (Ferasin 2009a), and in many cases are related to congestive heart failure (Ferasin et al. 2003, Abbott 2010). In general, the main clinical signs and/or findings of the clinical examinations in the present study were restrictive mixed dyspnea, hydrothorax, vomiting and lethargy, which are frequently described in the literature (Bright et al. 1992, Ferasin et al. 2003, Kimura et al. 2016). However, the clinical presentation did not differ significantly between cardiomyopathy classifications in cats (Ferasin et al. 2003). In addition to the clinical changes already described, some cats may be asymptomatic (Rush et al. 2002, Trehiou-Sechi et al. 2012, Payne et al. 2013) or experience sudden death (Abbott 2010, Trehiou-Sechi et al. 2012, Miller & Gal 2017). Heart disease is considered to be one of the most frequent causes of unexpected death in cats (Olsen & Allen 2001, Wilkie et al. 2015), and in the present study sudden death was reported in 15 cats. Stress is an important factor that can trigger or exacerbate clinical signs, or be related to sudden death, as there is a rapid release of catecholamines. This induces generalized vasoconstriction and increased cardiac output. In addition to stress, severe arrhythmias may be responsible for episodes of syncope and sudden death (Ferasin 2009a). Acute paresis of the pelvic limbs as a consequence of aortic thromboembolism in the aorta artery bifurcation in the internal iliac arteries were identified in eight cats of the present study, of which seven were diagnosed with HCM and one with RCM. Arterial thromboembolism is the most serious consequence of cardiomyopathies (Ferasin 2009a). Cats have aortic thromboembolism in 6% to 20% of cases of myocardial disease. The formation of this condition results from left atrial thrombosis, due to the change in blood flow (stagnant blood flow), which is released into the aorta with the consequent obstruction (Ferasin et al. 2003, Ferasin 2009a, Kimura et al. 2016, Ocarino et al. 2016, Robinson & Robinson 2016).

HCM causes a diastolic ventricular dysfunction, due to impaired ventricular filling, due to myocardial thickening, which makes the ventricle rigid, and not complacent (Ferasin 2009a, Robinson & Robinson 2016). The enlarged heart is

grossly observed as a consequence of ventricular hypertrophy, mainly on the left side, with reduction of ventricular lumen. Occasionally, both sides are affected (Fox 2003, Ferasin 2009a). These data corroborate the findings of the present study. According to Robinson & Robinson (2016), most cats present symmetrical hypertrophy, but in some cases there is asymmetrical septal thickening, where the ventricular septum is thicker than the free wall of the left ventricle. Atrial dilatation and thrombosis, as well as right ventricular dilatation resulting from predominantly left ventricular myocardial hypertrophy, are frequent in cases of HCM (Ferasin 2009a, Robinson & Robinson 2016, Miller & Gal 2017). The whitish areas visualized in the hearts of HCM used in the present study were related to multifocal fibrosis (Fox 2004, Ferasin 2009a).

The main histological lesions found in HCM were hypertrophied cardiomyocytes, fibers that are arranged in a disorganized pattern and varying degrees of fibrosis. These are findings that are frequently described in the literature (Fox 2003, Ferasin 2009b, Robinson & Robinson 2016). Hypertrophy is characterized as large cardiomyocytes, with enlarged and pleomorphic nuclei, and often prominent nucleoli (Fox 2003). Disarray is characterized by interweaving of the myofibers (Maron et al. 1981, Liu et al. 1981) and researchers suggest that this alteration constitutes the gold standard for the histological diagnosis of HCM (Fox 2003, Biasato et al. 2015). Proliferation of smooth muscle and connective tissue of the coronary vessel wall, fat infiltration, inflammatory infiltrate, degeneration and necrosis of cardiomyocytes are also described in HCM (Ferasin 2009b, Khor et al. 2015, Wilkie et al. 2015), and were frequent in the microscopic evaluation of the present study. Disarray of myofibers may predispose towards ischemia and, consequently, myocardial fibrosis, as disorganized cardiomyocytes promote insufficient contraction and increased energy demand (Iida et al. 1998, Varnava et al. 2001, Cesta et al. 2005). Narrowing of intramural coronary arteries may also contribute to the development of myocardial ischemia in these cases (Maron & Spirito 1998, Cesta et al. 2005).

RCM is characterized by impaired ventricular diastolic filling, due to increased stiffness of the heart muscle caused by intense endomyocardial fibrosis (Kushwaha et al. 1997, Ferasin et al. 2003, Fox 2004). In the present study, an increase in cardiac volume was grossly observed as a consequence of ventricular and atrial dilatations. This was mainly associated with diffuse whitish and thickened endocardium. Several researchers report that there are two morphological patterns of endomyocardial fibrosis, although a degree of feature overlap may occur (Fox 2004). Diffusely thickened and whitish endocardium or irregular trabeculae may be observed connecting the interventricular septum to the left ventricular free wall (Fox 2004, Ferasin 2009b, Kimura et al. 2016, Miller & Gal 2017). Trabeculae formation is the most common presentation of RCM in cats (Fox 2004, Kimura et al. 2016), but in the present study this presentation was less common than the diffuse form. Atrial enlargement, multiple whitish areas resulting from fibrosis and left ventricular myocardial hypertrophy, are frequent gross findings (Ferasin et al. 2003, Fox 2004, Ferasin 2009b, Ocarino et al. 2016, Locatelli et al. 2018). Aortic thromboembolism may occur in RCM, resulting from left atrial and ventricular filling defects and thrombus formation (Fox 2004, Kimura et al. 2016). This condition is subdivided into two forms: endomyocardial, when predominant endocardial lesions are observed, and myocardial form, with predominant myocardial involvement (Gallo & D'amati 2001, Fox 2004, Kimura et al. 2016). Researchers report that the endomyocardial form is more commonly found in cats (Ferasin et al. 2003, Fox 2004, Kimura et al. 2016), as is also identified in the present study.

Fibrosis was observed microscopically, predominantly in the endocardium, with cases involving the left ventricular myocardium, interventricular septum and left atrium. The characteristic lesion of CMR is a marked endocardial thickening due to fibrosis (Fox 2004, Ferasin 2009b, Kimura et al. 2016). In many cases, myocardial interstitial fibrosis is also evident (Fox 2004). Cardiomyocyte hypertrophy, mild inflammatory infiltrate and cartilaginous metaplasia were also histological findings identified in the present study and are described in the literature (Fox 2004, Kimura et al. 2016). In a study related to feline CMR cases, researchers suggested that false left ventricular tendons provide a framework for the development of irregular trabecular formation that connects the septum to the left ventricular free wall (Kimura et al. 2016).

DCM is characterized by systolic dysfunction as a result of lack of contractility, which results in bilateral congestive heart failure (Ferasin et al. 2003, Ocarino et al. 2016, Robinson & Robinson 2016). The hearts were grossly enlarged and globose as a result of the dilatation of all cardiac chambers, with thin and flaccid ventricular walls, and flattening of the papillary muscles. Similar data are described in the literature (Liu 1977, Tilley et al. 1977, Ferasin et al. 2003, Robinson & Robinson 2016). Histological lesions are often nonspecific, discrete or absent (Robinson & Robinson 2016, Miller & Gal 2017). In most of the diagnoses of DCM in the present study, no significant histological lesions were observed. These, when present, are characterized by discrete thinning of cardiac fibers, wavy fibers and fibrosis (Tilley et al. 1977, Robinson & Robinson 2016).

Pulmonary edema and congestion, hydrothorax and chronic passive congestion of the liver were frequent extra cardiac lesions. These changes are common in cases of heart failure in animals, and several researchers describe these findings in different classifications of cardiomyopathies in cats (Ferasin et al. 2003, Fox 2003, Payne et al. 2010, Kimura et al. 2016, Robinson & Robinson 2016). The formation of pulmonary edema, hydrothorax, ascites and hydropericardium occur mainly by increased hydrostatic pressure due to blood flow stasis in cats with cardiomyopathies (Robinson & Robinson 2016, López & Martinson 2017). Chronic passive congestion of the liver is also related to portal hypertension resulting from heart failure (Miller & Gal 2017). Cyanosis, in cases of heart failure, is due to deposition of unoxygenated hemoglobin in the mucosae (Miller & Zachary 2017) and was a relatively frequent finding in the cases of the present study.

Mutations in genes that encode proteins associated with the cardiac contraction process have been identified in cats with HCM (Robinson & Robinson 2016). In this condition, the hereditary character in the Maine Coon and Radgoll breeds, and their relationship with genetic mutations, is documented (Meurs et al. 2005, Meurs et al. 2007, Robinson & Robinson 2016). Researchers report that endomyocarditis of viral, bacterial (*Bartonella* sp.) and immunomediated origin, or as a consequence of end-stage HCM, are the possible etiological

factors of feline RCM (Fox 2004, Cesta et al. 2005, Robinson & Robinson 2016). Feline parvovirus genomic material has been isolated from the hearts of cats with cardiomyopathy and myocarditis (Robinson & Robinson 2016). Furthermore, researchers have suggested the possible involvement of the feline immunodeficiency virus in cats with lymphocytic myocarditis and hypertrophic cardiomyopathy, although the cause and effect have not been well understood (Robinson & Robinson 2016, Rolim et al. 2016). In recent studies, no related viral agents have been identified in HCM and RCM (Kimura et al. 2016, McEndaffer et al. 2017). Genetic inheritance is suggested as the cause of idiopathic DCM and secondary DCM-related taurine deficiency in cats (Pion et al. 1987, Robinson & Robinson 2016, Miller & Gal 2017).

CONCLUSIONS

Cardiomyopathies were related to the cause of death in 4.5% of cats referred for necropsy; HCM was the most frequent morphological classification, followed by RCM and DCM.

Cardiomyopathies showed great variation in age, consisted of predominantly affected cat mixed breed adult males with a median age of seven years.

Restrictive mixed dyspnea and hydrothorax were the major findings on clinical examination. Less frequently, cases of sudden death and acute paresis of the pelvic limbs due to aortic thromboembolism at the aorta artery bifurcation in the internal iliac arteries were reported.

Cats with HCM had myocardial thickening with consequent reduction in the ventricular chamber, which were histologically characterized by hypertrophy, disarray, and fibrosis.

In CMR, the endocardium was diffusely whitish and thickened, and was less common, forming irregular bands connecting the left ventricle to the interventricular septum.

Endocardial fibrosis was the predominant histological form of CMR. In CMD, the heart had dilated cardiac chambers, and microscopic examination revealed no significant findings.

The main extra cardiac lesions revealed pulmonary edema and congestion, hydrothorax and chronic passive congestion of the liver.

Cardiomyopathies are important causes of death in cats and should be included in the differential diagnosis of patients with cardio respiratory clinical signs and in cases related to sudden death and acute paresis of the pelvic limbs.

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Original Article
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Seroprevalence and incidence of *Leptospira* spp. in domestic dogs in the Southeast region of São Paulo State, Brazil[1]

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ABSTRACT.- Sevá A.P., Brandão A.P.D., Godoy S.N., Souza G.O., Souza Filho A.F, Jimenez-Villegas T., Heinemann M.B. & Ferreira F. 2020. **Seroprevalence and incidence of *Leptospira* spp. in domestic dogs in the Southeast region of São Paulo State, Brazil.** *Pesquisa Veterinária Brasileira 40(5):399-407*. Embrapa Suínos e Aves, Rodovia BR-153 Km 110, Cx. Postal 21, Distrito de Tamanduá, Concórdia, SC 89700-000, Brazil. E-mail: anaiaps@usp.br

Leptospirosis is a zoonotic disease caused by *Leptospira* and domestic dogs can act as host of some serovars. In order to analyze the transmission dynamics in a dog population, with and without immunization, a longitudinal study was carried out with a focus to evaluate antibody response and to identify serovars. Blood samples were collected in three consecutive years (2015 to 2017) from 331, 373 and 347 dogs respectively. The dog seroprevalence in each year was 11%, 7% and 14%, respectively, and the incidence in 2016 was 5% and in 2017, 14%. The most frequent serovars were Cynopteri and Butembo in 2015, Cynopteri, Butembo and Hardjoprajitno in 2016, and Canicola and Butembo in 2017. Dogs can play a role as sentinel animals and hosts of *Leptospira* serovars. The percentage of seropositive dogs due to vaccination was higher than the previous years without immunization and lower than in previous years for other serovars, which we interpret as evidence for the importance of immunization. These parameters associated with active canine population control are important for prevention and control of leptospirosis not only in dogs but alsoto inhibit the transmission between dogs and humans.

INDEX TERMS: Seroprevalence, *Leptospira* spp., domestic dogs, São Paulo, Brazil, program, immunization, vaccine, dogs, Cãoservação Program.

RESUMO.- [Soroprevalência e incidência de *Leptospira* spp. em cães domésticos na região sudeste do estado de São Paulo, Brasil.] A leptospirose é uma zoonose causada pelo agente etiológico *Leptospira*. Cães domésticos atuam como hospedeiro de diversos sorovares deste agente. Com intuito de analisar a dinâmica da leptospirose em uma população canina, com e sem imunização, um estudo longitudinal foi realizado avaliando a resposta sorológica destes animais e identificando seus sorovares. Foram coletadas amostras de 331, 373 e 347 cães em três anos consecutivos (2015 a 2017). As soroprevalências foram de 11%, 7% e 14%, respectivamente, e a incidência em 2016 foi de 5% e em 2017 de 14%. Os sorovares mais frequentes foram Cynopteri e Butembo em 2015, Cynopteri, Butembo e Hardjoprajitno em 2016, e Canicola e Butembo em 2017. Estes cães estão atuando como bio-indicadores da presença de *Leptospira* na região do estudo, incluindo sorovares zoonóticos, e contribuindo com a sua manutenção no ambiente. A soropositividade para sorovares protegidos pela vacina foi mais alta do que nos anos anteriores à imunização, enquanto para os sorovares não protegidos pela vacina diminuiu, demonstrando a importância da imunização para essa população de cães. Medidas de prevenção e controle para a leptospirose, como imunização e controle populacional canino, são recomendadas no local para inibir a transmissão do agente entre as populações de cães e humanos envolvidas.

TERMOS DE INDEXAÇÃO: Soroprevalência, *Leptospira* spp., cães domésticos, São Paulo, Brasil, imunização, vacina, caninos, Programa Cãoservação.

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INTRODUCTION

Leptospirosis is a zoonotic disease caused by the bacteria *Leptospira* and it affects most mammalian species (Acha & Szyfres 2001). Human leptospirosis in Brazil is considered a neglected disease and requires mandatory notification (Brasil 2017). Although the number of human cases has declined in recent years (from 4,676 in 2014 to 2,930 in 2017), disease occurrence is still high, being São Paulo State the most affected (18.6% in 2017) (Brasil 2019).

Domestic dogs contribute to the maintenance of pathogens such as the *Leptospira*, that can be transmitted to both wild animals and humans (Courtenay et al. 2001, Doherty et al. 2017). Canine leptospirosis can be a serious public health issue, due to the pathogenicity, extended shedding period (Ávila et al. 1998), and the potential contagion established by the proximity between humans and dogs (Brasil 1998, Polachini & Fujimori 2015).

The course of infection and severity of symptoms vary according to the serovar, host species and host immune status (Maria & Kimura 2002). Leptospirosis is commonly asymptomatic (Levett 2001) and the clinical manifestations vary from subclinical to lethal (Levett 2001, Polachini & Fujimori 2015), being the symptoms related to renal insufficiency and vascular disorders (Birnbaum et al. 1996, Levett 2001). The bacteria affects kidneys and is excreted with the urine, remaining at the environment mostly in water sources (Polachini & Fujimori 2015).

There are 21 reported species of *Leptospira* and more than 300 identified serovars (Faine et al. 1999). Each serovar has one or more specific host species and one mammalian species can host more than one serovar (Levett 2001). In Brazil, the most important serovar in humans is Icterohaemorrhagiae (Nascimento et al. 2004), which is also important for dogs, in addition to Canicola and Copenhageni (Sakata et al. 1992, Scanziani et al. 1994, Jorge et al. 2017, Pinto et al. 2017).

The occurrence of leptospirosis varies in different geographical areas and is affected by socioeconomic characteristics; climatic conditions, including high temperature, rainfall and by biologic features, such as the presence of wild and domestic reservoirs (Brod et al. 2005). Therefore, if humans and domestic dogs share the same environment, investigation leptospirosis in dogs is of utmost importance for successful prevention and control, including in humans (Brod et al. 2005, Polachini & Fujimori 2015). It is also important to point the fact that the transmission of the disease by dogs is especially common when they are unvaccinated, a usual situation in rural and low-income areas (Martinez et al. 2013).

This study was performed in a small district, close to a Conservation Unit of São Paulo State, Brazil, and aimed to analyze the dynamics of leptospirosis in a domestic dog population, evaluating their serological response in three consecutive years, being that in the second year, the animals were vaccinated in order to compare seroprevalence before and after immunization.

MATERIALS AND METHODS

Study area. Two neighborhoods of São Miguel Arcanjo municipality were included in the study, Abaitinga and Gaviões, located in the surrounding of the "Parque Estadual Carlos Botelho" (CBSP), São Paulo State, Brazil. Dogs had access to the street and to the Park and were non-vaccinated. The area is as a rural zone with a subtropical, humid climate (an average annual temperature of 18ºC and a monthly average rainfall over 40mm) without a dry season and with a hot summer .(Alvares et al. 2013).

Data and biological sample collection. In April of 2015, 2016 and 2017 all residences of the neighborhoods were visited for biological sampling of all domiciled dogs. (1st, 2nd and 3rd collection, respectively). During the visits, the owners signed a "free and informed consent form", which was approved by the Ethics Commission of Animal Use (CEUA) of the "Faculdade de Medicina Veterinária e Zootecnia" of the "Universidade de São Paulo" (FMVZ-USP) under protocol number CEUA 2452231014. After the owner's authorization, the residence was geo-referenced (Fig.1), dogs were identified and their blood samples were collected from the jugular, cephalic or femoral vein. Samples were stored at 5ºC for a maximum of five hours until serum was extracted by centrifugation at 1500rpm for 10 minutes. Sera were stored at -20ºC for serological analysis at the "Laboratório de Zoonoses Bacterianas" of the FMVZ-USP.

Laboratory analysis. A microscopic agglutination test (MAT) using live antigens Cole et al. 1973 was performed to measure binding antibody levels using cultures of 24 serovars of *Leptospira* maintained in EMJH (Ellinghausen McCullough Johnson Harris) medium as antigens (Table 1). Sera were diluted 1:50 in Sorensen buffered saline solution. Reactions were performed in polystyrene microplates with 50µl of antigen. All samples with agglutinant activity at a dilution of 1:100 (cutoff point) were considered positive and titrated in two-fold serial dilutions. The highest titer reached with at least 50% of agglutinated *Leptospira* was used as cut-off to identify the infective serogroup (Myers 1985).

Immunization. During the 2nd collection, the dogs were immunized with the polyvalent Biovet® vaccine, which contains a lysed concentrate of *Leptospira canicola, L. icterohaemorrhagiae, L. grippotyphosa, L. copenhageni* and *L. pomona.* The vaccine was administered according to the manufacturer's recommendations to all clinically healthy dogs over 4 months old.

Data analysis. Most probable serovar: when the animal presented seropositivity for more than one serovar, the serovar with the highest titer and with a dilution twice as high as that of the serovar with the second highest titer was considered. In cases where the animal presented more than one serovar having the highest titer at the same dilution or with a single dilution of difference, the result was considered undetermined, because it was not possible to confirm the causative agent of the infection, and was excluded.

The disease incidence was calculated for the 2nd and 3rd blood collections considering the population that remained throughout the currently and previous collection. Animals that were seropositive in two consecutive years but for different most probable serovars in each year were considered new cases. The annual seroprevalence of *Leptospira* spp. was calculated according to the numbers of seropositives by the total sampled population in each year.

Spatial analysis. To identify clusters of leptospirosis cases in dogs, a local spatio-temporal analysis was performed using the computational program SatScan® version 9.4.2. This estimate was evaluated according to the spatial scanning method developed by Kulldorff & Nagarwalla (1995), which considers qualitative data such as the geographical location of cases (dogs seropositive for *Leptospira* spp.) and controls (seronegative dogs) (Kulldorff & Nagarwalla 1995). For each cluster, we performed a likelihood ratio test comparing the hypothesis that the disease risk is higher within the circle with the hypothesis that the risk is equal for the areas inside and outside the circle, with a significance level of 5%.

The circle with the maximum likelihood ratio was considered the most likely cluster (Wheeler 2007). All maps were built using the QGIS program, version 2.18.

Statistical analysis. To compare results from groups according to age and sex, chi square test was performed using R software, version 3.5.0, considering significance level of 5%.

RESULTS

Over three years of the study, 189, 200 and 179 residences were visited. Differences in number of residences taken each year are due to unavailability of dog owners even after up to four attempts. Three owners refused to participate. There were collected blood of 331, 373 and 347 animals in each year, respectively, 166 were present in all years. Seventy-five percent of the residences had an area of less than 300m^2 and were in contact or close to paved streets (termed houses), 20% were rural properties larger than 300m^2 (termed country houses) and 5% were small properties located inside an agricultural farm (termed farm houses). At the 2nd and 3rd collections, some animals were not available for recollection, due to death or exit from the area. However, some new dogs were included in the studied population due to birth or entrance to the study area (Fig.2). One hundred sixty-six animals were sampled in all three years.

For the 1st, 2nd and 3rd collections, the seroprevalences were 10.9% (36/331, 95% CI 7.5-14.2), 7.2% (26/363, 95% CI 4.9-10.4) and 13.8% (48/347, 95% CI 10.2-17.5), respectively (Table 2). The incidence in the 2nd year (2016) was 10.13% (23/227, 95% CI 6.9-14.9) and in the 3rd year (2017) was 19.9% (95% CI 15.4-25.7). In the 2nd year, three animals were seropositive for the same serovar that they were seropositive for in the 1st year (and thus were not considered new cases), and in the 3rd year just one dog was seropositive for the same serovar as in the 2nd year.

Regarding the sex of the animals (Table 2), it was observed that over the two first years, much more males than females were infected; however, this difference was not statistically significant. Some owners did not collaborate in providing information.

Regarding the age of the animals (Table 2), in all years, the seropositivity was lower in young dogs (less than 1 year old). The seroprevalence was significantly higher for adult dogs (1-9 years old) than for old dogs (over 9 years old) at the 3rd collections.

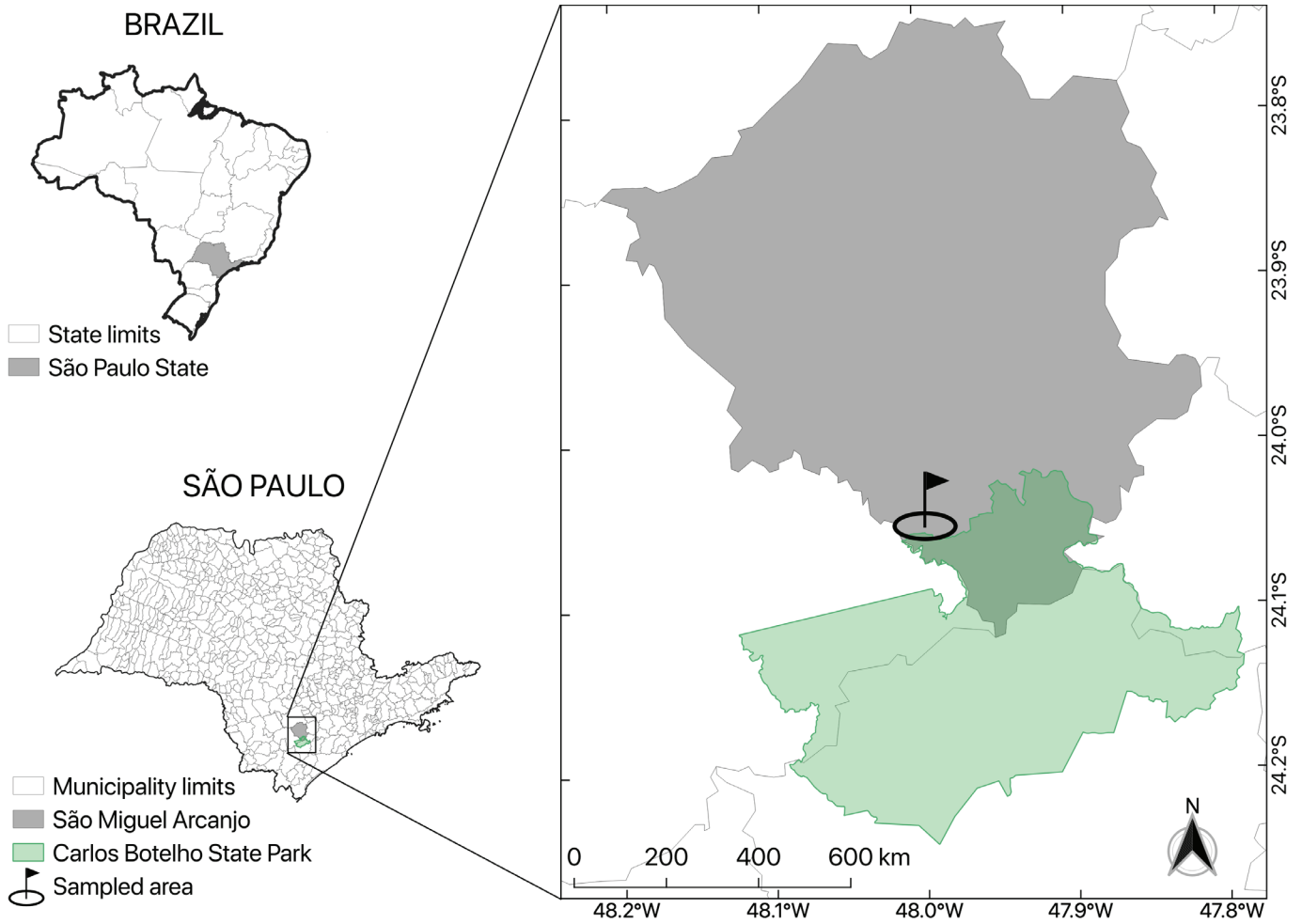


Fig 1. Residences in Abaitinga and Gaviões neighborhoods of São Miguel Arcanjo Municipality with dogs sampled for serological evaluation of *Leptospira* spp. in three sample collection periods.

In the 1st collection the most probable serovars were Cynopteri (71.4%, 25/35) and Butembo (14.3%, 5/35) and in the 2nd were Cynopteri (37.5%, 9/24) followed by Butembo (16.6%, 4/24) and Hardjoprajitno (16,6%, 4/24). In the 3rd collection, the most probable serovars were Canicola (53.6%, 22/41) and Butembo (14.6%, 6/41). Some of these positive animals at the 3rd sample collection have been vaccinated at the 2nd collection (Table 3). Eight seropositive samples were positive for an undetermined serovar.

When the geographic distribution of all seropositive animals for the serovar Canicola was evaluated, it was observed that these animals were not concentrated in a specific region included in the study (Fig.3). The distributions of the residences with the serological status of the dogs in each year are shown in Fig.4. It is important to highlight that if there was more than one dog at the residence, they overlap in the maps, since each point represents one household. Both seronegative and seropositive dogs are distributed over the whole study area. However, in 2017, a significant cluster was found (p=0.030) with a prevalence of 75% (6/8) and relative risk of 7.30 in an area where rural houses are near the CBSP (Fig.4C).

In the present study, few animals had high antibody titers (Fig.5 and 6): one had a high titer for Butembo (800) in the 1st collection; one had a high titer for Grippotyphosa (800) in the 2nd collection; and two had a high titer for Canicola (800 and 3200), one for Hebdomadis (1600), one for Pomona (1600) and one for Guaicura (1600) at the 3rd collection. More dogs presented antibody titer higher than 800 in the 3rd collection comparing with the other two, although this difference was not statistically significant (p>0.05).

Table 1. Serovars of live antigen strains of *Leptospira* used in microscopic agglutination test

Serogrup	Serologic variant
Australis	Australis
Australis	Bratislava
Sejroe	Guaicura
Autumnalis	Autumnalis
Autumnalis	Butembo
Ballum	Castellonis
Batavia	Bataviae
Canicola	Canicola
Celledoni	Whitcombi
Cynopteri	Cynopteri
Grippotyphosa	Grippotyphosa
Hebdomadis	Hebdomadis
Icterohaemorrhagiae	Copenhageni
Icterohaemorrhagiae	Icterohaemorrhagiae
Javanica	Javanica
Panama	Panama
Pomona	Pomona
Pyrogene	Pyrogenes
Sejroe	Hardjoprajitno
Shermani	Shermani
Tarassovi	Tarassovi
Pomona	Pomona
Hardjo	Bovis
Djasiman	Sentot

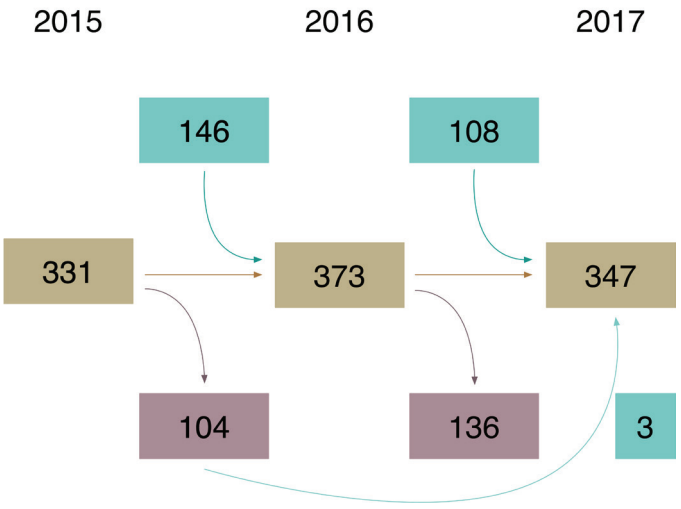


Fig 2. Population dynamic of sampled dogs at the 1st, 2nd and 3rd collection (years 2015, 2016 and 2017, respectively). Sampled dogs in each year (brown boxes), dogs that were not recollected in each year (wine boxes), new dogs included in each year (blue boxes).

Table 2. Seroprevalence of antibodies anti-*Leptospira* spp. in domestic dogs from the surroundings of Carlos Botelho State Park according to age and gender from 2015 to 2017

Age (years old)	2015					2016					2017				
	Pos	%	Neg	%	p-value	Pos	%	Neg	%	p-value	Pos	%	Neg	%	p-value
<1	2	5.6%	22	7.5%	0.399	2	7.7%	35	10.4%	0.194	2	4.2%	43	14.4%	0.043*
1-4	11	30.6%	99	33.6%		5	19.2%	101	30.0%		14	29.2%	87	29.1%	
≥4-9	11	30.6%	116	39.3%		13	50.0%	123	36.5%		19	39.6%	106	35.4%	
≥9	6	16.7%	25	8.5%		5	19.2%	30	8.9%		7	14.6%	17	5.7%	
WI	6		33			1		48			6		46		
Gender															
M	24	68.6%	154	52.9%	0.124	16	61.5%	164	49.0%	0.336	23	47.9%	151	51.0%	0.844
F	11	31.4%	137	47.1%		10	38.5%	171	51.0%		25	52.1%	145	49.0%	
WI	2		11			6		9			0		3		

Pos = positive, Neg = negative, WI = without information.

DISCUSSION

We found that more males than females were seropositive to *Leptospira*, however with no statistical significance. Rentko et al. (1992) found this result in dogs, and Andrade (2007) in wild animals which can be explained by the fact that males have the behavior of using larger areas to support reproduction (Andrade 2007).

In our study the seroprevalence was significantly higher for dogs from one to eight years old. Batista et al. (2005) and Sant'Anna et al. (2017) also found higher seroprevalence in dogs over one year old, suggesting that these animals have had more time of exposure to the infectious agent.

In Brazilian studies, it was observed that in Pelotas Municipality (Rio Grande do Sul State), domestic dogs presented a higher level of seropositivity for the Canicola and Icterohaemorrhagiae serovars in the urban zone (Furtado et al. 1997) and for the Icterohaemorrhagiae, Copenhageni, Australis and Canicola serovars in the rural zone (Brod et al. 2005). In Santana do Parnaíba Municipality (São Paulo State), the seroprevalence was 15%, with higher prevalence of Copenhageni, Canicola and Hardjo (Mascolli et al. 2002). In the same State, from 1984 to 1997, in 56.0% (14/24) of the municipalities studied, the seroprevalence in dogs was 17.9% (137/795), and the most likely serologic variants were Copenhageni and Icterohaemorrhagiae (Favero et al. 2002). In the area of the present study, the seroprevalence was lower than these others and only the serovar Australis was not identified.

Dogs are the main host of serovar Canicola (Rentko et al. 1992), and they can remain reservoirs for a long period of time (Furtado et al. 1997, Miotto et al. 2016). In non-vaccinated dog populations, the incidence of this serovar can reach 50% or 75% (Oliveira 2010). In the present study, this serovar was found at a high prevalence only for the 3rd collection (53.7%), but 91.0% of the seropositive dogs in that year were vaccinated one year earlier, and this seropositivity rate could be due to the vaccine response. According to Grosenbaugh and Pardo, 65% (13/20) of dogs seroconverted for this serovar post vaccine challenge, however titers declined to undetectable levels in all vaccinates 30 weeks after (Grosenbaugh & Pardo 2018). However, other authors found titers one year after vaccine challenge (Klaasen et al. 2003). The seropositivity for this serovar, in the present study, represents a low percentage (8.5%) of all 234 vaccinated animals that were reevaluated in 2017. It is important to consider that the vaccine is serovar-specific; that is, it only protects against infections caused by the homologous serovar or antigenically similar serovars (Oliveira 2010).

In addition, it could also be possible that serovar Canicola was present in the dog population before our immunization program but was not identified. Miotto et al. (2016) observed that dogs, even seronegative, eliminate the bacteria in the urine (Miotto et al. 2016). Another hypothesis for this increase in the seroprevalence of serovar Canicola is its entrance into this dog population between the 2nd and 3rd collection. There were only two dogs seropositive for Canicola who were not vaccinated, and these dogs were not present in the studied population before the 3rd collection. These dogs were adult dogs (5 and 15 years old) and lived in the center of the urban area of the municipality. One was a male who had free access to the street and lived in the same residence of another male seropositive for Cynopteri in the previous year. The other one was a female with no access to the street and with no dogs in the same residence.

The fact that the animals seropositive for Canicola were not concentrated in a specific geographic region of the study means that if an outbreak had occurred, it had already spread during the period between the two evaluations (2nd and 3rd). The possible spread of the Canicola serovar by the migration of dogs could be explained by the fact that many animals that live in the study area had free access to the street. A condition that can represent a risk factor for *Leptospira* infection in dogs (Querino Martins et al. 2003).

In the 3rd year, incidence and prevalence of leptospirosis in dogs were higher than those in the 2nd year. It was observed that among the new cases and vaccinated, 20 were caused by serovar Canicola one by Icterohaemorrhagiae and one by Grippotyphosa. If we exclude these vaccinated and seropositive animals we could consider a lower incidence, and comparing with the previous years could be justified by the effectiveness of the vaccine, however we cannot affirm that because the efficacy for these serovars are not ever observed.

Of all immunized dogs in our study 8.5% were seropositive for the serovar Canicola. Seroconversion to *Leptospira* serovar Grippotyphosa, Canicola and Icterohaemorrhagiae followed by vaccination was observed in a total of 50% (10/20), 65%

Table 3. Seropositive samples for the most probable serovars of *Leptospira* from 2015 to 2017

Serogroup	Serovars	2015	2016	2017 (vaccinated)	Total (%)
Cynopteri	Cynopteri	25	9	1	34 (32)
Canicola	Canicola	0	0	22 (20)	22 (21)
Autumnalis	Butembo	5	4	5	15 (14)
Sejroe	Hardjo (Hardjoprajitno)	0	4	4	8 (8)
Grippotyphosa	Grippotyphosa	2	1	4 (1)	7 (7)
Sejroe	Guaicura	2	1	2	4 (4)
Pomona	Pomona	0	3	1	4 (4)
Hebdomadis	Hebdomadis	0	0	2	2 (2)
Icterohaemorrhagiae	Copenhageni	0	1	1 (1)	2 (2)
Pyrogene	Pyrogenes	0	1	0	1 (1)
Ballum	Castellonis	1	0	0	1 (1)
TOTAL (%)		35 (10)	24 (6)	41 (13)	

(13/20) and 20% (4/20) respectively, and with a decrease after 30 weeks (Grosenbaugh & Pardo 2018).

The non-vacinated animals presented lower serological titers than the vaccinated animals, which was also found by Wilson et al. (2013). However, serologic data is not sufficient to differentiate vaccine response and infection, since in both cases the serological titers can vary widely, depending on the period after the exposure to the agent or immunization (Ahmad et al. 2005, Grosenbaugh & Pardo 2018). Thus, in the present study, it is not possible to confirm that the serovar Canicola was present at the area, different from the serovars Grippotyphosa and Icterohaemorrhagiae, which were detected prior to the vaccination.

On the other hand, if we consider the incidence increase in the 3rd collection as a result of the entrance of the serovar Canicola into the dog population, we could also consider that more frequent vaccination (every six months) could be a preventive measure for dogs and other hosts. Although vaccination doesn't prevent the infection, but rather reduces the

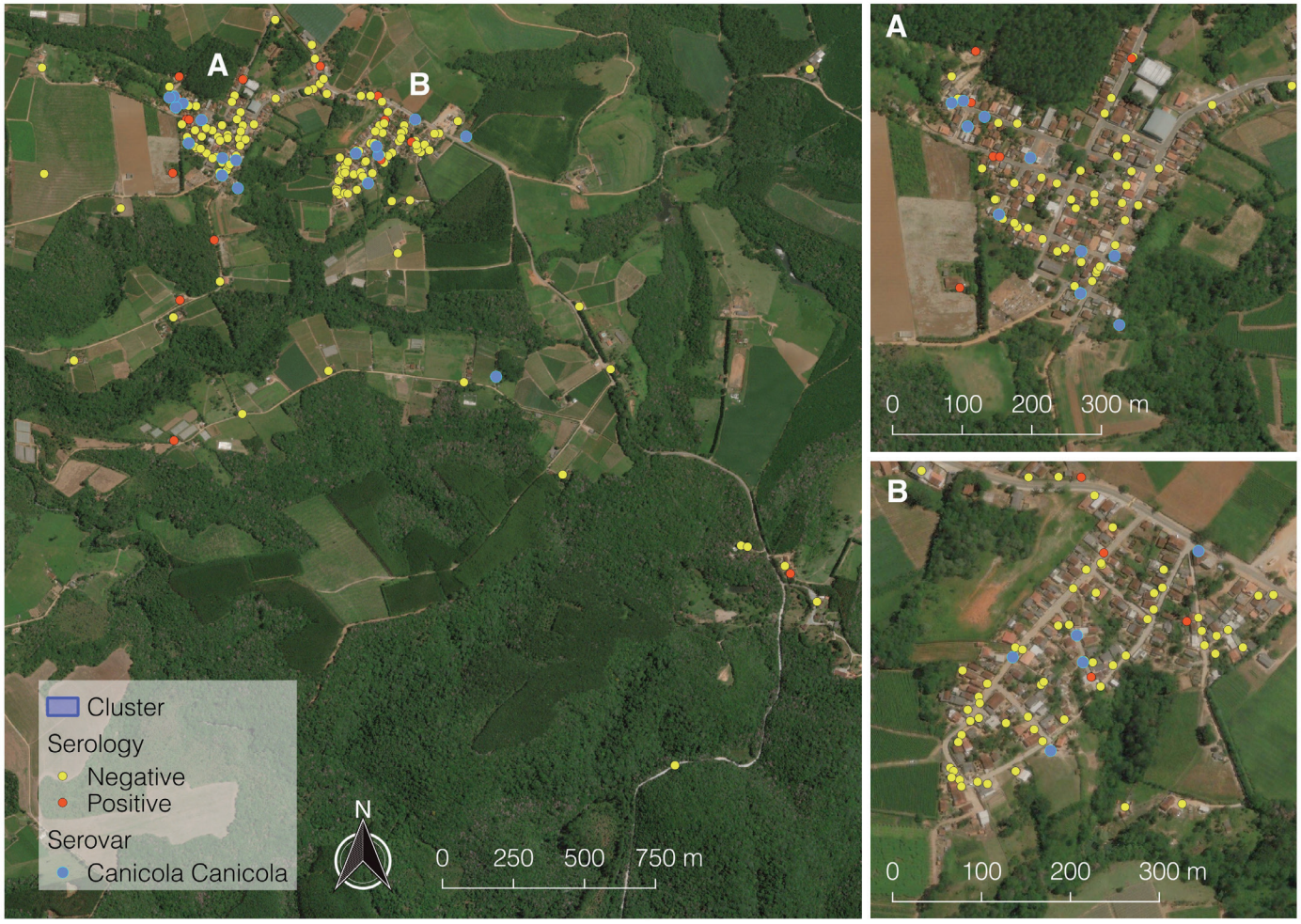


Fig.3. Seronegative and seropositive animals for serovar Canicola in 2017. Abaitinga (A), Gaviões (G). No cluster was found.

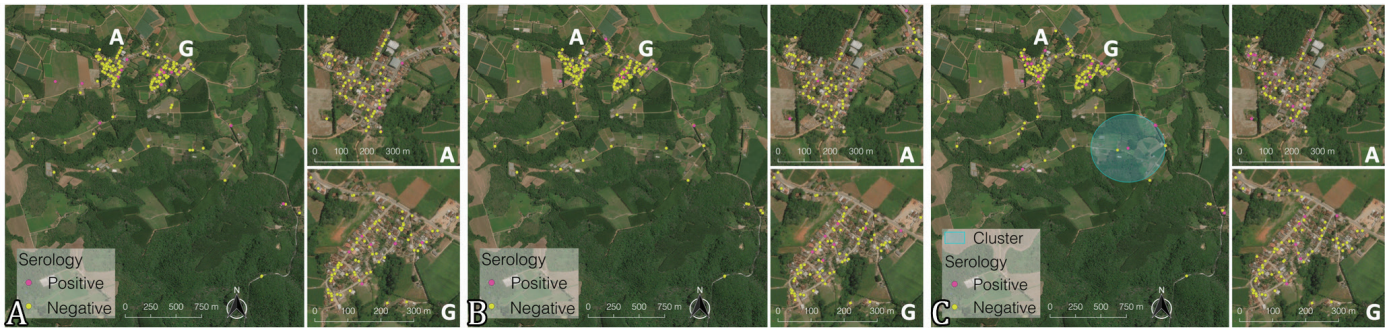


Fig 4. Residences with seronegative dogs and those with dogs seropositive for anti-*Leptospira* antibody. (**A**) At the 1st collection (2015), (**B**) at the 2nd collection (2016), (**C**) at the 3rd collection (2017). Abaitinga (A), Gaviões (G). The cluster found has 0.36km of radius.

level of shedding, respectively the likelihood of transmission (Polachini & Fujimori 2015).

Antibody titers equal to or greater than 800 indicate active infection (Grosenbaugh & Pardo 2018). In the present study, the majority of animals had a low titer; however, the agent could still be being eliminated in the environment, since dogs with low antibody titers.

The serogroup Icterohaemorrhagiae, which contains Copenhageni serovar, is most important in terms of public health, since it is more frequently related to human infections than other serovars (Nascimento et al. 2004). The sewer rat (*Rattus norvegicus*) is the most relevant host of the serogroup Icterohaemorrhagiae and is the main reservoir for humans (Nascimento et al. 2004). Although dogs are not considered to be the main reservoirs, they are frequently accidental hosts (Brod et al. 2005). In Brazil serovars in humans cases infected and hospitalized with leptospirosis from 1986 to 1989 in São Paulo State, Brazil, were Copenhageni (77.8%), and Canicola (11.1%) (Sakata et al. 1992).

In dogs, most of the studies also found high frequence of Icterohaemorrhagiae and Canicola (Scanziani et al. 1994, Favero et al. 2002, Mascolli et al. 2002, Jorge et al. 2017, Pinto et al. 2017). The present study found few infected dogs by Icterohaemorrhagiae.

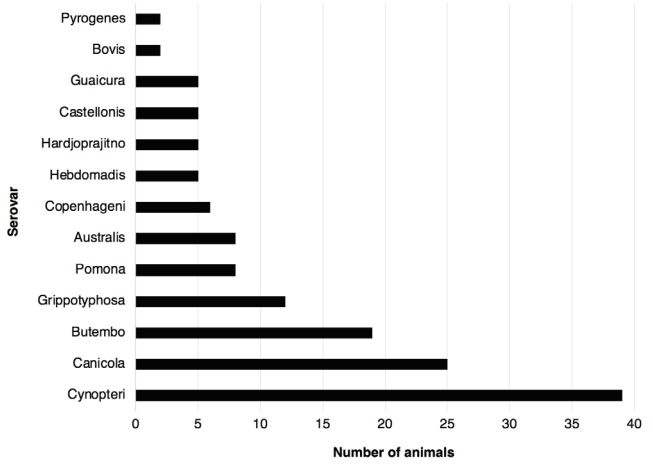


Fig.5. Serovar frequencies for all the three years of study.

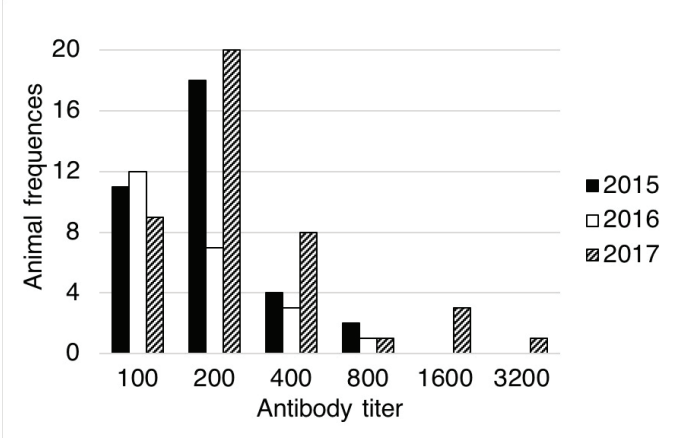


Fig.6. Number of animals and serologic titer variations by year. The median values of titers was 200 in 1st and 3rd collection and 100 at the 2nd collection.

Nascimento et al. (2004) mentioned that serogroups Pomona and Hardjo have a higher tropism for swine and cattle, respectively (Nascimento et al. 2004). That would not be a problem in the study area, since there were not many dogs seropositive for these serovars and they were geographically distant from the only property with cattle and the only one with swine. The specific *Leptospira* serovars involved in leptospirosis depend on both the geographic location and the ecology of local hosts (Levett 2001). One route of *Leptospira* transmission between domestic dogs, wild animals and humans is the contamination of water supplies with infected urine of the hosts. Infected water supplies have already resulted in notable outbreaks of leptospirosis in humans (Levett 2001). The entry of the agent into the host may be through skin lesions, conjunctive membranes or intact skin after prolonged immersion in water (Levett 2001). The contact of dogs with ponds, dams and small pools of water is the main risk factor for canine leptospirosis (Jouglard & Brod 2000). Thus, the dogs sampled can be considered as bio-indicators of the presence of the serovares in the study area.

Due to the kind of *Leptospira* transmission by contact, a high density of animals per residences could increases the chance of infection between them. During the year the average ratio of dogs per residence was increasing 1.75, 1.86 and 1.95 such as the incidence. However, only two years is not sufficient to affirm this positive association. There is a high flow of animals at that studied population, with entrance of animals at residences, which can affect in more level the contact between them, than change their density per residences through the period. In addition, the high rate of exit of animals can reduce the contact between them.

The control of Leptospirosis is based on blocking the transmission of the agent (Grosenbaugh & Pardo 2018). Specific methods can be applied according to the animal population and vaccine availability (Faine et al. 1999, Grosenbaugh & Pardo 2018). We suggest for the studied area vaccines campaigns for dogs. In addition, there are some domiciled dogs created freely, and, as well as stray dogs, they have access to both street and Park. Hence, they are able to urine in some areas where humans, sometimes barefoot, and wild and other domestic animals also frequent. An indirect way to control leptospirosis is controlling the stray dogs, by sterilization surgery campaigns, and promoting responsible ownership of domiciled dogs.

CONCLUSIONS

Leptospira spp. are present in the study region, and dogs with high antibody titers were found, mainly in the 3rd collection. The seroprevalence of *Leptospira* in dogs before and after immunization against *Leptospira* was evaluated in a naturally exposed population. The high levels of antibodies induced by vaccine can last for months up to a year. The dog seropositivity for serovars protected by vaccine in the 3rd collection, higher than the previous ones, is not necessarily a result of vaccine protection. Therefore, the seropositive dogs found for these serovars can suggest the need of more frequent application intervals of the dog vaccine, as an efficient measure to prevent and control the disease in domestic dogs and consequently in humans and wild animals in the area.

The serovars found in the dogs of this study can also infect humans. So, there is the possibility of a two-way transmission route. Therefore, stimulating the responsible ownership to

control dog population size, maintain the health of the dogs and vaccinate the dogs against *Leptospira* can assist in disease prevention in the study area.

The results presented here can contribute to other studies related to the understanding of the leptospirosis dynamic and transmission among different hosts. Analyzing the disease in the dog population, before and after vaccination, consequently helps to plan prevention and control measures to be carried out by public authorities and dog owners.

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Conflict of interest statement.- The authors declare that they have no conflict of interest.

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Colégio Brasileiro de Patologia Animal

Em 15 de agosto de 2019

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Exemplos de Referências

➢ Artigos publicados em periódicos:

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Rondelli L.A.S., Silva G.S., Bezerra K.S., Rondelli A.L.H., Lima S.R., Furlan F.H., Pescador C.A. & Colodel E.M. 2017. Doenças de bovinos no Estado de Mato Grosso diagnosticadas no Laboratório de Patologia Veterinária da UFMT (2005-2014). Pesq. Vet. Bras. 37(5):432440.

Hooiveld M., Smit L.A., Wouters I.M., Van Dijk C.E., Spreeuwenberg P., Heederik D.J. & Yzermans C.J. 2016. Doctor-diagnosed health problems in a region with a high density of concentrated animal feeding operations: a cross-sectional study. Environ. Health 17:15-24.

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(Notem: Os iniciais dos autores devem ser colocados sem espaço. O sinal "&" é usado para separar o penúltimo do último autor. As primeiras letras das palavras do título de artigos publicados em periódicos científicos devem ser de preferência minúsculas. A palavra "Revista" deve ser abreviada como "Revta" em diferença a "Rev.", do inglês "Review". Deve-se indicar o número do respectivo volume do periódico e, se possível, também do fascículo. Somente abreviações tem um ponto, exceto as que terminam com a última letra da palavra em extenso. O traço entre as páginas é curto (-) e não comprido. Não devem ser usados "pontovírgulas" (;) em lugar de vírgulas.

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Marsh P. & Martin M. 1992. Oral Microbiology. 3rd ed. Chapman and Hall, London, p.167-196.

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Tokarnia C.H., Brito M.F., Barbosa J.D., Peixoto P.V. & Döbereiner J. 2012. Plantas que afetam o funcionamento do coração, p.27-94. In: Ibid. (Eds), Plantas Tóxicas do Brasil para Animais de Produção. 2ª ed. Helianthus, Rio de Janeiro.

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- Dissertações e Teses:

Rech R.R. 2007. Alterações no encéfalo de bovinos submetidos à vigilância das encefalopatias espongiformes transmissíveis. Tese de Doutorado, Universidade Federal de Santa Maria, Santa Maria. 228p.

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Mendonça F.S., Almeida V.M., Albuquerque R.F., Chaves H.A.S., Silva Filho G.B., Braga T.C., Lemos B.O. & Riet Correa F. 2016. Paralisia laríngea associada à deficiência de cobre em caprinos no semiárido de Pernambuco (IX Endivet, Salvador, BA). Pesq. Vet. Bras. 36(Supl.2):50-51. (Resumo)

Pierezan F., Lemos R.A.A., Rech R.R., Rissi D.R., Kommers G.D., Cortada V.C.L.M., Mori A.E. & Barros C.S.L. 2007. Raiva em equinos. Anais XIII Encontro Nacional de Patologia Veterinária, Campo Grande, MS, p.145-146. (Resumo)

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Exemples for References:

➢ Articles published in scientific journals:

Ubiali D.G., Cruz R.A., De Paula D.A., Silva M.C., Mendonça F.S., Dutra V., Nakazato L., Colodel E.M. & Pescador C.A. 2013. Pathology of nasal infection caused by *Conidiobolus lamprauges* and *Pythium insidiosum* in sheep. J. Comp. Pathol. 149(2/3):137-145.

Hooiveld M., Smit L.A., Wouters I.M., Van Dijk C.E., Spreeuwenberg P., Heederik D.J. & Yzermans C.J. 2016. Doctor-diagnosed health problems in a region with a high density of concentrated animal feeding operations: a cross-sectional study. Environ. Health 17:15-24.

(Note: The first letters of the words in the title of papers published in journals are small. It is preferable to indicate the number of the respective issue.)

➢ Books:

Marsh P. & Martin M. 1992. Oral Microbiology. 3rd ed. Chapman and Hall, London, p.167-196.

Tokarnia C.H., Brito M.F., Barbosa J.D., Peixoto P.V. & Döbereiner J. 2012. Plantas Tóxicas do Brasil para Animais de Produção. 2ª ed. Helianthus, Rio de Janeiro, p.305-348.

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➢ Chapters of books:

Uzal F.A., Plattner B.L. & Hostetter J.M. 2016. Alimentary system, p.1-257. In: Maxie M.G. (Ed.), Jubb, Kennedy and Palmer´s Pathology of Domestic Animals. Vol.2. 6th ed. Elsevier, St Louis, Missouri.

Barros C.S.L. 2007. Doenças víricas: leucose bovina, p.159-169. In: Riet-Correa F., Schild A.L., Lemos R.A.A. & Borges J.R.J. (Eds), Doenças de Ruminantes e Equídeos. Vol.1. 3ª ed. Pallotti, Santa Maria, RS.

Tokarnia C.H., Brito M.F., Barbosa J.D., Peixoto P.V. & Döbereiner J. 2012. Plantas que afetam o funcionamento do coração, p.27-94. In: Ibid. (Eds), Plantas Tóxicas do Brasil para Animais de Produção. 2ª ed. Helianthus, Rio de Janeiro.

➢ Dissertations and Theses:

Rech R.R. 2007. Alterações no encéfalo de bovinos submetidos à vigilância das encefalopatias espongiformes transmissíveis. Tese de Doutorado, Universidade Federal de Santa Maria, Santa Maria. 228p.

(Note: Use articles which originated from dissertations or theses instead of these).

➢ Abstracts published in Events:

Massa A.T., Potter K.A. & Bradway D. 2016. Epizootic bovine abortion outbreak in Eastern Nevada cattle. Annual Meeting American College of Veterinary Pathologist (ACVP), New Orleans, Lousiana. (Abstract D-50)

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Mendonça F.S., Almeida V.M., Albuquerque R.F., Chaves H.A.S., Silva Filho G.B., Braga T.C., Lemos B.O. & Riet Correa F. 2016. Paralisia laríngea associada à deficiência de cobre em caprinos no semiárido de Pernambuco (IX Endivet, Salvador, BA). Pesq. Vet. Bras. 36(Supl.2):50-51. (Resumo)

Pierezan F., Lemos R.A.A., Rech R.R., Rissi D.R., Kommers G.D., Cortada V.C.L.M., Mori A.E. & Barros C.S.L. 2007. Raiva em equinos. Anais XIII Encontro Nacional de Patologia Veterinária, Campo Grande, MS, p.145-146. (Resumo)

(Note: Consult entire papers instead of only Abstracts)

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