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INFECÇÃO POR *Sarcocystis* EM BÚFALOS NO ESTADO DO RIO GRANDE DO SUL, BRASIL

Santa Maria, RS, Brasil
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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Sanidade e Reprodução Animal, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutor em Medicina Veterinária**

Orientador: Fernanda Silveira Flores Vogel

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Santa Maria, RS.
2019

RESUMO

INFECÇÃO POR *Sarcocystis* EM BÚFALOS NO ESTADO DO RIO GRANDE DO SUL, BRASIL

AUTOR: Luiza Pires Portella

ORIENTADOR: Fernanda Silveira Flores Vogel

Os testes sorológicos são frequentemente utilizados para detecção de anticorpos e são úteis tanto para avaliação de um grande número de amostras como para diagnóstico em animais vivos. Exame direto e testes moleculares proporcionam a detecção e caracterização de espécies envolvidas nas infecções, porém necessitam de tecidos-alvo, como o miocárdio, para que sejam realizados, sendo possível então, apenas após a morte dos animais infectados. Portanto, os objetivos deste estudo foram: (1) verificar a presença de anticorpos contra *N. caninum*, *T. gondii* e *Sarcocystis* spp. em búfalos do Rio Grande do Sul e (2) esclarecer a infecção de búfalos por *Sarcocystis*, bem como realizar a identificação molecular de espécies que acometem esses animais no Estado. No Capítulo 1 desta tese apresenta-se um estudo no qual amostras de soro de 220 bubalinos foram analisadas para presença de anticorpos, através de reação de imunofluorescência indireta (RIFI). Foram consideradas positivas as amostras que apresentaram títulos de anticorpos maiores ou iguais a 100, para os protozoários estudados. Um total de 60,5% (133/220) das amostras sorológicas dos búfalos foram positivas para pelo menos um dos parasitas pesquisados. Anticorpos para *N. caninum*, *Sarcocystis* spp. e *T. gondii* foram encontrados em 36,4% (80/220); 25,5% (56/220) e 16,8% (37/220) dos búfalos respectivamente, indicando que houve uma maior frequência de infecção de *N. caninum* em relação aos demais protozoários (p=0.0133). No estudo apresentado no Capítulo 2 foram coletadas amostras de miocárdio 80 búfalos submetidos a exame microscópico, seguido de análise molecular. Microcistos estavam presentes em 23,75% (19 de 80) das amostras. O DNA genômico foi extraído dos 19 isolados e submetido à PCR. Seis sequências legíveis foram obtidas após o sequenciamento em ambas as direções, todas as quais todas eram de *Sarcocystis levinei.*

Palavras-chave: *Sarcocystis,* diagnóstico sorológico, diagnóstico molecular, análise filogenética.

ABSTRACT

Sarcocystis INFECTION IN BUFFALOES IN THE STATE OF RIO GRANDE DO SUL, BRAZIL

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Serological tests are often used for the detection of antibodies and are useful both for the evaluation of large numbers of samples and for diagnosis in live animals. Direct examination and molecular testing provide the detection and characterization of species involved in infections but require target tissues, such as the myocardium, to be carried out only after the death of the infected animals. Therefore, the objectives of this study were: (1) to verify the presence of antibodies against *N. caninum, T. gondii* and *Sarcocystis* spp. in buffalo from Rio Grande do Sul and (2) to clarify the infection of buffalo by *Sarcocystis*, as well as to carry out the molecular identification of species that affect these animals in the State. In Chapter 1 of this thesis is presented a study in which serum samples of 220 buffaloes were analyzed for the presence of antibodies, through indirect immunofluorescence reaction (IFAT). Samples with antibody titers greater than or equal to 100 were considered positive for the protozoa studied. A total of 60.5% (133/220) of the buffalo serological samples were positive for at least one of the parasites surveyed. Antibodies to *N. caninum, Sarcocystis* spp. and *T. gondii* were found in 36.4% (80/220); 25.5% (56/220) and 16.8% (37/220) of the buffalo, respectively, indicating a higher frequency of *N. caninum* infection than the other protozoa ($p = 0.0133$). In the study presented in Chapter 2, myocardial samples were collected 80 buffaloes submitted to microscopic examination, followed by molecular analysis. Microcysts were present in 23.75% (19 of 80) of the samples. Genomic DNA was extracted from the 19 isolates and submitted to PCR. Six readable sequences were obtained after sequencing in both directions, all of which were from *Sarcocystis levinei*.

Key words: *Sarcocystis,* serological diagnosis, molecular diagnosis, phylogenetic analysis.

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1 – INTRODUÇÃO

O gênero *Sarcocystis* pertence ao filo Apicomplexa e à família Sarcocystidae. (PESCADOR et al., 2007). Esse filo caracteriza-se por apresentar protozoários intracelulares obrigatórios que apresentam um ciclo biológico onde se alternam a reprodução sexuada e a reprodução assexuada. Caracterizam-se ainda por possuírem no polo anterior de seu corpo alongado, uma estrutura celular chamada de complexo apical, utilizada para a penetração nas células dos seus hospedeiros (REYS, 2008).

Os parasitas do gênero *Sarcocystis* são protozoários heteroxenos obrigatórios que apresentam ciclo de vida consistindo nas seguintes fases: esquizogonia, gametogonia e esporogonia. Sendo assim, são necessários tanto hospedeiros intermediários como hospedeiros definitivos para completarem seu ciclo (DUBEY, 1976; TENTER, 1995).

No hospedeiro intermediário se formam sarcocistos no tecido muscular que servem de forma infectante para o hospedeiro definitivo, onde evoluem diretamente para gametas no intestino. Outra característica desse gênero é que a esporogonia dos oocistos ocorre no intestino do hospedeiro definitivo com a excreção dos oocistos esporulados ou de esporocistos nas fezes (NEVES, 2005; REYS, 2008; STELMANN & AMORIM, 2010).

Os hospedeiros intermediários se infectam ao ingerir esporocistos no alimento ou na água; esses esporocistos passam por, pelo menos, três gerações assexuadas. Na primeira, esporozoítos liberados dos esporocistos, invadem a parede intestinal e penetram nos capilares onde permanecem nas células endoteliais realizando dois ciclos esquizogônicos. O terceiro ciclo assexuado ocorre em linfócitos circulantes, produzindo merozoítos que penetram nas células musculares. Os merozoítos liberados iniciam a formação do cisto que são encontrados na musculatura estriada esquelética e cardíaca, bem como no sistema nervoso central e em fibras de Purkinje no coração dos hospedeiros intermediários (POWELL et al., 1986; DUBEY et al., 1989). O protozoário se encista dando origem a bradizoítos contidos nos cistos, que são os *Sarcocystis* maduros, estágios infectantes para os carnívoros (TAYLOR et al., 2007).

Nem todas as espécies causam doenças em seus hospedeiros, geralmente, aquelas que usam canídeos como hospedeiros definitivos são mais patogênicas do que aquelas

que usam felinos. A patogenicidade das espécies manifesta-se usualmente no hospedeiro intermediário, e de maneira geral não causa doença em hospedeiros definitivos (DUBEY & LINSDAY, 2006).

Os cistos variam em tamanho e forma, dependendo da espécie do parasita. Alguns sempre permanecem microscópicos, enquanto outros se tornam macroscópicos. Os cistos microscópicos variam de longos e estreitos a curtos e largos, enquanto os macroscópicos aparecem filamentosos, tipo grãos de arroz, ou globulares (DUBEY, 1992).

A infecção por sarcocistos microscópicos geralmente é assintomática e esses microcistos não são detectáveis pelas técnicas rotineiramente utilizadas em inspeção da carne em frigoríficos e o mesmo animal pode estar infectado por múltiplas espécies de *Sarcocystis* (LOPES, 2004; MORÉ et al., 2013, 2014), algumas delas não são morfologicamente distinguíveis, exceto através da microscopia de transmissão eletrônica (GJERDE et al., 2016), mas esta não é aplicável para diagnóstico de uma grande quantidade de amostras. Com isso, a identificação de *Sarcocystis* spp., em carnes destinadas a consumo humano através de métodos moleculares é essencial no diagnóstico e consequentemente no que diz respeito à saúde pública e produção (MORÉ et al., 2011; BUCCA et al.,2011).

A patogenia da sarcocistose é mais bem conhecida nos animais de produção, uma vez que pode gerar perdas econômicas relevantes devido à condenação de carcaça, e está associada aos efeitos dos esquizontes nos vasos sanguíneos. Nessas espécies, a infecção pode causar anemia, anorexia, caquexia, encefalomielite, hemorragias, aborto e morte em casos de infestação massiva (DOMENIS et al., 2001; PESCADOR et al., 2007; RUAS et al., 2001).

O gênero *Sarcocystis* compreende grande variedade de espécies que geralmente possuem características morfológicas distintas e especificidade aos hospedeiros. No entanto, há algumas exceções em que não é possível estabelecer distinção morfológica entre as espécies (MATUSHIMA et al., 2009; TENTER & HECKEROTH, 1999), ou mais de uma espécie é capaz de infectar o mesmo hospedeiro (MATUSHIMA et al., 2009).

Fatores esses que dificultam um diagnóstico específico da espécie do parasito presente em seus hospedeiros. Para melhor identificação das espécies de *Sarcocystis*,

métodos de diagnóstico molecular como as técnicas de reação em cadeia pela polimerase (PCR), nested-PCR, polimorfismo do comprimento de fragmentos de restrição (RFLP) e sequenciamento, entre outros, são empregados (DOMENIS et al., 2011; DUBEY et al., 2010; OLIAS et al., 2010; PRAKAS et al., 2011). Esses métodos de detecção do parasito visam superar as dificuldades encontradas nos métodos diagnósticos comuns. As técnicas são aplicadas no estudo da sistemática, em particular na resolução do problema taxonômico, em ecologia dos parasitos, evolução biológica, genética de população, estrutura de comunidades, epidemiologia e interação com seus hospedeiros, melhorando o entendimento da relação parasito hospedeiro (DOLEZEL et al., 1999; MUDRIDGE et al., 2000;).

A bubalinocultura é uma alternativa na produção animal, visto que os búfalos são animais que possuem múltiplas funções: dentre elas temos a produção de carne, leite e tração, e essa última é uma prática comum em muitos países (OLIVEIRA et al., 2013). No Brasil as criações de búfalos visam principalmente à produção de carne e leite (OLIVEIRA et al., 2013).

Existem várias doenças que podem interferir na produção e na qualidade sanitária da reprodução, do leite e da carne (OLIVEIRA, 2006). Dessa forma o manejo sanitário na produção animal tem a finalidade de impedir que doenças interfiram no desempenho produtivo e reprodutivo do rebanho, e consequentemente perdas econômicas (FUJII, 2001)

Quatro espécies de *Sarcocystis* já foram relatadas RM búfalos, sendo elas, *S. fusiformis, S.buffalonis, S.levenei e S.dubeyi* (DISSANAIKE & KAN 1978; HUONG et al., 1997; DUBEY et al., 2014). *S. fusiformis* e *S. buffalonis* formam cistos macroscópicos e apresentam felídeos como hospedeiros definitivos enquanto a espécie *S. levinei* formam cistos microscópicos e apresentam canídeos como hospedeiros definitivos. Em relação ao *S. dubeyi*, esta espécie também forma microcistos porém o hospedeiro definitivo ainda não foi identificado (ORYAN et al., 2010).

Tendo em vista a importância da infecção pelo *Sarcocystis* em animais domésticos e, somado ao fato que no Brasil não existem estudos que demonstrem a infecção por *Sarcocystis* em búfalos, bem como as espécies envolvidas na infecção, este trabalho foi desenvolvido com o intuito de: i) verificar a presença de anticorpos *contra N. caninum*,

T. gondii e Sarcocystis spp. em búfalos do Rio Grande do Sul. ii) isolar e caracterizar molecularmente as espécies de *Sarcocystis* que infectam búfalos. Esta tese está dividida em dois capítulos. Sendo o primeiro capítulo intitulado: “Antibodies against *Neospora caninum*, *Sarcocystis* spp. and *Toxoplasma gondii* detected in buffaloes from Rio Grande do Sul, Brazil. ” e o segundo: “First molecular detection and characterization of natural *Sarcocystis* infection among buffaloes in Rio Grande do Sul, Brazil ”.

2– CAPÍTULO 1 - Artigo científico

Este capítulo originou um artigo científico que foi publicado na revista: Pesquisa Veterinária Brasileira.

Antibodies against *Neospora caninum*, *Sarcocystis* spp. and *Toxoplasma gondii* detected in buffaloes from Rio Grande do Sul, Brazil[1]

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2 Laboratório de Doenças Parasitárias, Departamento de Medicina Veterinária Preventiva (DMVP), Universidade Federal de Santa Maria (UFSM), Santa Maria, RS 97105-900, Brazil. *Corresponding author: fefevogel@ gmail.com

3 Laboratório de Virologia, Departamento de Veterinária Preventiva (DVP), Universidade Federal de Pelotas (UFPel), Campus Porto, Pelotas, RS 96010-610, Brazil.

ABSTRACT. - Portella L.P., Cadore G.C., Lima M., Sangioni L.A., Fischer G. & Vogel F.S.F. 2016. **Antibodies against *Neospora caninum*, *Sarcocystis* spp. and *Toxoplasma gondii* detected in buffaloes from Rio Grande do Sul, Brazil.** *Pesquisa Veterinária Brasileira 36(10):947-950*. Laboratório de Doenças Parasitárias, Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria, Camobi, Santa Maria, RS 97105-900, Brazil. E-mail: fefevogel@gmail.com. The presence of antibodies against *Neospora caninum*, *Sarcocystis* spp. and *Toxoplasma gondii* was evaluated in buffaloes (*Bubalus bubalis*) from Rio Grande do Sul state (RS), southern Brazil. Serum samples (n=220) were analyzed for antibodies by indirect fluorescent antibody test (IFAT). Antibody presence was considered when the titers were equal or higher than 100 for these protozoa. A total of 60.5% (133/220) buffalo serum samples were positive for at least one of the protozoa evaluated in this study. Antibodies for *N. caninum*, *Sarcocystis* spp. and *T. gondii* were found in 36.4% (80/220), 25.5% (56/220) and 16.8% (37/220) of the buffaloes respectively, indicating a higher frequency of *N. caninum* infection (p=0.0133). The IFAT is a suitable method to diagnose *N. caninum*, *Sarcocystis* spp. And

T. gondii infection in buffaloes for detecting IgG antibodies. This study demonstrates the presence of these three protozoa in buffalo herds in RS, Brazil, which may be source of infection to other animals. The high frequency of animals positive for *N. caninum* is important and could be related to reproductive problems. Additionally, the presence of *Sarcocystis* spp. and *T. gondii* in buffaloes can be a possible public health issue.

INDEX TERMS: Antibodies, *Neospora caninum*, *Sarcocystis* spp., *Toxoplasma gondii*, IFAT, protozoa, Apicomplexa, Sarcocystidae, *Bubalus bubalis*, buffaloes, Rio Grande do Sul, Brazil.

RESUMO. - [**Anticorpos contra *Neospora caninum*, *Sarcocystis* spp. e *Toxoplasma gondii* detectados em búfalos no Rio Grande do Sul.**] A presença de anticorpos contra *Neospora caninum*, *Sarcocystis* spp. e *Toxoplasma gondii* foi avaliada em búfalos (*Bubalus bubalis*) no estado do Rio Grande do Sul (RS), Região Sul do Brasil. Amostras de soro de 220 bubalinos foi analisada para presença de anticorpos, através de reação de imunofluorescência indireta (RIFI). Foram consideradas positivas as amostras que apresentaram títulos de anticorpos maiores ou iguais a 100, para os protozoários estudados. Um total de 60,5% (133/220) das amostras sorológicas dos búfalos foram positivas para pelo menos um dos parasitas pesquisados. Anticorpos para *N. caninum*, *Sarcocystis* spp. e *T. gondii* foram encontrados em 36,4% (80/220); 25,5% (56/220) e 16,8% (37/220) dos búfalos respectivamente, indicando que houve uma maior frequência de infecção de *N. caninum* em relação aos demais protozoários (p=0.0133). A RIFI é um método adequado para o diagnóstico sorológico da infecção por *N. caninum*, *Sarcocystis* spp. e *T. gondii* em búfalos. Este estudo demonstrou a presença destes três protozoários em bubalinos no RS, Brasil, que pode ser fonte de infecção para outros animais. A elevada ocorrência de animais positivos para *N. caninum* é importante e pode estar relacionada a problemas reprodutivos. Adicionalmente, a presença de *Sarcocystis* spp. e *T. gondii* em búfalos, pode significar um possível problema de saúde pública.

TERMOS DE INDEXAÇÃO: Anticorpos, *Neospora caninum*, *Sarcocystis* spp., *Toxoplasma gondii*, RIFI, protozoários, Apicomplexa, Sarcocystidae, *Bubalus bubalis*, búfalos, Rio Grande do Sul.

INTRODUCTION

Buffaloes are fairly healthy animals even though live in hot and humid regions that are favorable to the development of illness. They are susceptible to most diseases, specially infectious and parasitic, that similarly affect cattle. However, the effects of these is eases in buffaloes are less deleterious than in bovine (Michelizzi et al. 2010). *Sarcocystis* spp., *Neospora caninum* and *Toxoplasma gondii* are Apicomplexa protozoa that have worldwide distribution andrequire an intermediate and a definitive host to complete the life cycle and also have been reported in buffaloes (Dubey et al. 1998, Silva et al. 2010, Konrad et al. 2013). Ruminants can be infected by these parasites through ingesting of the sporocysts or sporulated oocysts present in food or water (Dubey & Lindsay 2006).

Antibodies against *N. caninum* and *T. gondii* have been show in buffaloes in various countries, nevertheless in Southern Brazil the evidence of these protozoa are still scarce. Seroprevalence studies for *N. caninum* in buffaloes showed a positivity ranging from 1.5% to 70.9% and are described in southeastern and northern region of Brazil (Fujii et al. 2001, Gennari et al. 2005), Argentina (Campero et al. 2007, Konrad et al. 2013), Egypt (Dubey et al. 1998), India (Meenakshi et al. 2007), Vietnam (Huong et al. 1998), and Italy (Guarino et al. 2000). Seroprevalence to *T. gondii* are described in Brazil (Silva et al. 2010), Argentina (Konrad et al. 2013), Vietnan (Huong et al. 1998), Iran (Navidpour & Hoghooghi-Rad 1998), and India (Sharma et al. 2008) with the seropositivity ranging from 1.1% to 25.4%. Studies showing the seroprevalence for *Sarcocystis* spp. in buffaloes they are rare, but in Argentina Konrad et al. (2013) reported an occurrence of 50.8% of seropositive animals for this protozoan.

T. gondii and *N. caninum* are parasites that play important roles as cause of abortions and congenital diseases in ruminants (Uggla & Buxton 1990, Buxton 2014). Infections by *Sarcocystis* spp. affect ruminants but are frequently asymptomatic. Buffaloes are intermediate hosts for *Sarcocystis fusiformis*, *Sarcocystis levinei*, *Sarcocystis dubeyi*, *Sarcocystis sinensis* and *Sarcocystis buffalonis* (Dubey et al. 2014). Toxoplasmosis and sarcocystosis are important zoonoses transmitted to the humans, beings by ingestion of raw meat from intermediate hosts, including the buffaloes (Fayer 2004, Jones & Dubey 2010). The aim of this research was to verify the presence of

antibodies against *N. caninum*, *T. gondii* and *Sarcocystis* spp. in buffaloes from Rio Grande do Sul, Southern Brazil.

MATERIALS AND METHODS

Blood samples were collected for convenience from 220 healthy buffaloes (*Bubalus bubalis*) at a slaughterhouse from Pelotas, Rio Grande do Sul, Brazil. The samples were collected between May and July 2014. The buffaloes were of both genders, Mediterranean breed, aging 2 to 8 years and raised on extensive system, and originated from different parts of RS. Blood was centrifuged for 10 minutes at 1000g and serum was stored at-20°C until analysis by indirect fluorescence antibody test (IFAT). For detection of antibodies, *Sarcocystis neurona* (strain SN-37R) merozoites were used as antigen cultivated in CV-1 cells (African Green Monkey kidney cells). *Neospora caninum* (strain NC-1) and *Toxoplasma gondii* (strain RH) tachyzoites were maintained in VERO cells (African Green Monkey kidney cells). The cell cultures were maintained in RPMI 1640 culture medium (Invitrogen, Brazil), supplemented with 10% fetal bovine serum (Nutricell, Brazil) under 5% CO2 at 37°C.

Serum samples were diluted in PBS at 1:100 (Konrad et al. 2013) positive and negative buffalo serum was used as control for all protozoa tested. Commercial fluorescein-labeled anti-bovine IgG© (Goat Anti-Bovine IgG FITC®, 160A, Southern Biotech, Oxmoor Blvd, Birmingham, USA) was used as the secondary antibody. Slides were observed at 400x magnification under fluorescent microscope (Leica CTR 4000/EBQ 100, Leica Microsystems, Germany). Titers samples equal to 100 were considered positive for all parasites tested (Fig.1).

All data were analyzed using SAS software (SAS Institute Inc., Cary, NC) to statistical analysis. To evaluate the statistical frequencies of infected animals with different protozoa was used chi-square and Fisher exact test with a 95% confidence.

RESULTS AND DISCUSSION

Figure 2 shows *Neospora caninum*, *Sarcocystis* spp. and *Toxoplasma gondii* infections detected by IFAT in this study. A total 60.5% (133/220) of the buffalo serum samples were positive for at least one of the tested protozoa. Antibodies to *N. caninum*,

Sarcocystis spp. and to *T. gondii* were found in 80 (36.4%), 56 (25.5%) and 37 (16.8%) of the 220 buffaloes respectively, indicating a higher frequency of *N. caninum* infection (p=0.0133). Infection by the three protozoa was found in 1.4% (3/220) of the animals. However, *N. caninum* and *Sarcocystis* spp. were detected in 9.5% (21/220); *N. caninum* and *T. gondii* in 3.2% (7/220); and *Sarcocystis* spp. and *T. gondii* 2.7% (6/220). Statistical difference

was found in regard to infection of *N. caninum* associated with *Sarcocystis* spp. compared with other mixed infections (p=0.006). Presence of antibodies against these protozoa suggests that the buffaloes from RS were infected by these agents and they may be an important reservoir of these pathogens.

Generally, the difference of seropositive animals is variable, compared to other studies. The lack of standardization in the cut-off point of IFAT as well as the use of different diagnostic techniques complicates comparison with other studies (Dubey 2003). In this study were found antibodies to *N. caninum* in 36.4% of the serum samples from buffaloes.

The prevalence of antibodies against *N. caninum* in buffaloes was variable in the Brazilian regions. In Northern region of Brazil, were found 48.9% of 4796 buffaloes (Silva et al. 2014), in the Southeastern region of Brazil was found in 64% of 222 buffaloes (Fujii et al. 2001). Similar results to our study were found in Northeastern Brazil, with seropositivity 35.9% of 117 buffalo sera (Gondim et al. 2007). *N. caninum* infection has been reported in buffalo fetus which provides evidence of naturally occurring vertical infection (Chryssafidis et al. 2011), and exists reports indicating an increasing exposure to *N. caninum* infection in buffalo with age (Moore et al. 2014).

Studies evaluating antibody detection of *Sarcocystis* in buffaloes are rare, and in southern Brazil this is first report of exposure. The seropositivity to *Sarcocystis* spp. by IFATfound was of 25.5% (56/220). Sarcocystosis is widespread in livestock throughout the world, and most livestock species may harbor these species (Latif et al. 1999). Infection of *Sarcocystis* spp. in buffaloes have been reported in various countries and detected by different assays. The seroprevalence in Argentina was 50.8% detected by IFAT (Konrad et al. 2013). In Iran were detected 54.3% animals positive by ELISA (Ghorbanpoor et al. 2007), from Egypt was verified that 67.6 % and 63.6 % of the serum samples were seropositive to sarcocystosis by ELISA and indirect haemagglutination assay (IHA), respectively (Ashmawy et al. 2014). Furthermore, highest infection rates of

Sarcocystis spp. in buffaloes have been reported by using direct examination and histopathology (Oryan et al. 2010, El-Dakhly et al. 2011).

The occurrence of buffaloes positive to *T. gondii* may indicate a potential risk for the infection of humans. Given that unpasteurized buffalo milk and meat when consumed inadequately cooked from infected animals is a potential source of human toxoplasmosis (Lundén & Uggla 1992, Dehkordi et al. 2013). In Brazil, States of the Bahia (Gondim et al. 1999), Pará (Silva et al. 2010) and São Paulo (Souza et al. 2001) found that 3.8% (4/104), 1.1% (4/374) and 49.9% (205/411) of the buffaloes had antibodies to *T. gondii* respectively, lower results compared to 16.8% (37/220) of the buffaloes reported in this study. Similar results were described in Iran with 14.3% of the buffaloes seropositive to *T. gondii* (Hamidinejat et al. 2010). The toxoplasmosis transmission occurs often following ingestion of sporulated oocysts, or bradyzoites within cysts present in the tissues of numerous food animals (Dubey & Jones 2008) and products derived from these animals may be infective to man.

CONCLUSIONS

This study evidences the detection of IgG antibodies against *Neospora caninum*, *Sarcocystis* spp. and *Toxoplasma gondii* in buffalo herds in RS, Brazil. The high frequency of animals positive for *N. caninum* is important and could be related to reproductive problems. The presence of *Sarcocystis* spp. and *T. gondii* in buffaloes can be a possible public health issue.

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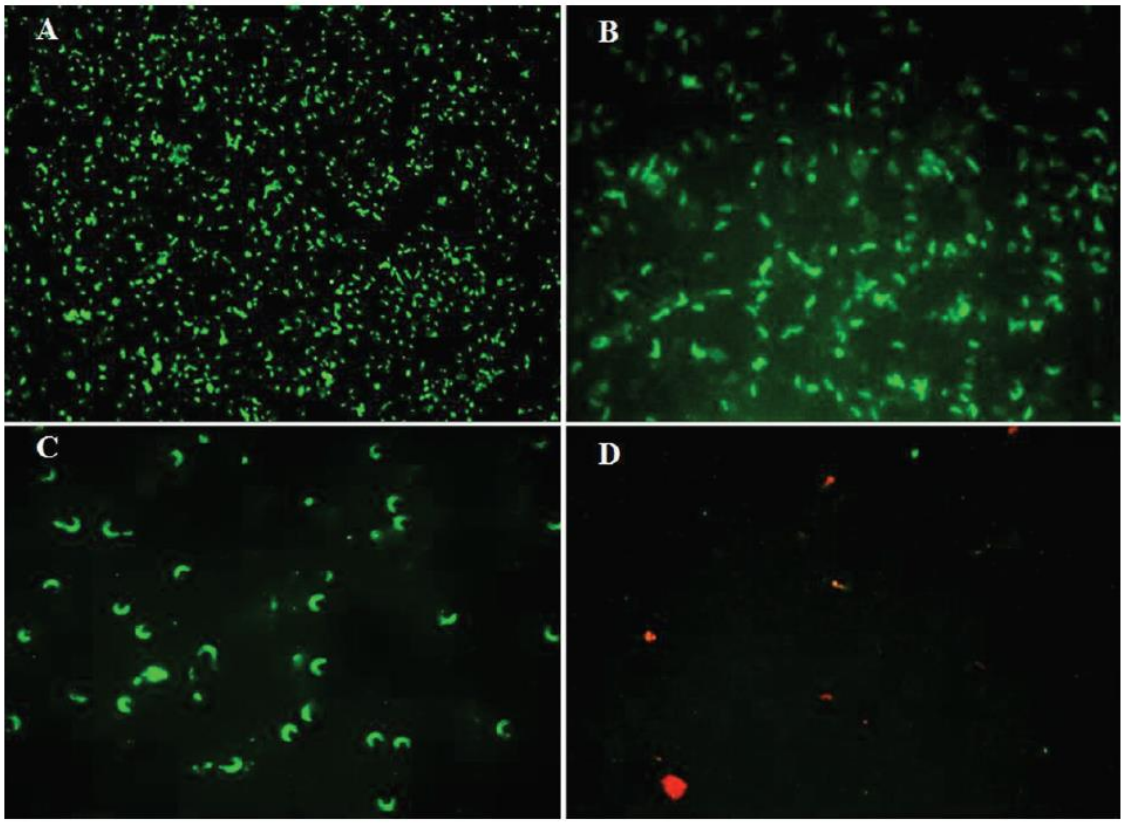

Fig.1. (**A**) Indirect fluorescence antibody test in buffaloes. Positive titer with tachyzoite of the *Neospora caninum.* 100x. (**B**) Positive titer with tachyzoite of the *Toxoplasma gondii.* 400x. (**C**) Positive titer with merozoites of *Sarcocystis* spp. 400x. (**D**) Negative control with absence of fluorescence. 400x.

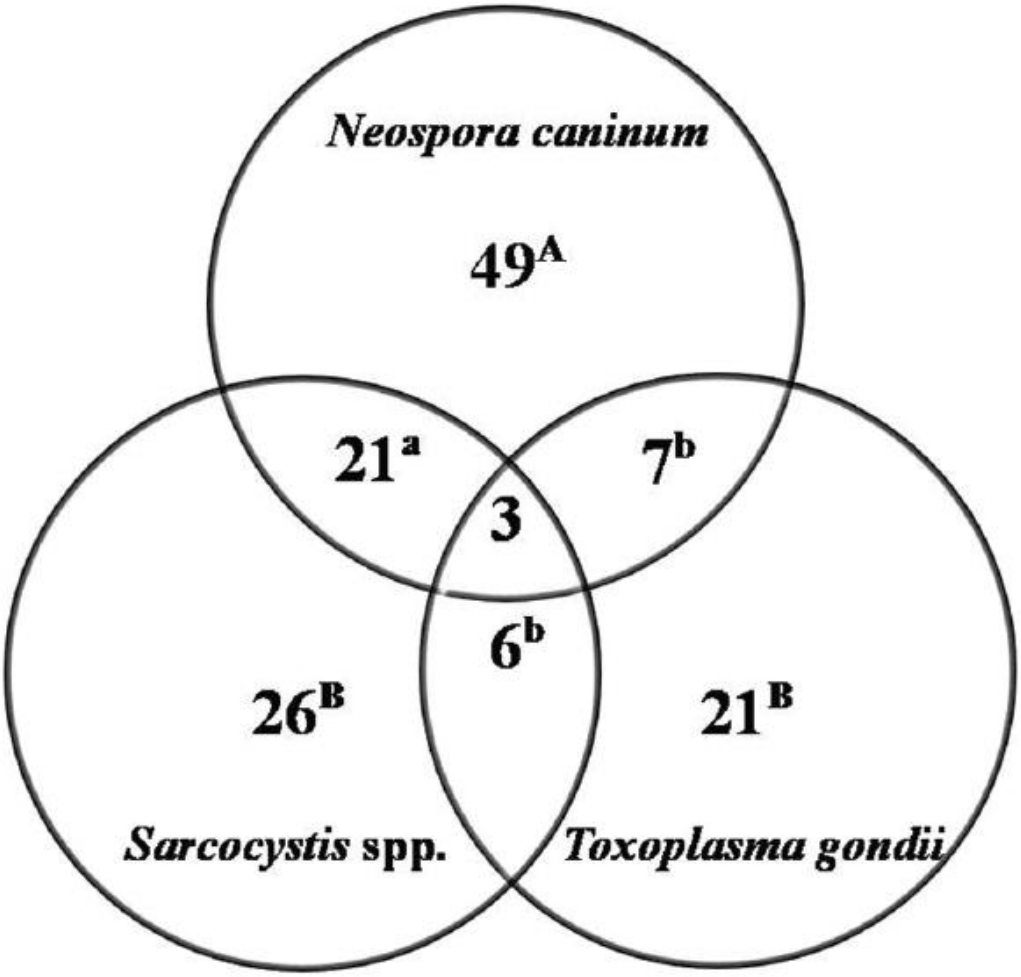

Fig.2. Detection of antibodies against *Neospora caninum*, *Sarcocystis* spp. and *Toxoplasma gondii*, by IFAT, in buffaloes in Rio Grande do Sul, Brazil. Distinct letters indicate statistical differences by Chi-square (capital letters) and Fisher exact test (small letters).

CAPÍTULO 2 - Artigo científico

Este capítulo originou um artigo científico que foi submetido na revista: Parasitology Research

First molecular detection and characterization of natural *Sarcocystis* infection among buffaloes in Rio Grande do Sul, Brazil

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Abstract

Sarcocystosis is a disease caused by a variety of *Sarcocystis* species, which can infect humans and animals, particularly ruminants. Although *Sarcocystis* can be found distributed all over the world, the species that infects buffaloes in Brazil remains unknown. This study aimed to estimate the molecular prevalence of natural infection with *Sarcocystis* spp. in buffaloes using molecular identification techniques. In addition, phylogenetic analyses were used for the first time to identify the different species of this protozoan infecting buffaloes in the south of the country. Heart samples from 80 buffaloes were subjected to microscopic examination, followed by molecular analysis. Microcysts were present in 23.75% (19 out of 80) of the samples. Genomic DNA was extracted from

the 19 isolates, and subjected to PCR. Six readable sequences were obtained after sequencing in both the directions, all of which were from *Sarcocystis levinei.*

Keywords: *Sarcocystis levinei*, buffaloes, Brazil, phylogenetic

1. Introduction

Sarcocystosis is caused by species of *Sarcocystis,* a tissue cyst-forming coccidian protozoan parasite with a wide geographic distribution and host range, affecting humans as well as wild and domesticated animals (Abu-Elwafa et al. 2015). The genus *Sarcocystis* contains more than 200 named species that infect mammals, birds, marsupials, and poikilothermic animals (Dubey and Lindsay 2006). *Sarcocystis* species require two obligatory hosts to complete their life cycle, carnivores and humans as definitive hosts and herbivores as intermediate hosts (Soulsby 1982).

Buffaloes are usually infected with *Sarcocystis fusiformis, Sarcocystis levinei, Sarcocystis dubeyi,* and *Sarcocystis buffalonis*. Some *Sarcocystis* species induce weight loss, general weakness, fever, anorexia, abortion, and death in domestic animals but, macrocyst-inducing *Sarcocystis* species are often considered as economic loss producers in slaughterhouses (Dubey et al. 1989a).

Among these, *S. fusiformis* and *S. buffalonis* use cats as their definitive host. Dogs are known to be the definitive hosts for *S. levinei*; however, the definitive host(s) for *S. dubeyi* has not yet been identified (Hilali et al. 2011). Several studies have already been conducted to investigate the prevalence and molecular characterization of *Sarcocystis* in buffaloes in many countries (Oryan, et al. 2010; Chen et al. 2017), but in Brazil few studies have been done on these animals. In a previous study with anti-*Sarcocystis* antibodies, of 220 animals surveyed, 56 (25.5%) tested positive in an indirect fluorescent - antibody test (Portella et.al, 2016). Therefore, the present study was performed to better clarify the infection of buffaloes by *Sarcocystis* and for the molecular identification of species that affect these animals in the Rio Grande do Sul state, Brazil.

2. Materials and methods

The animals included in this study were all slaughtered in officially inspected abattoirs. The authors were not directly involved in the animals slaughtering and

evisceration. All of these procedures were performed in accordance with Brazilian legal protocols of ethics and animal welfare, under the supervision of Official Veterinary Inspection Service technicians.

2.1 Animals and samples

Myocardium samples were collected from 80 buffaloes that were slaughtered for human consumption in abattoir, located in the Rio Grande do Sul state, Brazil. During the inspection of the buffalo's viscera, a piece of approximately 50 g of the heart was collected from each animal. Samples were individually stored in plastic bags identified with numbers corresponding to each buffalo, and were maintained at 4°C until analysis. Collections were performed once a week during the months of June to September, 2018.

2.2 Microscopic examination

Fresh microscopic examination was performed using 50 g of each tissue sample that was cut, minced, and macerated in a separate petri dish using a sterile scalpel blade. Subsequently, 20 g of each macerated tissue sample was individually processed, being mixed with 20 ml of phosphate buffered saline (PBS, pH 7.3), filtered through a gauze to a sterile petri dish, and examined with light microscopy at 400x magnification. Each sample was classified as positive or negative for the presence of sarcocysts. During microscopic examination, five microcysts from each tissue sample were collected with a micropipette and stored in microtubes at −80°C until further DNA extraction.

2.3 Molecular assays

2.3.1 DNA extraction

Genomic DNA was extracted from each pool of microcysts from each heart sample using a commercial kit (Wizard Genomic DNA Purification Kit, Promega, USA) according to the manufacturer´s instructions. Briefly, 600 µl of chilled Nuclei Lysis Solution was added to each of the previously collected pools of microcysts or an individual macrocyst, homogenized, and then incubated at 65°C for 30 min. After incubation, 3 µl of RNase solution was added. However, the lysis step was slightly modified (performed at 55°C for 16 h) to improve the sarcocyst disruption efficiency

(Bräunig et al., 2016). This was followed by the addition of 200 µl of protein precipitation solution. Samples were then vortexed, chilled on ice for 5 min, and centrifuged at 13,000 × g for 4 min. The supernatant was transferred to a fresh new tube and 600 µl of isopropanol was added before mixing and centrifuging at 13,000 × g for 1 min. After centrifugation, the supernatant was removed, 600 µl of 70% ethanol was added to the pellet, and this mixture was centrifuged under the same conditions described above. The ethanol was aspirated, the pellet was air-dried, and the yielded DNA was rehydrated in 100 µl of DNA rehydration solution for 1 h at 65°C. After rehydration, the concentration and purity of the DNA extracted from each sample were analyzed using spectrophotometer NanoDrop 1000 (using 260/280 nm absorbance ratio for purity evaluation) (Thermo Scientific, USA). Finally, DNA samples were stored at −80°C until PCR analysis.

2.3.2 Polymerase chain reaction

The amplification of different fragment sizes was done using 18S rRNA gene primers 2L–Forward (GGATAAACCGTGGTAATTCTATG); 3H–Reverse (GGCAAATGCTTTCGCAGTAG), amplifying a fragment of 915 bp (Rosenthal, 2010). Each PCR was performed in a total volume of 25 µl, containing 1X buffer (Promega, USA); 10 mM dNTPs (Ludwig Biotec, Brazil); 10 pmol of primers (IDT, USA); and 1 U Taq DNA polymerase (Promega, USA). A 50 ng aliquot of extracted DNA was used as a template in the PCR. A *Sarcocystis* DNA sample sequenced in previous studies was used as a positive control for subsequent PCR analysis, and MilliQ water was used as a negative control. PCR was performed using a thermocycler, with an initial denaturation step of 94°C for 2 min; followed by 40 cycles of 94°C for 40 s, 56°C for 50 s, and 72°C for 60 s; and a final extension step of 72°C for 6 min. PCR products were analyzed by electrophoresis on a 1.5% agarose gel stained with SYBR Safe DNA stain (Invitrogen, USA) and visualized by UV illumination.

2.3.3 Gene sequencing and phylogenetic analysis

Sarcocystis species identification was performed using gene sequencing. Amplified DNA product was subjected to DNA sequencing. For this purpose, amplicons from the PCR were purified using a QIAquick PCR Purification Kit (Qiagen, USA)

according to the manufacturer's instructions. Briefly, PB buffer was added to PCR product (5:1), mixed, applied to a column placed in a microtube, centrifuged (1 min at 17,900 × g), and the flow-through was discarded. Next, 750 µl of Buffer PE was added to the column and centrifuged (1 min at 17,900 × g). Thereafter, the flow-through was discarded and the centrifugation step was repeated. The column was placed in a clean tube, 50 µl of Buffer EB was added, and the tubes were then centrifuged (1 min at 17,900 × g). Subsequently, the column was discarded and the eluted DNA concentration and purity (at 260/280 nm absorbance ratio) were analyzed using NanoDrop 1000 spectrophotometer.

Purified DNA samples (45 ng) were mixed with 5 pmol of each internal primer (in separate microtubes) and MilliQ water was added up to a final volume of 6 µl. This mixture was dehydrated at 60°C for 2 h and subjected to gene sequencing (performed by ACTGene - Sequencing Service, Brazil). The results obtained were analyzed using Staden Package software and the generated nucleotides sequences were analyzed using the Genbank database (http://www.ncbi.nlm.nih.gov/BLAST).

The sequences were compared with previously published sequences of *Sarcocystis* species, *Neospora caninum*, and *Toxoplasma gondii* (GenBank). The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are expressed as the number of base substitutions per site. All ambiguous positions were removed for each sequence pair (pairwise deletion option). Evolutionary analyses were conducted in MEGA X.

3. Results

The result of microscopic examination of 80 samples of buffalo heart revealed that 19 of the examined samples (23.75%) were positive for *Sarcocystis* spp. Microcysts were found in all samples, numerous elongated cysts. Genomic DNA was extracted from the 19 isolate samples. The PCR yielded an amplicon of an approximate length of 915 bp for cyst isolates. Of the 19 DNA samples extracted, all were positive for the amplicon, allowing DNA amplification with the primer used in this study.

Ten samples were sequenced but only six readable sequences were obtained after sequencing in both the directions. In the present study, all the sequenced samples

indicated were of *Sarcocystis levinei* (Figure 1). Nucleotide sequences showed homology of 98.3–100% and the matrix results of identities are shown in Table 1.

4. Discussion

Sarcocystis spp. are common parasites with a worldwide distribution that infects the skeletal and the cardiac muscles of humans, as well as many species of wild and domestic animals (Fayer 2004). Buffaloes are frequently exposed to infection due to their close relationships with dogs, cats, and even wild animals that act as hosts for these protozoa. However, most of the animals infected with sarcocystosis appear normal and healthy at ante-mortem examination (Soulsby 1982).

In Brazil, the prevalence of *Sarcocystis* spp. infection in cattle herds is ~90-100% (Ruas et al., 2001; Ferreira et al.,1018). However, there are no studies demonstrating infection by *Sarcocystis* in buffaloes. In this study, microscopic examination and molecular characterization were used to investigate *Sarcocystis* infection in buffalo heart. Usually, macroscopic lesions are not observed in infected animals and diagnosis depends on microscopic examination of muscle samples taken from target tissues, such as the heart, tongue, esophagus, and diaphragm (Dubey et al., 2016).

In buffaloes, Sarcocystosis has been observed worldwide, but in this study, infection rates of 23.75% were found. This value is relatively low compared to other countries, such as 69% in Egypt (Nahed et al. 2014), 65% in the Philippines (Claveria et al. 2000), 87% in India (Mohanty et al. 1995), and 82.9% in Iraq (Latif et al. 1999).

Sarcocystis fusiformis was first found in the muscles of a water buffalo in Vietnam and was named *Balbiania fusiformis*, which was later classified as *Sarcocystis* (Levine 1977). Dissanaike and Kan (1978) described a second species of *Sarcocystis*, *Sarcocystis levinei*, in buffaloes from Malaysia. Dubey et al. (1989b) found a third species in buffaloes from India, based on its distinctive cell wall, but did not name it. Huong and Uggla (1999) found the same parasite in buffaloes in Vietnam and consequently named it *Sarcocystis dubeyi*. Huong et al. (1997) described a fourth species, *Sarcocystis buffalonis*, in buffaloes from Vietnam.

In the recent years, the 18S rDNA gene has been extensively used as a suitable target to differentiate the closely related species of the *Sarcocystis*, as well as for phylogenetic analysis (Jeffries et al. 1997; Yang et al. 2002; Dahlgren and Gjerde 2007;

Kia et al. 2011). The differentiation between species that affect animals is of great importance, because it allows us to better understand the epidemiology of these infections, identify the definitive hosts involved, and investigate species with zoonotic importance. Pritt et al. (2008) reported that using molecular methods (PCR) may allow greater detection of *Sarcocystis* species in beef.

The comparison of sequences obtained by DNA sequencing, with published data on Genbank using the NCBI Blast program, demonstrated that 100% of the samples were *Sarcocystis levinei*, through the partial sequencing of the 18S rRNA gene. In addition, it was possible to evaluate these sequences through phylogenetic tree and identity matrix analyses, which represent the similarity between the sequences of species in the database, with those of the species being studied. The results of these studies showed 98.3–100% identity between the study isolates and accession numbers MG957195, MG957192, MG957194, KU247922, and KU247918. In relation to the phylogenetic tree (Figure 1), it is possible to observe how the study samples are similar to *S. cruzi* and *S. heydorni* species, which infect cattle as intermediate hosts. The isolated samples did not present high similarity with other species that affect the buffalo, being *S. buffalonis* 86.2 – 87.5%; *S. fusiformis* 88-88.9%; *S. senensis* 90-91.4%.

5. Conclusion

The results of this study demonstrate the occurrence of *Sarcocystis levinei* infection in buffaloes in the Rio Grande do Sul state. They suggest that further studies should be carried out in the state, and in the country, to identify other species of *Sarcocystis* involved in buffalo infection and to better understand the clinical and economic importance of these protozoa.

Conflict of interest

The authors declare no conflict of interest in relation to this work.

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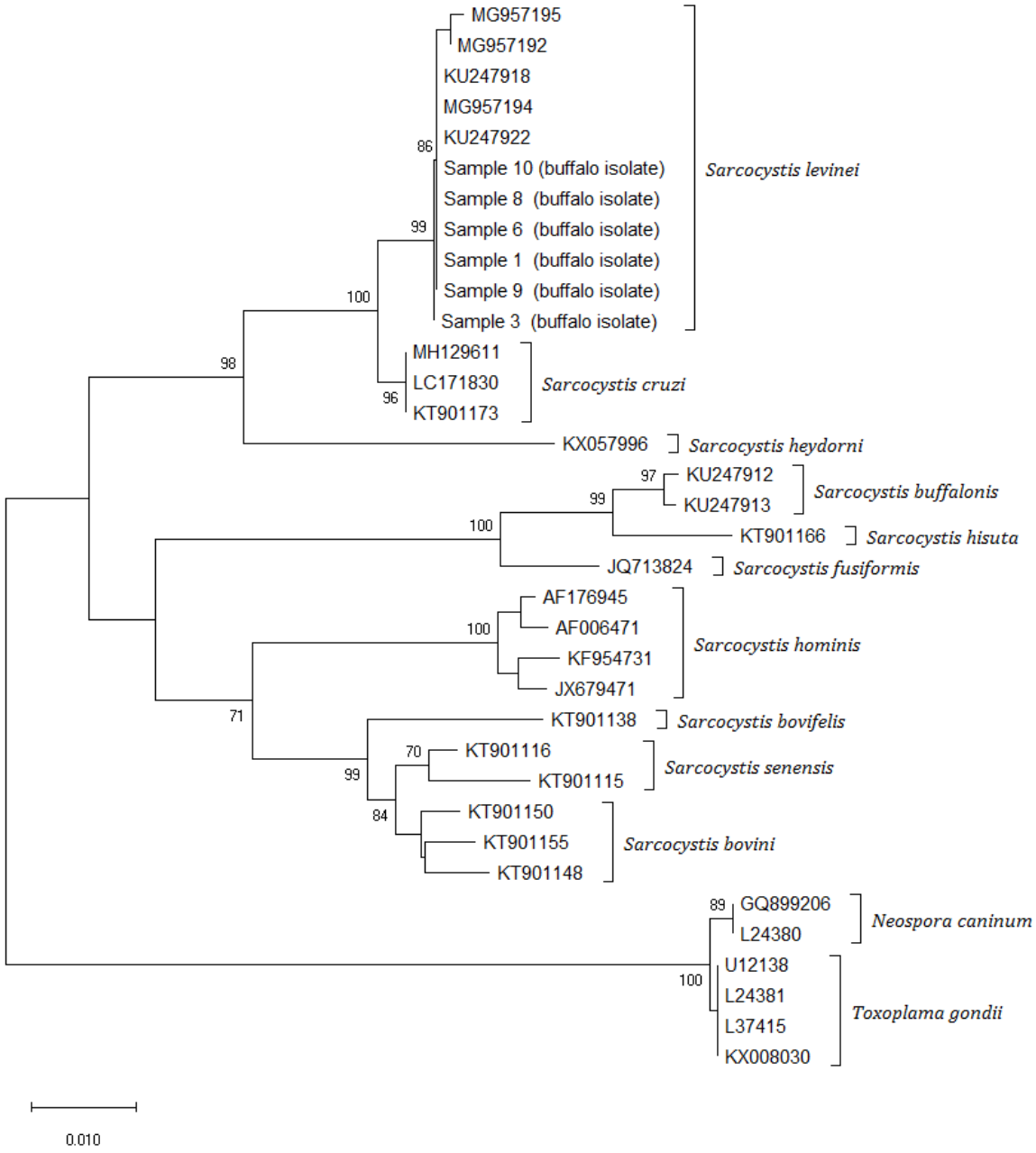

Figure 1- Phenogram construction of the 18S rRNA gene of Sarcocystis isolates from buffaloes sourced in this study with GenBank reference strains. Numbers shown at different nodes represent percentage confidence limits obtained in the bootstrap analysis. The optimal tree with the sum of branch length = 0.33757427 is shown

Table 1: Results of the identity matrix of the isolated samples at work and in relation to the species of *Sarcocystis*

Identity Matrix	
	(%)
Sarcocystis levinei	98.3 - 100
Sarcocystis buffalonis	86.2 – 87.5
Sarcocystis fusiformis	88 – 88.9
Sarcocystis senensis	90 – 91.4
Sarcocystis cruzi	97.2 – 98.7
Sarcocystis hirsuta	86.7 – 87.6
Sarcocystis hominis	89.2 – 90.7
Sarcocystis heydorni	91.9 – 93.3
Sarcocystis bovifelis	89.7 – 90.6
Sarcocystis bovini	90 – 91.2
Neospora caninum	82.1 – 83.1
Toxoplasma gondii	81.9 – 83.1

CONSIDERAÇÕES FINAIS

Em um primeiro momento, foi realizado um estudo, através da detecção de anticorpos utilizando a imunofluorescência indireta (RIFI), para verificar a infecção de búfalos por *Neospora, Sarcocystis* e *Toxoplasma gondii.* Como conclusão dos estudos realizados, no capítulo 1, demonstramos a detecção de anticorpos IgG contra *Neospora caninum*, *Sarcocystis* spp. e *Toxoplasma gondii* em rebanhos de búfalos no RS, Brasil. A alta frequência de animais infectados por *Neospora caninum* é importante pois pode estar intimamente ligada a problemas reprodutivos nestes animais. Em relação a detecção de anticorpos para *Sarcocystis* e *T. gondii,* salientamos a importância do ponto de vista de saúde pública, com o consumo de carne e também das perdas econômicas que podem ser causadas pela condenação de carcaças acometidas por sarcocistose.

No capítulo 2, observamos a presença de cisto em miocárdio de animais avaliados, e através de técnicas moleculares, foi possível caracterizar a espécie envolvida na infecção. Demonstrando então a ocorrência da infecção por *Sarcocystis levinei* em bubalinos no Estado do Rio Grande do Sul.

Após a realização dos nossos estudos, foi possível verificar a infecção de búfalos por *Sarcocystis* no Rio Grande do Sul. Estes resultados não foram uma surpresa visto que a infecção por este protozoário é comum em búfalos em vários países no mundo. Somado a isto, estudos realizados anteriormente no estado, demonstraram a infecção pelo protozoário em diversas espécies animais, tais como bovinos e ovinos.

Sugerimos que mais estudos sejam realizados no estado e no país, para identificar outras espécies de *Sarcocystis* envolvidas na infecção por búfalos, e para entender melhor a importância clínica e econômica deste protozoário.

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ANEXOS

Anexo I - Artigo cientifico a ser submetido para a revista: **Small Ruminant Research**

Original research paper

Association of immunostimulants with anthelmintic in the treatment of animals experimentally infected with *Haemonchus contortus*

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Abstract

Due to the importance of *Haemonchus contortus* infection in sheep herds, in addition to the problem of parasite resistance, the goal of this study was to evaluate the influence of the administration of adjuvants alone, or in combination with anthelmintic treatment, for the control of *H. contortus* in experimentally infected sheep. The results obtained from the excretion of eggs per gram of feces and parasite count at necropsy suggest that the use of adjuvants associated with anthelmintics is related to a better drug

of reason, lower rate of reinfection and retardation in the development of anthelmintic resistance in *H. contortus*.

Key words: gastrointestinal nematodes, sheep, adjuvants

1. Introduction

Infection with gastrointestinal nematodes is one of the main sanitary problems in sheep herds (Sczesny-Moraes et al., 2010), and causes economic losses in sheep farming. Nematodes affecting sheep in Brazil include the species *Haemonchus contortus*, a direct life-cycle hematophagous parasite, which is the most prevalent and pathogenic (Amarante, 2001).

The severity of the disease caused in the host is directly related to the number of *H. contortus* larvae that are established in the abomasum, since there is a strong correlation between blood loss and the number of adult parasites (Le Jambre, 1995). The clinical signs of *H. contortus* infection also depends on other factors, such as individual susceptibility, nutritional status and age. The disease is characterized by severe anemia, with paleness of the mucous membranes, submandibular edema and progressive weight loss. When blood loss exceeds hematopoietic capacity, hemonchosis can lead to death (Onyiah and Arslan, 2005; Bowman et al., 2006).

Control of these parasites is based, almost exclusively, on the use of anthelmintics. However, intensified breeding makes this control difficult, because with increase in animal stocking rate per area, there is greater environmental contamination and, consequently, an increase in the rate of infection of the animals. In these conditions the high frequency use of anthelmintic treatments is common, leading to the selection of parasite populations, resistant to the active principles available in the market (Kotze and Prichard, 2016).

Anthelmintic resistance is a mechanism whereby the drug cannot display the same therapeutic efficacy after a certain period, when used under the same conditions (Buzzullini et al., 2007). Parasite resistance to anthelmintics is known to be widely disseminated and includes multi resistant populations (Cezar et al., 2010; Bartley et al., 2012; Keane et al., 2014; Martinez- Valladares et al., 2015). Due to this problem, many studies have sought to find alternatives to the use of antiparasitics, how to identify and activate the natural mechanisms of resistance of the organism, or ways of selecting naturally resistant animals (Pugh and Baird, 2012).

However, tolerance of helminths in the gastrointestinal tract has not yet been completely elucidated. It is known as a multifactorial feature, with genetic, immunological, physiological, and nutritional components, varying among races and between individuals, and which determines three distinct animal biotypes: resistant, sensitive and resilient (Albers et al., 1987; Amarante et al., 2004).

H. contortus infection causes inflammation of the abomasal mucosa and activation of the immune system, that leads to the production of cytokines, which are molecules involved in the emission of signals between defense cells and are responsible for directing immune response. Resistant animals have a greater efficiency in developing the Th2 response, while sensitive animals mostly present the Th1 response (Lawrence, 2003; Alba-Hurtado and Muñoz-Guzman, 2013; Amarante, 2014).

In view of the above, it is essential to search for therapeutic alternatives that prolong the effectiveness of treatments, delay the process of parasitic resistance and ensure the sustainability of anthelmintic control in sheep herds. In that sense, the stimulus to the immune response can act synergistically with anthelmintics for reducing the parasite burden on animals. Adjuvants are molecules or substances capable of amplifying

and prolonging the cascade of immunological events that make up the immune response (Audibert, 2003).

Thus, considering the importance of *H. contortus* infection in herds, and the problem of parasite resistance, we evaluated the influence of the administration of adjuvants alone, or in combination, with anthelmintics for controlling of *H. contortus* in experimentally infected sheep.

2. Material and Methods

2.1 Location and animals

Thirty sheep of the Texel breed, of both genders, aged between 6 and 8 months, were used for the study. Each was individually identified with numbered earrings and raised in a herd located in the subtropical region of Brazil. The animals were housed in collective bays, five animals per bay, where all the animals remained in the same shed throughout the period of the experiment. For all the animals, the same diet was offered, with about 14% of rough protein. These animals remained for a period of 20 days of adaptation, where they were treated for elimination of the parasite load (Monepantel, Zolvix® - Novartis Animal Health, 2.5 mg/kg). Water was supplied *ad libitum* in suspended buckets.

Procedures involving the use of animals (sample collection, anthelmintic treatment, identification, body weighting, and general management) were submitted and approved by the Committee of Ethics in Animal Experimentation of the Federal University of Santa Maria, ensuring the welfare of the animals throughout the experiment.

2.2 Production of H. contortus larvae experimental infection

For experimental infection of the animals, infective larvae (L3) of *H. contortus* cultures were prepared. For this, fecal collections were made directly from the rectal ampulla of a donor animal, with a monoinfection, gently administered at the Laboratory

of Parasitology, UNESP Bioscience Institute, Botucatu campus, under the supervision of Prof. Alessandro Francisco Talamine do Amarante. The infective larvae (L3) were obtained using the technique of *Roberts* O'*Sullivan*. Copro-parasitological examination of all the lambs was carried out, to certify that they were all negative.

The animals were inoculated with third stage larvae, which were diluted in distilled water and administered orally. Three administrations were carried out, each at two-day intervals. The number of administered larvae was based on the animals' weight. For each kg of animal live weight, 300 third stage larvae were administered. The first day of infection was established as day 0 of experimental infection (D0), and after 14 days (D + 14), the animals were reinfected.

2.3 Experimental groups

The animals were individually weighed on a digital scale to ensure accurate estimation of the doses to be administered. In relation to the application of adjuvants and active ingredients, these were standardized for better monitoring of possible changes in the region of application on the animals. All treatments were done individually in animals administered with Moxidectin (Mox), and hydroxyl aluminum gel was also administered subcutaneously. The lipopolysaccharide (LPS) associated with *Propionibacterium acnes* was administered via intra-muscular. All animals remained under observation for 60 minutes after the treatments to evaluate possible clinical changes. Groups treatments: G1, Control; G2, Moxidectin; G3, Moxidectin + Aluminum Hydroxide Gel; G4, Aluminum Hydroxide Gel; G5, Moxidectin + LPS associated with *P. acnes*; and G6, LPS associated with *P. acnes*.

2.4 Antiparasitic drug and immunostimulants

Moxidectin (1%, Cydectin®, Zoetis Industria de Produtos Veterinários Ltda. Campinas-SP, Brazil) was used at a dose of 0.2 mg/kg (subcutaneously) as indicated by

the manufacturer. Moxidectin was chosen as the antiparasitic to be tested because, in a previous study, the drug was found ineffective against *H. contortus* (Almeira et al., 2010). Adjuvants tested in combination with Moxidectin were aluminum hydroxide gel (0.04 ml/animal, subcutaneous) and LPS associated with *P. acnes* (0.025 mg/kg, intramuscular) (Infervac®, Calier SA, Barcelona, Spain).

2.5 FAMACHA and sample collection

On D0, D7, D14, D21, D28 and D35, all animals were contained individually, to evaluate the ocular mucosa. The examination was carried out by scoring the different shades, from pinkish red to the white pale, of the conjunctiva, on a scale of 1 to 5 and comparing these with a standard field guide card. A general clinic evaluation of the presence of submandibular edema and diarrhea in animals was also carried out, and blood samples were collected by jugular vein puncture using a vacutainer, packed in tubes with EDTA. Simultaneously, fecal samples were collected from the rectal ampulla and packed in individual plastic bags, labelled and refrigerated until processing.

2.6 Blood processing and parasitological examinations

To determine the hematocrit, the microhematocrit technique, involving

The use of 75 × 1 mm capillary tubes, was employed, with two-thirds of their total volume filled, labelled and placed in a microcentrifuge at 10,000 rpm for five minutes and taken to the reading table of the equipment.

Two blood smears for differential leukocyte counting were distended. These smears, after drying, were dyed using a fast dye. In each blood smear, 100 leukocytes, classified and observed under a microscope at a 1000× magnification were differentiated, according to its morphological and tannic characteristics, in neutrophils, eosinophils, basophils, lymphocytes, and monocytes.

To obtain EPG (eggs per gram of feces) counts, the McMaster modified technique, with a sensitivity of 100 EPG, was performed. Animals that had an EPG count ≥ 500 on D-2 were selected. At all farms, the animals were distributed into six randomized blocks on the basis of pre-treatment EPG and weight.

In addition, feces from all sheep were pooled, mixed with sterile wood shavings, and stored for larval cultures (moisturized daily with sterile water) on D7, D14, D21, and D28. These cultures were incubated for 7 days at 22–27 °C and 80% humidity as recommended by Coles et al. (2006). After incubation, the larvae were recovered by baermanization, and100 third-stage larvae were identified (by genera) in each culture, following the criteria described by Van Wyk and Mayhew (2013).

2.7 Necropsy of the animals

At D21 after reinfection (D35), three animals from each group were euthanized, with 10 mg of acepromazine intravenous [IV], 2 g of thiopental sodium [IV] and 100 ml of potassium chloride [IV], and their abomasa were collected to quantify the number of adult parasites (L5). After opening each abomasum at the major curvature, the organ was carefully washed with 1 liter of water and the entire contents were collected. After the contents were homogenized, a 100 ml aliquot was collected for parasite counting, as recommended by the World Association for the Advancement of Veterinary Parasitology.

2.8 Eosinophil tissue counting

After washing the abomasum, fragments were also collected to count eosinophil tissues. Samples from the abomasal fundus area were sectioned, fixed in 10% formaldehyde for 24h and included in paraffin. The sections were stained with hematoxylin-eosin for subsequent counting of eosinophils. The methodology for obtaining the number of eosinophils was based on the total quantification of these cells obtained in 10 random fields, focused from the muscular layer to the surface of the

mucosa at 400× magnification. Individual counting was performed for each animal and the results expressed as an average of the number of eosinophils.

2.9 Statistical analysis

The efficacy of each treatment was estimated based on the fecal egg reduction counts test, using pre- and post-treatment EPG counts for each group (Neves et al. 2014). In the statistical analysis of the data obtained from FAMACHA, hematocrit, leukocytes, larval counts and tissue eosinophil counts, a Tukey test was performed, with a 95% confidence interval.

3. Results

The average efficacy of the different treatments or groups on days 7, 14, 21, 28, and 35 is shown in Table 1. According to the criteria proposed by WAAVP (Coles et al., 1992), at day 7 of the experiment, no group had shown efficacy in the treatments, except the combination of Mox + LPS associated with *P. acnes* (LPS assoc. *P. acnes*), the one which has shown the best results (79%). When we evaluated the animals on day 14, the combination of Mox + $Al(OH)_3$ showed 95% efficacy, followed by treatments with Mox + Assoc. *P. acnes* with 93% efficacy. On day 21, treatments with Mox, Mox + $Al(OH)_3$ and Mox + LPS assoc. *P. acnes* presented desirable efficacy (96%, 99% and 95%).

Regarding the evaluation of reinfection, on day 28 the Mox group, Mox + $Al(OH)_3$ and Mox + LPS assoc. *P. acnes* (97%, 98%, 97%) also showed the best efficacy, but by day 35, the groups that presented the best efficacy were Mox + $Al(OH)_3$, $Al(OH)_3$, and Mox + LPS Assoc. *P. acnes*, where all three groups showed 100% efficacy. The results of FAMACHA, hematocrit, leukocyte, leukocyte differential, and eosinophil counts or evaluations, in the abomasal mucosa, were not demonstrated since they did not present

statistical differences between the study groups and remained within the normal levels presented in the literature for the species.

Total parasite counts of aliquots collected following the abomasal washing of the euthanized sheep is presented in Table 2. These results show that the only group that maintained a high population of parasites was the control group, in all the other groups, treated with anthelmintics, adjuvants or their associations showed low parasite count.

4. Discussion

Reports of parasitic resistance to Moxidectin are common worldwide (Love et al. 2003; Howel et al. 2008), including Brazil, where ~80% moxidectin efficacy was verified by Cunha Filho et al. (1998), in an evaluation of the region of Londrina, Paraná, by Buzzulini et al. (2007), in Jaboticabal, São Paulo, and by Rosalinsk-Moraes et al. (2007), in Santa Catarina, as well as, other studies conducted in the region where the experiment was carried out (Almeida et al., 2010; Cezar et al., 2010).

The chronic failure of anthelmintic treatments results in increased parasite load in the animals and continued contamination by eggs and larvae in the pastures. Low productivity and the appearance of characteristic clinical signs, such as submandibular edema or diarrhea, especially in animals under stress conditions and in lambs in general, are usually the first obvious signs of a decrease in efficacy of the drugs used against the parasites. Producers often take the wrong steps in reestablishing the efficacy of antiparasitic treatments, such as increasing the frequency of treatments, the use of high drug concentrations (with long action) and the rapid alternation of chemical bases. This results in a greater selection pressure and in the rapid development and manifestation of parasitic resistance, increased environmental contamination by drug residues, greater risk of deposition of these residues in products intended for human consumption, increased

costs of treatments without proportional improvement in its effectiveness, and a tendency towards the total dysfunction in anthelmintic control in these sheep herds (Cezar, 2010).

Once parasite resistance is established, it can be anticipated that most conventional anthelmintics are compromised or, if not, they will only be effective in a medium to long term. These frameworks tend to evolve dramatically if specific measures are not taken to decelerate and solve this problem. Knowing this, the need for pharmacological alternatives to improve the effectiveness of treatments and ensure the sustainability of anthelmintic control and productive viability in herds affected by multi resistant gastrointestinal nematodes, is clear. Among these alternatives, is the modulation of the immune response (Quinn et al., 1990).

The immune response against nematodes mainly involves the recognition and processing of parasite antigens, the action of CD4 + Th2 lymphocytes, the increase in inflammatory cells present in the mucosa of the animals, the number of circulating eosinophils, the production of specific antibodies, and the increased mucus production with inhibitory substances (Hohenhaus and Outteridge, 1995; Amarante, 2003). Substances, such as immunostimulants, that have the capacity to stimulate the nonspecific immune response, can be used to aid the general resistance of animals that are exposed to the risk of infection (Quinn et al., 1990). The use of two adjuvants in the non-associated form reduced the infection rate in animals in this study, as well as, reinfection (evaluated mainly at day 35 post-infection) It was observed that groups treated with immunostimulants alone (Group 4 and Group 6) had a reduction in EPG count on days 7, 14, 21, 28, and 35, with the best results on day 28, 87% and 85%, and day 35, 100% and 84%, for groups 4 and 5, respectively. These data corroborate the report of Abel et al (2009), who used and LPS-based compound adjuvant associated with *P. acnes* in

experimental models infected with nematodes and observed a significant reduction in infection rate. This reduction is probably associated with the proliferation of lymphocytes T and an increase in the number of eosinophils in nonspecific form.

Based on these evidences, we expected that the association of immunostimulants with an antiparasitic of compromised efficacy could improve the efficacy of the drug. The results obtained prove this hypothesis correct. In groups where moxidectin combinations with adjuvant were used, improvement in the efficacy of the treatments was observed. In Group 3, where the animals received 1% Moxidectin associated with aluminum hydroxide, improved efficacy of treatment was observed from day 14 (95%) after treatment, and increased by days 21 (99%), 28 (98%) and 35 (100%). In group 5, where animals received 1% Moxidectin combined with LPS associated with *P. acnes*, improved treatment efficacy was observed on days 14 (93%), 21 (95%), 28 (97%), and 35 (100%). In the group treated with only Moxidectin (G2), a lower efficiency was observed on days 7 and 14, compared with groups G3 and G4. Furthermore, reinfection evaluated on day 35 was higher on group G2 when compared to G3 and G5 groups. From the results above, better treatment efficacy, indicated by a reduced parasite count, was achieved in groups that received anthelmintic administration associated with immunostimulants, when compared to their isolated use. Although these data do not present statistical differences, they strongly suggest that the association of antiparasitics with immunostimulants are able to reduce and/or eliminate nematode infection of the GI tract, as well as, reduce reinfection. Similarly, Martins et al. (2012), using a group of sheep treated with antiparasitics and another group treated with antiparasitics associated with immunostimulants, found no significant differences in the EPG values, although lower values were obtained in all samples from animals treated with the combinations.

In general, it is known that the use of immunostimulants is associated with the appearance of neutrophilia and eosinophilia, and an increase in the response of T and B lymphocytes, with important changes in bone marrow activity (Mendoza et al., 2000). An increase in the number of eosinophils or immunoglobulin E (IgE) levels, has a negative correlation compared to the number of OPG found in the animals (Kooyman et al., 1997), thus indicating a certain antiparasitic resistance in sheep (Shakya et al., 2009). However, in this study no statistical differences were detected in relation to blood and tissue eosinophil counts between the experimental groups.

The reduction in egg count per gram of feces and reinfection is associated with the immunostimulating effect of the adjuvants. Braga et al., 2003 demonstrated the action of a suspension of *P. acnes* on the modulation of immune response and characterized this late-acting adjuvant, which justifies the gradual increase in treatment efficacy, as observed with animals receiving Moxidectin associated with aluminum hydroxide (G3) in this study.

Following hematocrit and FAMACHA evaluations, there was no statistical difference between the treated and untreated groups ($p > 0.05$). In a study conducted by STEAR et al. (1995), they demonstrated that, through consecutive *H. contortus* infections, animals may present different hematological behaviors. They showed that the first infection may have a marked decrease in red blood cells, however, these animals recover and after the second and third infections, these parameters continue to improve. They also showed that at the end of 23 weeks, the number of red blood cells between treated and untreated may be similar.

In addition to EPG, necropsy techniques are important ways of revealing the main nematode lesions, as well as, the amount and species of parasites present (Climeni et al.,

2008). The results of the parasite counts are shown in Table 3. All the parasites identified in the counts were *H. contortus*.

After counting the parasites, it was evident that animals subjected to anthelmintic and immunostimulant treatments had a low larvae count, differing statistically ($p < 0.05$) from the control group, which presented the highest larvae count. The parasitological necropsy result is very important, mainly because of the density-dependent phenomenon that *H. contortus* presents. Although EPG is still the most used technique in the scientific environment (Cezar et al., 2010; Domke et al. Mitchell et al., 2010; Pivoto et al., 2014; Santos et al., 2014), its sensitivity presents a limitation, as it is characteristic of ovines with high parasitic load to present feces with little or no eggs of the parasite, because *H. contortus* females cease in egg production due to the high parasitic load present in the abomasum of the animals (Zajac, 2006).

5. Conclusion

The results obtained from excretion of eggs per gram of feces and parasite count at necropsy, suggest that the use of adjuvants associated with anthelmintics is related to better drug efficacy, lower rate of reinfection and a retardation in the development of anthelmintic resistance by *H. contortus*. In the current context, an improvement in the efficacy of products in herds that present populations of multidrug resistant parasites is an extremely important alternative that is directly linked to the maintenance of sheep. In view of the discovery of a new active principle, the commercialization of this approach, involving adjuvants, could delay the process of nematode resistance among sheep herds and improve the efficacy of existing antiparasitic products.

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Table 1. Efficacy of treatment in experimentally infected sheep with *H. contortus* on different days after treatment of the animals.

Groups (n= 5) and treatments	Eficácia (%)				
	Day 7	Day 14	Day 21	Day 28	Day 35
G2. Mox	65	85	96	97	90
G3. Mox + $Al(OH)_3$	68	95	99	98	100
G4. $Al(OH)_3$	61	72	59	87	100
G5. Mox + LPS assoc. *P. acnes*	79	93	95	97	100
G6. LPS assoc. *P. acnes*	39	56	64	85	84

Table 2. Parasite count of the sample collected in the abomasum wash.

Groups (n= 5)	Parasite count
	H. contortus
G1. Cont	206
G2. Mox	5
G3. Mox + $Al(OH)_3$	0
G4. $Al(OH)_3$	9
G5. Mox + LPS assoc. *P. acnes*	1
G6. LPS assoc. *P. acnes*	8

Anexo II - Artigo cientifico submetido para a revista: **Pesquisa Veterinária Brasileira**

Aspectos histológicos e confirmação molecular de *Sarcocystis* em tecidos de bovinos e ovinos condenados em abatedouro-frigorífico

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ABSTRACT - Portella L.P., Minuzzi C.E., Sangioni L.A., Flores, M., Vogel, F.S.F. **[Histological aspects and molecular confirmation of *Sarcocystis* in tissues of cattle and sheep condemned in slaughterhouse].** *Pesquisa Veterinária Brasileira 00(0):00-00*. Laboratório de Doenças Parasitárias, Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria, Av. Roraima n. 1000, Camobi, Santa Maria, RS 97105-900, Brazil. e-mail: lupiresportella@gmail.com.

Sarcocystis is one of the most prevalent protozoa in striated muscle of animals slaughtered for consumption, such as cattle and sheep. The objective of this work is to describe the macro and microscopic characteristics of the lesions found in cattle and sheep condemned in the slaughterhouse by sarcocystosis, as well as molecularly evaluating the species of Sarcocystis involved in these infections. It was possible to verify that all tissues of sheep referred had macroscopic cysts characterized by being white, round to oval and striated, ranging from 0.3 to 1cm in diameter. On the other hand, bovine tissues did not present macroscopic cysts, but had whitish lesions characteristic of Sarcocystis infection. All samples from both cattle and sheep had microscopic cysts. In molecular analysis, DNA specific for Sarcocystis spp. From the genetic sequencing and evaluation of these consensus sequences were identified the species *S. gigantea* for sheep and *S.cruzi* for bovines.

INDEX TERMS*: Sarcocystis gigantea, Sarcocystis cruzi,* PCR

RESUMO- O *Sarcocystis* é um dos protozoários mais prevalentes no músculo estriado de animais abatidos para consumo, tais como como bovinos e ovinos. O objetivo deste trabalho é descrever as características, macro e microscópicas, das lesões encontradas em bovinos e ovinos condenados em frigorifico por sarcocistose, bem como avaliar molecularmente quais as espécies de *Sarcocystis* envolvidas nessas infeções. Foi possível

verificar que todos os tecidos de ovinos encaminhados apresentavam cistos macroscópicos caracterizados por serem brancos, redondos a ovais e estriados, que variavam de 0,3 a 1cm de diâmetro. Em contrapartida, os tecidos dos bovinos não apresentavam cistos macroscópicos, porém apresentavam lesões esbranquiçadas características da infecção por *Sarcocystis*. Todas as amostras, tanto dos bovinos como dos ovinos, apresentavam cistos microscópicos. Na análise molecular, em todas as amostras foi detectado DNA específico para *Sarcocystis spp.* A partir do sequenciamento genético e avaliação destas sequencias consensos foram identificadas as espécies *S. gigantea* para ovinos e *S.cruzi* para bovinos.

TERMOS DE INDEXAÇÃO**:** *Sarcocystis gigantea, Sarcocystis cruzi,* PCR.

INTRODUÇÃO

O *Sarcocystis* é um dos protozoários mais prevalentes no músculo estriado de animais abatidos para consumo, tais como como bovinos e ovinos, sendo uma infecção muito comum em todo o mundo, mesmo em países desenvolvidos (Savini et al. 1992). Ovinos podem ser hospedeiros intermediários de quatro espécies de *Sarcocystis* (*S. gigantea, S. medusiformis, S. tenella e S. arieticanis*) e, de acordo com as espécies, podem formar cistos macroscópicos ou microscópicos em diferentes tecidos desses animais (Dubey et al. 1989). Em ovinos, os principais sinais clínicos da infecção por *Sarcocystis* são anorexia, perda de peso e hipertermia (Banerjee 1998, Heckeroth & Tenter 2007).

Bovinos podem ser infectados por pelo menos sete espécies de *Sarcocystis,* sendo elas*: Sarcocystis cruzi, S. hirsuta, S. hominis, S. rommeli, S. heydorni, S. bovini e S. bovifelis.* A infecção por qualquer uma destas espécies é potencialmente capaz de causar perdas produtivas em bovinos, incluindo menor produtividade e condenação de carcaça em frigorífico. O diagnóstico da sarcocistose em bovinos é difícil, uma vez que os animais raramente apresentam sinais clínicos e estes, quando presentes são inespecíficos. Visto que, *S. hominis* e *S. heydorni* são espécies potencialmente zoonóticas é importante a realização de inquérito epidemiológico, incluindo a implementação de testes de diagnóstico (Thompson 2013).

Somado a isto, após 60 anos de vigência do Decreto nº 30.691/1952, foi publicado o Novo RIISPOA **(**regulamento da inspeção industrial e sanitária de produtos de origem animal) através do Decreto nº 9.013/2017. Com ela, grande parte das regras sobre a

inspeção industrial e sanitária de produtos de origem animal foi renovada, tornando imprescindível o correto diagnóstico de Sarcocistose no Brasil, principalmente em abatedouros e frigoríficos. Através do "Art. 168", fica definido que: "carcaças com infecção intensa por *Sarcocystis* spp (sarcocistose) devem ser condenadas. § 1º Entende-se por infecção intensa a presença de cistos em incisões praticadas em várias partes da musculatura. § 2º Entende-se por infecção leve a presença de cistos localizados em um único ponto da carcaça ou do órgão, devendo a carcaça ser destinada ao cozimento, após remoção da área atingida.". Sendo assim, tem sido crescente a busca por diagnóstico no Laboratório de Doenças Parasitarias, em Santa Maria, Rio Grande do Sul.

O presente trabalho é parte de uma investigação no aumento no aparecimento de lesões e cistos macroscópicos em tecidos musculares de ovinos e bovinos encaminhadas para diagnóstico no Laboratório de Doenças Parasitárias-UFSM. Todos os tecidos foram coletados em abatedouro-frigorífico. O objetivo deste trabalho é descrever as características, macro e microscópicas, das lesões encontradas, bem como avaliar molecularmente quais as espécies de *Sarcocystis* envolvidas nessas infeções, para avaliar o potencial zoonótico das espécies envolvidas, e contribuir para um correto destino das vísceras e carcaças.

MATERIAL E MÉTODOS

Foram recebidos no Laboratório de Doenças Parasitárias (Ladopar) da Universidade Federal de Santa Maria (UFSM) fragmentos de tecidos de ovinos e bovinos para a confirmação do diagnóstico de *Sarcocystis.* Esôfago, diafragma, língua e laringe de 4 ovinos e fragmentos dos músculos peitoral e cleido mastoideo, masseter e coração de 2 bovinos que tiveram suas carcaças condenadas em frigorifico por apresentarem lesões características de Sarcocistose.

Os tecidos foram encaminhados para o laboratório, pois houve um aumento acentuado no número de condenações por sarcocistose em um determinado município do Estado do Rio Grande do Sul, porém por questões éticas, não serão fornecidas mais informações sobre a origem geográfica dessas amostras. Todos os dados relatados foram fornecidos pelos médicos veterinários responsáveis pela inspeção e prefeitura.

As amostras foram recebidas frescas e refrigeradas, analisadas quanto a presença de cistos e lesões macroscópicas, sendo posteriormente divididas em dois fragmentos

sendo um para realização de exame direto seguido de molecular e outro para realização de exame histopatológico. Para o histopatológico, os fragmentos coletados foram fixados em formol a 10% tamponado, posteriormente, clivados, processadas e incluídas em parafina para secção. Esses cortes foram então corados por hematoxilina e eosina para análise das alterações morfológicas do tecido.

Para detecção de cistos teciduais, foi realizado exame direto conforme descrito por Minuzzi et al. (2018). Quando visualizados, os cistos eram coletados para posterior extração de DNA e realização da técnica da reação em polimerase em cadeia (PCR). O DNA total dos cistos foi extraído utilizando kit comercial (Wizard genomic DNA purification, Promega, Madison, WI, USA), de acordo com recomendações do fabricante, com modificações no passo de lise conforme descrito por Moré et al. (2011). Após a extração, a concentração de DNA em cada amostra foi mensurada através de absorbância por luz ultravioleta (UV) a 260nm, ficando o DNA total armazenado a temperatura de -20°C até sua utilização. O DNA total extraído das amostras teciduais foi submetido a reações de PCR, utilizando os iniciadores específicos para o gene18S rRNA, sendo eles: *2L–Forward* (GGATAAACCGTGGTAATTCTATG); *3H–Reverse* (GGCAAATGCTTTCGCAGTAG) amplificando um fragmento de aproximadamente 915pb (Rosenthal, 2010).

Cada reação de PCR foi realizada em um volume total de 25µL, contendo tampão de PCR 5X; 10mM de dNTPs; 10 pmol de cada iniciador; 1,5 unidades de Taq polimerase e 50ng de DNA total utilizado como molde. Os produtos de PCR foram analisados através de luz UV, após eletroforese em gel de agarose a 1% corado com GelRed® (Biotium Inc. , CA, USA). As condições utilizadas para realização do PCR para o gênero *Sarcocystis* foram: desnaturação inicial 94 °C for 2 min; seguidos por 40 ciclos de 94 °C por 40 s, 56°C por 50 s, e 72 °C por 6 s; com uma extensão final de 72 °C for 6 min. A identificação das espécies de *Sarcocystis* foi realizada utilizando o sequenciamento de DNA. Para tanto, os produtos das reações de PCR foram purificados, através de kit comercial de Purificação de PCR QIAquick (Quiagen, EUA) de acordo com as instruções do fabricante. Posteriormente, amostras de DNA purificado (45 ng) foram misturadas com 5 pmol de cada primer e água MilliQ. Essa mistura foi desidratada a 60°C por 2 horas e submetida ao serviço de sequenciamento de genes (realizado pelo ACTGene - Sequencing Service, Brasil). Os resultados obtidos foram analisados usando o software

Standen Package e as sequências consensos de nucleotídeos gerados foram analisadas usando o banco de dados *Genbank* (http://www.ncbi.nlm.nih.gov/ BLAST).

RESULTADOS

Após a avaliação macroscópica dos tecidos encaminhados ao laboratório, foi possível verificar que todos os tecidos de ovinos encaminhados apresentavam cistos macroscópicos (figura 1, seção A B e D), estes eram caracterizados por serem brancos, redondos a ovais e estriados, que variavam de 0,3 a 1cm de diâmetro. Ao corte, as estruturas císticas apresentavam uma cápsula branca e lúmen preenchido por material gelatinoso translúcido, este material ao ser visualizado em microscópio apresentava incontáveis bradizoítos. Em contrapartida, os tecidos dos bovinos não apresentavam cistos macroscópicos, porém apresentavam lesões esbranquiçadas características da infecção por *Sarcocystis* (figura 1, seção C). Todas as amostras, tanto dos bovinos como dos ovinos, apresentavam cistos microscópicos.

Na investigação histológica dos ovinos, pode-se observar microscopicamente, estruturas alongadas, encapsuladas, hipereosinofílicas, contendo múltiplos bradizoítos em formato de banana ou meia-lua, medindo 3mm de comprimento x 1 mm de diâmetro, entremeados às fibras musculares (figura 2). Observam-se áreas multifocais de infiltrado inflamatório neutrofílico. Em outros fragmentos, também foram observados cistos teciduais, histologicamente semelhantes às descritas para o fragmento 1, porém de tamanho menor, apresentando área focal e discreta necrose da gordura e necrose flocular (fragmentação do sarcoplasma) de miócitos com quantidade discreta de neutrófilos, o diagnóstico patológico apontou: miosite neutrofílica multifocal discreta, presença moderada de cistos teciduais multifocais morfologicamente compatíveis com *Sarcocystis* spp. Não foi observada inflamação grave o suficiente para ser visualizada macroscopicamente. Em relação aos bovinos em todos os fragmentos analisados observou-se deposição de tecido conjuntivo fibroso maduro focalmente extenso acentuado (fibrose) por vezes com infiltrado linfoplasmocítico multifocal leve, nas amostras de miocárdio, foi possível observar múltiplas estruturas redondas basofílicas em meios às fibras cardíacas e no interior de algumas fibras de Purkinje (compatíveis com cistos de *Sarcocystis)*. No entanto, não foi possível distinguir as espessuras da parede do cisto usando coloração HE (figura3).

Na análise molecular, em todas as amostras foi detectado DNA específico para *Sarcocystis spp.* A partir do sequenciamento genético e avaliação destas sequencias consensos foram identificadas as espécies *S. gigantea* para ovinos e *S.cruzi* para bovinos foram encontradas.

DISCUSSÃO

Ainda existem muitas lacunas a respeito da epidemiologia da infecção pelo *Sarcocystis* nas mais diferentes espécies incluindo bovinos e ovinos. No entanto, sabemos que a infecção de bovinos e ovinos apresenta uma alta ocorrência em diversos países do mundo (Dubey et al., 2016; Hornok et al., 2015; Latif et al., 2015) incluindo o Brasil (Ruas et al., 2001; Ferreira et al.,1018; Minuzzi et al.,1018). A identificação de *Sarcocystis* é rotineiramente realizada através da observação da parede do cisto, tanto por microscopia de luz, como eletrônica (Dubey et al., 2015), porém alguns relatos têm demonstrado que algumas espécies de *Sarcocystis* são morfologicamente indistinguíveis (Gjerde 2016) e, portanto, só podem ser identificados inequivocamente por métodos moleculares. Além disso, os métodos de diagnóstico molecular são mais eficazes em termos de tempo/custo/confiabilidade do que a observação microscópica (Stojecki et al. 2012).

Salientamos que o abatedouro frigorífico é um relevante instrumento de diagnóstico de enfermidades em animais, principalmente em relação as enfermidades de caráter zoonótico (Ungar et al., 1990). Pois através de inspeção nos abatedouros é possível observar e examinar as carcaças e vísceras, em busca de condições anormais que, de alguma maneira, limitem ou impeçam o aproveitamento desses produtos ou matéria prima, e desta forma não cheguem ao consumo humano (Prata & Fukuda, 2001). Existem estudos desenvolvidos em diversos países demonstraram que a falta de acurácia na linha de inspeção tem elevado os custos de produção e perdas dos frigoríficos (Alberton 2000). Uma grande dificuldade enfrentada pelos inspetores oficiais em estabelecimentos de abate tem sido relacionada à falta de segurança em diagnosticar as diversas enfermidades e, em seguida, estabelecer o destino apropriado e confiável para as carcaças e vísceras desses animais (Freitas 1999).

Com a nova regulamentação, em que exige a condenação total de carcaças que apresentem infecção intensa por *Sarcocystis* e, carcaças que apresentem lesões leves,

devem ser destinadas ao cozimento, aumentou a importância do correto diagnóstico da Sarcocistose. Muitos abatedouros/frigoríficos não possuem a tecnologia necessária para dar correto destino as carcaças com infecção leve, levando ainda a maiores perdas econômicas tanto para o produtor como para a indústria.

Em relação aos ovinos, nossa pesquisa demonstrou a presença cistos macroscópicos, confirmados como *S. gigantea* por sequenciamento, nas amostras estudadas. Em trabalho realizado anteriormente na Espanha, avaliou-se o abate de 5720 ovinos durante um ano e encontrou cistos macroscópicos em 12% (712) das carcaças do abatedouro. Dos 712 animais que apresentaram os cistos, 564 (79%) tiveram condenação total de carcaça (Martínez et al., 2012). Panziera e colaboradores (2018) em estudo realizado em 1.479 ovinos de diferentes categorias encontra cistos macroscópicos em 31 animais abatidos e Minuzzi e colaboradores (2018) encontra 10 animais de 130 avaliados, também abatidos em abatedouro para consumo. A situação em ovinos é um pouco complicada, uma vez que até o presente não existem espécies que infectam pequenos ruminantes que apresentem caráter zoonótico, porém a condenação da carcaça é justificada com base no impacto visual negativo que os cistos podem ter sobre o consumidor (Martínez et al., 2012).

Após o sequenciamento genético nos bovinos, apenas a espécie de *S. cruzi* foi encontrada. A maioria dos estudos no Brasil, apontam o *S. cruzi* como principal espécie envolvida (Ferreira et al.,1018). Ainda assim, deve-se considerar que são recentes as técnicas moleculares para diferenciação entres as espécies de *Sarcocystis*. Embora possam existir lesões macroscópicas, muitas infecções por *Sarcocystis* passam despercebidas nos abatedouros e podem potencialmente ser fonte de infecção para os humanos. Esses dados questionam a indicação da condenação total de carcaças com infecção por *Sarocystis* por dois motivos: i. a maioria das infecções são determinadas por espécies que não apresentam caráter zoonótico e ii. A condenação de carcaças com lesões macroscópicas não impede uma potencial fonte de infecção para os seres humanos uma vez que a maior parte dos bovinos infectados não apresenta lesões macroscópicas.

Desta forma, mesmo com a preocupação em saúde pública, novas técnicas devem ser utilizadas para minimizar as perdas ao produtor e a indústria devido à alta prevalência deste protozoário nos rebanhos brasileiros. Como mostram outros trabalhos (Ruas et al.,

2001; Ferreira et al.,1018), mais de 90% dos bovinos apresentam infecção pelo *Sarcocystis* o que sugere que muitos tecidos que chegam à mesa do consumidor apresentam infecção por este protozoário e que aparentemente a condenação parcial/total de carcaças não resolve o problema. Ao nosso entendimento, medidas de saúde pública aplicáveis no controle do *Sarcocystis* e de outros protozoários com potencial zoonótico, como *Toxoplasma gondii*, envolvem a conscientização da população em não consumir tecidos cárneos crus ou malpassados ou indicar o congelamento dos tecidos prévios ao consumo.

CONCLUSÃO

A partir dos achados encontrados nas análises realizadas neste trabalho, foi possível verificar que as amostras foram corretamente condenadas por sarcocistose no frigorifico, conforme a nova legislação recomenda. E após análises moleculares as espécies *S. gigantea*, em ovinos e, *S. cruzi* em bovinos foram confirmadas.

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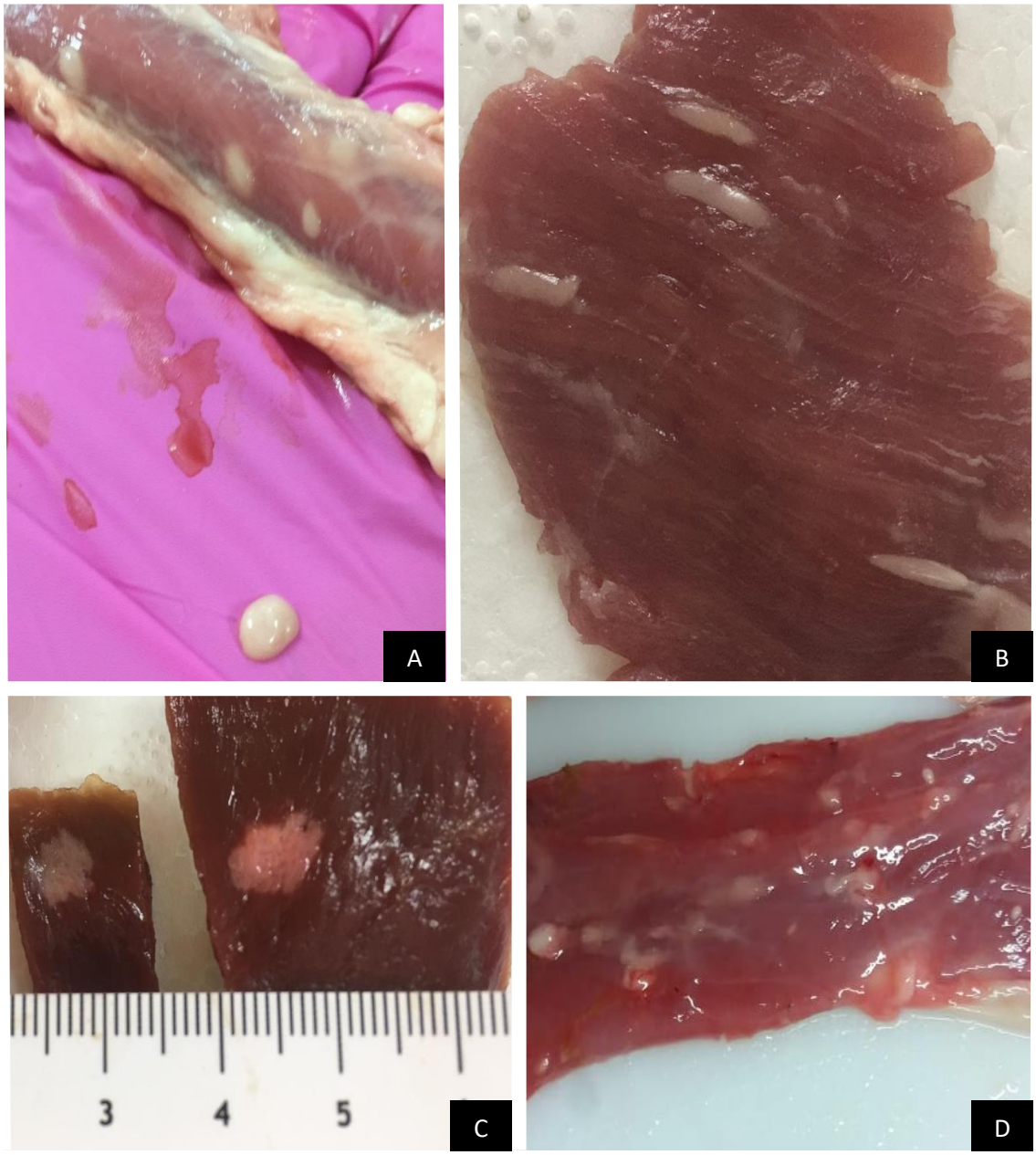

Fig.1 - Tecidos encaminhados para confirmação de sarcocistose. “A” Esôfago “B” e “D” Diafragma – ovinos com cistos macroscópicos. “C” Coração – Bovino com lesões macroscópicas.

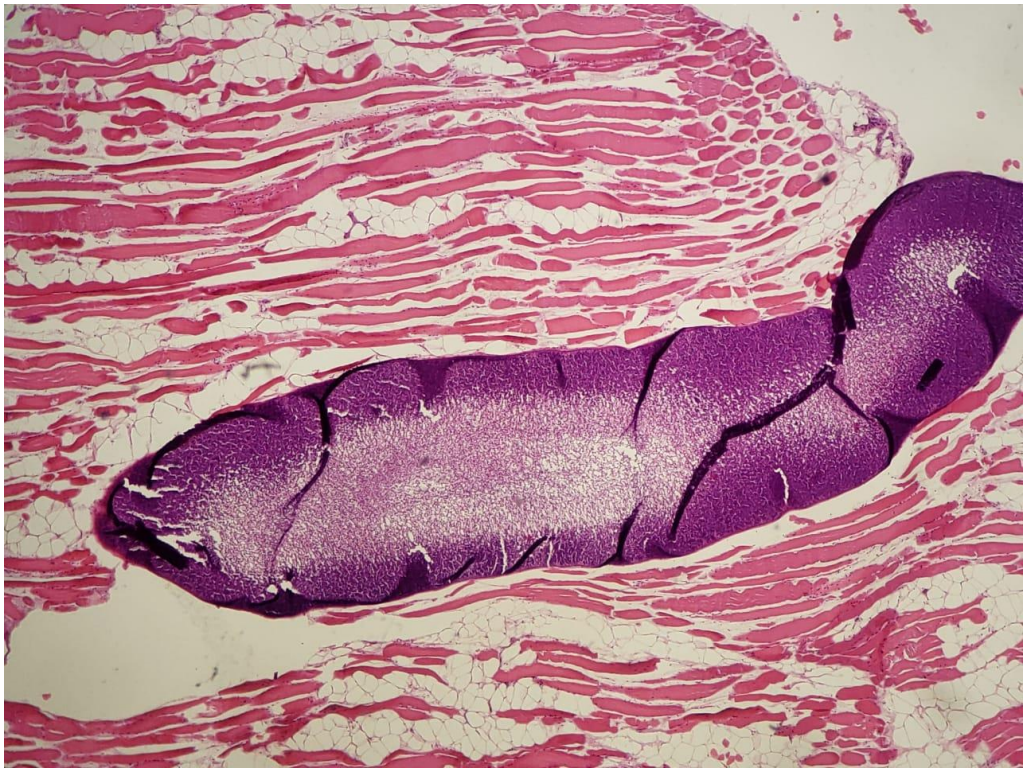

Fig.2 – Cisto apresentando caracteristica alongada, encapsulada, hipereosinofílica, contendo múltiplos bradizoítos em formato de banana ou meia-lua, medindo 3mm de comprimento x 1 mm de diâmetro, entremeados às fibras musculares

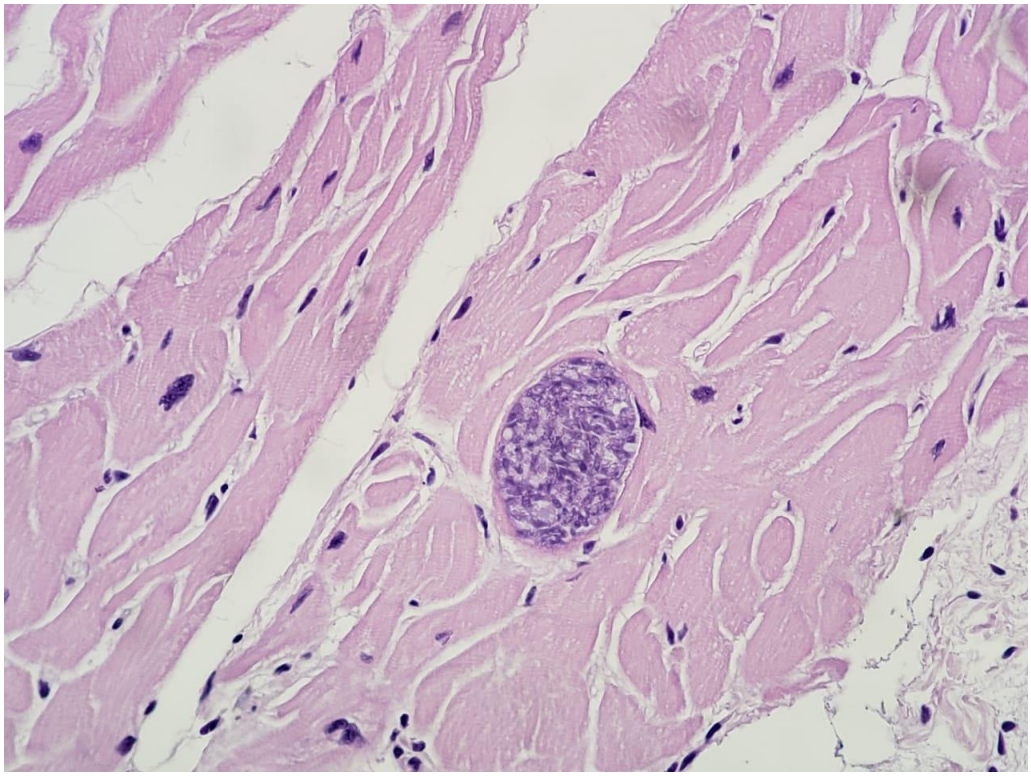

Fig.3 - Estrutura redonda basofílica em meios às fibras cardíacas (compatíveis com cistos de *Sarcocystis)*, com deposição de tecido conjuntivo fibroso maduro

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Fig. 3 –Estrutura redonda basofílica em meios às fibras cardíacas (compatíveis com cistos de *Sarcocystis),* com deposição de tecido conjuntivo fibroso maduro