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Sarcocystis spp. detection in cattle using different diagnostic methods[1]

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ABSTRACT.- Ferreira M.S.T., Fernandes F.D, Bräunig P., Guerra R.R., Sangioni L.A. & Vogel F.S.F. 2023. ***Sarcocystis* spp. detection in cattle using different diagnostic methods.** *Pesquisa Veterinária Brasileira 43:e07206, 2023.* Universidade Federal de Santa Maria, Av. Roraima 1000, Prédio 63D, Bairro Camobi, Santa Maria, RS 97105-900, Brazil. E-mail: fagner.fernandes@acad.ufsm.br

Cattle are considered intermediate hosts of *Sarcocystis,* which can cause clinical signs and lower performance in the acute phase of infection. *Sarcocystis* spp. are usually not visible to the naked eye during the *post mortem* inspection. Moreover, fresh microscopic examination and transmission electron microscopy techniques are difficult to apply to large samples. Therefore, extensive studies on *Sarcocystis* infection in cattle using molecular and serological methods are required. Here, we investigated *Sarcocystis* spp. infection in cattle using fresh microscopic examination and polymerase chain reaction of myocardium samples and compared the results with the presence of antibodies against *Sarcocystis* spp. in corresponding serum samples detected using indirect fluorescent antibody test. Microscopic *Sarcocystis* were observed in 100% of the myocardial samples, and *Sarcocystis* DNA was present in 86% (43/50) of these samples. Antibodies against *Sarcocystis* spp. were detected in 96% (48/50) and 80% (40/50) of the serum samples at 1:25 and 1:200 dilutions, respectively. The three associated methods (fresh microscopic examination, PCR and serology) showed good sensitivity and detection for *Sarcocystis* spp. compared with fresh microscopic examination (only), and they may facilitate diagnosis in live animals on a large scale as well as monitoring of the herd status.

INDEX TERMS: Sarcocystosis, bovine, Indirect Fluorescent Antibody Test (IFAT), fresh microscopic examination, PCR.

RESUMO.- [Detecção de *Sarcocystis* spp. em bovinos usando diferentes métodos de diagnóstico.] Os bovinos são considerados hospedeiros intermediários de *Sarcocystis*, podendo causar sinais clínicos e menor desempenho na fase aguda da infecção. *Sarcocystis* spp. geralmente não são visíveis a olho nu durante a inspeção *post mortem*. Além disso, o exame microscópico a fresco e as técnicas de microscopia eletrônica de transmissão são difíceis de aplicar a uma amostras de grande tamanho. Portanto, são necessários extensos estudos sobre a infecção por *Sarcocystis* em bovinos usando métodos moleculares e sorológicos. Aqui, investigamos a infecção de *Sarcocystis* spp. em bovinos por meio de exame microscópico a fresco e reação em cadeia da polimerase de amostras de miocárdio e comparado os resultados com a presença de anticorpos contra *Sarcocystis* spp. em amostras de soro correspondentes detectadas usando o teste de anticorpos fluorescentes indiretos. Sarcocistos microscópicos foram observados em 100% das amostras de miocárdio, e o DNA de *Sarcocystis* estava presente em 86% (43/50) dessas amostras. Anticorpos contra *Sarcocystis* spp. foram detectados em 96% (48/50) e 80% (40/50) das amostras de soro nas diluições 1:25 e 1:200, respectivamente. Os três métodos associados (exame microscópico a fresco, PCR e sorologia) mostraram boa sensibilidade e detecção para *Sarcocystis* spp. em comparação com o exame microscópico fresco (apenas) e podem facilitar o diagnóstico em animais vivos em larga escala, bem como o monitoramento do status do rebanho.

TERMOS DE INDEXAÇÃO: Sarcocistose, bovino, Teste de Imunofluorescência Indireta (IFAT), exame microscópico a fresco, PCR.

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INTRODUCTION

Sarcocystosis is one of the most prevalent parasitic diseases in cattle in several regions worldwide (Bucca et al. 2011, Akhlaghi et al. 2016). The prevalence of *Sarcocystis* spp. in naturally infected cattle has been investigated in many countries, and infection rates can vary depending on several factors, including the breeding system, animal age, and anatomical site of diagnosis (Dubey et al. 2016). Cattle are intermediate hosts of important *Sarcocystis* species: *S. hirsuta* and *S. hominis,* macrocyst-forming species, and *S. cruzi, S. bovifelis*, *S. bovini*, *S. rommeli*, and *S. heydorni*, as well as microcyst species (Moré et al. 2011, Hamidinejat et al. 2015). Sarcocystosis can induce weight loss, general weakness, fever, anorexia, abortion, and death in domestic animals, especially those infected with *S. cruzi*, where clinical signs and lower performance can be observed in the acute phase of infection (Hamidinejat et al. 2015).

During *post mortem* inspection in cattle, sarcocystosis is usually not visible to the naked eye, and diagnosis can be made by microscopic observation of muscular tissues (Bucca et al. 2011). Fresh examination using light microscopy is employed to detect the occurrence of cysts in tissues, and transmission electron microscopy is required to establish the presence of *Sarcocystis* based on the cyst wall ultrastructure (Akhlaghi et al. 2016). However, transmission electron microscopy has not been applied in large detection studies. Molecular diagnostic methods, such as polymerase chain reaction (PCR), are important to confirm *Sarcocystis* infection and perform epidemiological studies (Bucca et al. 2011, Moré et al. 2011, Vangeel et al. 2013). Subclinically infected animals may remain as infection sources, and serological diagnosis is crucial for large-scale identification of these animals to establish control measures in farms (Álvarez-García et al. 2014, García-Lunar et al. 2015). The indirect fluorescent antibody test (IFAT) has been widely applied to determine specific antibody levels (Moré et al. 2008, 2011, García-Lunar et al. 2015). Therefore, the aims of the present study were: a) to investigate cattle infection by *Sarcocystis* spp. in myocardium samples through direct microscopic examination and detection of *Sarcocystis* spp. of the cysts using PCR; b) to compare the frequency of parasite detection in the myocardium samples to the frequency of anti-*Sarcocystis* antibody detection in the corresponding serum samples at two different dilutions.

MATERIALS AND METHODS

Samples. Myocardial and blood samples from adult beef cattle (n=50) of both sexes were randomly collected once a week from April to July 2017 from an officially inspected abattoir located in Santiago city, Rio Grande do Sul state, southern Brazil. All slaughtered animals were obtained from commercial herds located in the central region of Rio Grande do Sul. Moreover, ten bovine fetal heart samples were collected during the viscera inspection of the slaughtered cows in the first trimester of pregnancy and analyzed using fresh microscopic examination and molecular methods.

Microscopic examination. Approximately 50g of each myocardium sample was minced for fresh microscopic examination. Each muscle was first scarified using scalpel blades and macerated in a Petri dish into 10 pieces, approximately 5g each, diluted, and homogenized with 20mL phosphate-buffered saline solution (PBS; phosphate 0.1M, NaCl 0.33M, pH 7.2). The homogenate was filtered using sterile gauze, transferred to another Petri dish, and observed using light microscopy at 400× magnification to visualize *Sarcocystis*. Samples were classified as positive or negative for *Sarcocystis* (Saito 1984) if at least one sarcocyst was observed. Ten cysts were collected from each sample using a micropipette and stored in microtubes at -20°C until DNA extraction for further molecular analysis (Moré et al. 2011). In addition, 10 negative bovine fetal heart samples were used as negative controls for PCR. Whole fetal heart tissue was cut and microscopically inspected as described above, and the absence of *Sarcocystis* was confirmed. Aliquots of 1mL of PBS solution containing fetal myocardium fragments were stored at -20°C until the next step (DNA extraction and PCR).

Nucleic acid extraction and polymerase chain reaction (PCR). According to the manufacturer's recommendations, DNA was isolated from 10 collected cysts using Wizard® Genomic DNA Purification Kit (Promega™, Madison/WI, USA). The lysis step was slightly modified (carried out at 55°C for 16 h) (Moré et al. 2011). The final concentration of the total extracted DNA was estimated using a NanoDrop ND-1000 spectrophotometer- Thermo Fischer Scientific. Then PCR was performed to amplify an approximately 700 bp fragment from the 18S ribosomal DNA (rDNA) gene using the specific primer pair: SarcoF 5′-CGCAAATTACCCAATCCTGA-3′ and SarcoR 5′-TTTCTCATAAGGTGCAGGAG-3′, as described by Yang et al. (2002). PCR cycling conditions were as described by Moré et al. (2011), using a T100™ Thermal Cycler (Bio-Rad, USA). DNA isolated from previously characterized *Sarcocystis* spp. was included as a positive control for the reaction, DNA extracted from a bovine fetal heart was negative for *Sarcocystis*, and DNA-free MilliQ water was used as a negative control. The PCR products were visualized after electrophoresis on 1% agarose gels containing SYBR® Safe DNA Gel Stain (Invitrogen, Carlsbad, USA) and visualized using an ultraviolet transilluminator.

Antibody detection. Ten milliliters of blood were collected from each animal at slaughter. Similarly, 10 blood samples from bovine fetal hearts were collected during the viscera inspection of the slaughtered cows in the first trimester of pregnancy and used as negative controls for IFAT. Sera obtained from blood samples were stored at -20°C until processing by IFAT for the detection of anti-*Sarcocystis* antibodies. Bradyzoites obtained from naturally infected bovine hearts were purified and used as antigens in a previously described procedure (García-Lunar et al. 2015). Serum samples were diluted 1:25 and 1:200 in PBS and analyzed for *Sarcocystis* spp. antibodies (García-Lunar et al. 2015). Rabbit anti-bovine IgG fluorescein isothiocyanate conjugate (Sigma BioSciences, St. Louis/MO, USA) was used as the secondary antibody. As described above, negative sera from bovine fetal serum and positive sera from a naturally infected bovine with *Sarcocystis* spp. were used as controls for IFAT. The slides were examined under a fluorescence microscope Leica CTR 4000/EBQ 100 (Leica Microsystems GmbH, Germany) using 400× magnification. Complete peripheral but not apical fluorescence of cystozoites was considered to indicate a positive result.

For statistical analysis, the Kappa coefficient and sensitivity and specificity tests were applied to compare the results among the techniques (Thrusfield 2007).

RESULTS AND DISCUSSION

The frequency of *Sarcocystis* detection by fresh examination was 100% in adult bovine heart samples (50/50). This finding is in agreement with those of studies from other countries that reported a high prevalence of sarcocystosis (Fukuyo et al. 2002, Moré et al. 2008, Bucca et al. 2011); studies in the

same region showed a 100% prevalence in cattle cardiac muscle analyzed using the same technique (Ruas et al. 2001). Therefore, the present study, following the results of previous studies, suggests that fresh microscopic examination is ideal for diagnosing infection by *Sarcocystis* spp., especially when it comes to *post mortem* diagnosis. This technique has a lower cost than PCR and serology techniques and time for its execution. A high number of cysts in adult cattle can be used to indicate *Sarcocystis* infection intensity. According to other studies, the sarcocyst number may increase with age, probably because of prolonged exposure to sporocysts or the lack of immunological control of reinfections (Ono & Ohsumi 1999, Ghisleni et al. 2006, Bucca et al. 2011). Cattle with high levels of *Sarcocystis* infection demonstrate close contact between intermediate and definitive hosts, which can be related to the appropriate sanitary conditions of husbandry practices (Bucca et al. 2011, Moré et al. 2008, 2011).

Specific antibodies against *Sarcocystis* spp. were detected in 96% (48/50) and 80% (40/50) of serum samples examined by IFAT, with titers of 25 and 200, respectively. Moré et al. (2008) showed that titers of 25 indicate infection by *Sarcocystis*, as they could be confirmed with parasitological findings. At the same time, they also observed a high number of bovines with antibody levels equal to or higher than 1:200.

Serological methods are important for detecting subclinically infected animals, representing an important infection source (Álvarez-García et al. 2014). None of the bovine fetal serum samples (10/10) demonstrated antibodies against *Sarcocystis* spp. PCR amplification products were detected in 86% (43/50) of the samples, which could be due to insufficient parasite DNA obtained from extraction and used for PCR (Moré et al. 2011), since samples with concentrations lower than 9ng/μL did not allow the amplification of *Sarcocystis* DNA. In addition, cysts were not species-characterized before PCR; therefore, mixed infections are possible (Rosenthal et al. 2008).

Two animals tested negative for IFAT at 1:25 dilution (2/50, 4%), and 10 animals were negative for IFAT at 1:200 dilutions (10/50, 20%), but these results were not in agreement with results obtained using molecular methods (7/50, 14%). The low titer of antibodies present in the serum samples probably resulted in negative IFAT results. However, some animals positive for IFAT were not positive for *Sarcocystis* DNA detection using PCR (7/48, 14.5% at 1:25 and 5/40, 12.5% at 1:200 dilutions), probably due to an insufficient amount of DNA extracted from the cysts, as discussed above (Moré et al. 2011). The high level of infection reported in the present study may cause severe economic losses due to the possibility of reduced meat quality, weight gain, and carcass condemnation (Daugschies et al. 2000, Bucca et al. 2011). The high frequency of infection in cattle is probably caused by the consumption of food or water contaminated with feces from infected dogs infected with contaminated bovine viscera and organs or carcasses (Ford 1986).

Although the microscopic examination is a qualitative diagnostic method and is ideal for detecting *Sarcocystis* infection because of its high sensitivity, PCR is extremely important for species identification, characterization, and epidemiological studies (Bucca et al. 2011, Moré et al. 2011, Vangeel et al. 2013). The statistical analysis results comparing fresh microscopic examination and IFAT techniques demonstrated greater sensitivity and specificity of the former (Table 1). In addition, fresh microscopic examination allows visualization of intact and ruptured cysts and merozoites with movement (Ruas et al. 2001). IFAT fails in *Sarcocystis* diagnostics because this technique does not always detect floating antibody levels in infected animals. Although IFAT has demonstrated a lower sensitivity than fresh microscopic examination, serological tests have great value in evaluating a large number of samples, revealing the serological status of the herd and detecting both acute and chronically infected live animals that either present clinical signs or do not (García-Lunar et al. 2015).

Table 1. Sensitivity, specificity, and Kappa value comparing IFAT 1:25 and IFAT 1:200 with direct microscopic examination

Diagnostic method	Sensitivity	Specificity	Kappa
IFAT 1:25	100%	83%	0.89
IFAT 1:200	80%	50%	0.35

CONCLUSION

Fresh microscopic examination is ideal for diagnosing infections in adult cattle, but it can only be performed *post mortem,* making it difficult to test many samples. However, it does not differentiate the species involved in the infection. Although PCR in this study did not show 100% sensitivity, this technique can be used to identify *Sarcocystis* species and be a possibility for *post mortem* diagnosis. Although fresh microscopic examination showed better sensitivity in this study, an indirect fluorescent antibody test (IFAT) can be used for live animals. Therefore, the three associated methods allowed the detection and epidemiology studies of *Sarcocystis* spp. infection.

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