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Short Communication

Macroscopic, histological, and molecular aspects of *Sarcocystis* spp. infection in tissues of cattle and sheep

Aspecto Macroscópico, histológico e molecular de *Sarcocystis* spp. em tecidos de bovinos e ovinos

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Abstract

The macroscopic, histological, and molecular aspects of *Sarcocystis* spp. were examined in the tissues of two cattle and four sheep, 16 and eight fragments analyzed respectively, condemned in the slaughterhouse. All 24 samples were collected and analyzed for detecting macrocysts and macroscopic lesions. Subsequently, subdivided for direct examination, polymerase chain reaction and histopathological examination. All sheep tissues samples had grossly white round to oval tissue cysts, ranging from 0.3 to 1 cm in diameter. In contrast, cattle tissues did not present grossly visible cysts but had randomly distributed white-yellow foci with irregular contours. All samples from cattle and sheep had microscopic cysts. In the histological examination of sheep tissues, circular to elongated, encapsulated, basophilic structures ranging from 30 to 3,000 µm in length and 20 to 1,000 µm in width were observed within the skeletal muscle fibers. In cattle tissues, all cardiac muscle four fragments analyzed contained circular to elongated basophilic structures inside cardiomyocytes and in some Purkinje fibers. PCR were performed using the primers: 2L and 3H. In conclusion, all 24 tissues were infected with *Sarcocystis* spp., and *S. gigantea* (in sheep) and *S. cruzi* (in cattle). were the identified species by sequencing.

Keywords: *Sarcocystis gigantea, Sarcocystis cruzi,* PCR, cysts, detection.

Resumo

Os aspectos macroscópicos, histológicos e moleculares de *Sarcocystis* spp. foram examinados nos tecidos de dois bovinos e quatro ovinos, 16 e oito fragmentos analisados, respectivamente, condenados no matadouro. Todas as 24 amostras foram coletadas e analisadas para detecção de macrocistos e lesões macroscópicas. Posteriormente, subdivididas para exame direto, reação em cadeia da polimerase e exame histopatológico. Todas as amostras de tecidos de ovelha apresentavam cistos grosseiramente visíveis, caracterizados como brancos, de redondos a ovais e estruturas variando de 0,3 a 1 cm de diâmetro. Em contraste, os tecidos de bovinos não apresentavam cistos grosseiramente visíveis, mas tinham focos branco-amarelos com contornos irregulares, distribuídos aleatoriamente. Todas as amostras de bovinos e ovinos apresentavam cistos microscópicos. No exame histológico de tecidos ovinos foram observadas estruturas basofílicas circulares a alongadas, encapsuladas, variando de 30 a 3.000 µm de comprimento e 20 a 1.000 µm de largura dentro das fibras do músculo esquelético. Nos tecidos de bovinos, todos os quatro fragmentos de músculo cardíaco analisados continham estruturas basofílicas circulares a alongadas, dentro dos cardiomiócitos e em algumas fibras de Purkinje. PCRs foram realizadas utilizando-se os "primers" 2L e 3H. Em conclusão, todos os 24 tecidos estavam infectados com *Sarcocystis* spp., sendo *S. gigantea* (em ovinos) e *S. cruzi* (em bovinos) as espécies identificadas por sequenciamento.

Palavras-chave: *Sarcocystis gigantea*, *Sarcocystis cruzi*, PCR, cistos, detecção.

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Introduction

Sarcocystis spp. are distributed worldwide and are the most frequent protozoa detected in the muscle tissue of animals intended for human consumption (Savini et al., 1992; Ruas et al., 2000; Moré et al., 2011). Different species of *Sarcocystis* can infect cattle and sheep (Dubey et al., 1989; Thompson, 2013). Sheep can be an intermediate host of four species of *Sarcocystis* (*S. gigantea, S. medusiformis, S. tenella,* and *S. arieticanis*), which form macrocysts or microcysts in tissues (Dubey et al., 1989). Cattle may be infected by at least seven species of *Sarcocystis* (*S. cruzi*, *S. hirsuta*, *S. hominis*, *S. rommeli*, *S. heydorni*, *S. bovini*, and *S. bovifelis*). Of these, *S. hominis* and *S. heydorni* are zoonotic species (Thompson, 2013). *Sarcocystis* infections can reduce animal productivity, can be a source of zoonotic infections for some species, and are the main reason for losses in slaughterhouses where? (Martínez-Navalón et al., 2012).

The new Regulation of Industrial and Sanitary Inspection of Animal Products (RIISPOA) was recently enacted (Brazilian Decree No. 9.013/2017). The Article 168 of RIISPOA states that "carcasses with intense *Sarcocystis* spp. infection (sarcocystosis) should be condemned and in those with mild infection the affected area should be removed, and the carcass submitted to heat treatment". Intense infection is characterized by the presence of cysts in various parts of the musculature or organ. Mild infection is defined as the presence of cysts located at a single point in the carcass or organ (Brasil, 2017).

The objective of this study was to describe the macroscopic, histological, and molecular aspects of *Sarcocystis* spp. in the tissues of cattle and sheep condemned in slaughterhouses, decreasing the rate of discards, in the case of infections of non-zoonotic species.

Materials and Methods

Samples were collected from sheep and cattle that had their carcasses condemned in the slaughterhouse Centro Sul, Dom Pedrito- RS and Frigorífico Silva, Santa Maria-RS, respectively, due to presentation of intense lesions and characteristics of sarcocystosis. The esophagus, diaphragm, tongue, and larynx were collected from four sheep, totalizing 16 samples. Skeletal muscle (pectoral, sternocleidomastoid, and masseter) and heart fragments were collected from two cattle, totalizing eight samples.

All the 24 samples (16 sheep samples and 8 sheep samples) were analyzed for the presence of macrocysts and macroscopic lesions. Subsequently, each sample was divided into two fragments. One fragment was used for direct microscopic examination followed by polymerase chain reaction (PCR). The other fragment was used for histopathological examination.

For the detection of microscopic tissue cysts, a direct examination was performed the tissues are separated individually, mixed with PBS (pH 7.3), filtered with gauze in a sterile petri dish and examined under a light microscope at 400x magnification, as described previously by Minuzzi et al. (2019). Positive samples were separated, and the cysts were harvested for DNA extraction and PCR. Total DNA from the cysts was extracted using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's recommendations, with modifications during the lysis step as described by Moré et al. (2011). After extraction, the DNA concentration in each sample was measured, and the samples were stored at -20 °C until use.

PCR reactions were performed using the 18S rRNA gene-specific primers: 2L – Forward (GGATAAACCGTGGTAATTCTATG) and 3H – Reverse (GGCAAATGCTTTCGCAGTAG), which amplified a fragment of approximately 915 bp (Rosenthal, 2010). Each PCR reaction was performed in a total volume of 25 µL containing 5× PCR buffer, 10 mM dNTPs, 10 pmol of each primer, 1.5 U Taq polymerase, and 50 ng total DNA used as a template. The conditions used to perform PCR for the genus *Sarcocystis* were initial denaturation at 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, along with a final extension at 72°C for 6 min. PCR products were analyzed using agarose gel electrophoresis (Biotium Inc., Freemont, CA, USA).

Sarcocystis spp. were identified via DNA sequencing. PCR products were purified using a commercial QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Subsequently, purified DNA samples (45 ng) were mixed with 5 pmol of each primer and MilliQ water. This mixture was dehydrated at 60°C for 2 h and subjected to gene sequencing (performed by ACTGene Sequencing Service, Alvorada- RS, Brazil) with BigDye Terminator method on a genetic analyzer (ABI 3500, Applied Biosystems, Foster City, CA). Results were analyzed using the Standard Package software, and the generated nucleotide consensus sequences were analyzed using the Genbank database (http://www.ncbi.nlm.nih.gov/BLAST).

For histopathological examination, the 24 collected fragments were fixed in 10% buffered formalin, processed for routine histopathology, and stained with hematoxylin and eosin (HE).

Results

All fragment tissues collected from sheep presented macroscopic cysts (Figure 1A, 1B). The macrocysts were white, round to oval, and striated, and ranged from 0.3 to 1 cm in diameter. On the cut surface, the cysts presented a white capsule and lumen filled with translucent gelatinous material. Macroscopic lesions were not observed in these tissues. In cattle tissues, macrocysts were observed, but irregularly contoured white-yellow foci were randomly distributed in the tissues (Figure 1C).

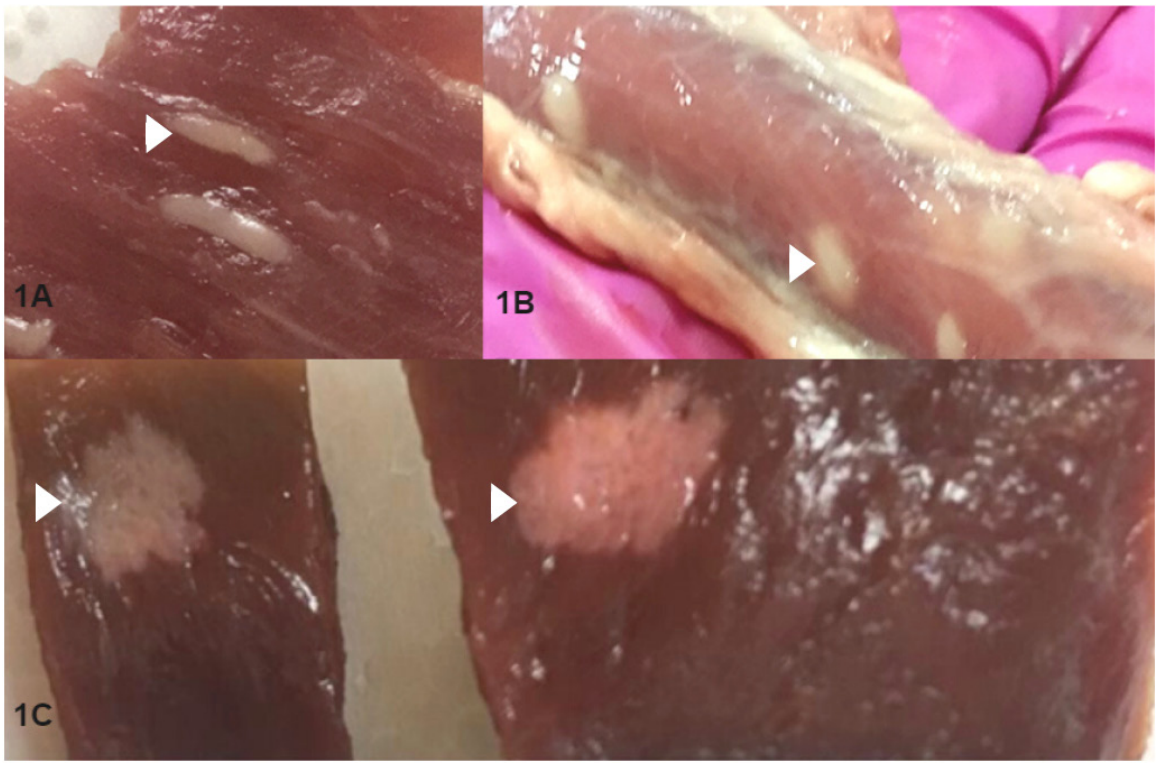

Figure 1. Sarcocystosis in ruminants. (1A) In the diaphragm of sheep; (1B) In the esophageal muscle of sheep; (1C) heart, tissues of cattle, presenting irregular white-yellow lesions.

On direct examination, microscopic cysts were observed in all samples of both cattle and sheep (Figure 2A). The microcysts had a thin wall containing some villar protrusions and septa dividing the internal compartments of the sarcocysts.

In the histopathological examination of sheep tissues, circular to elongated, encapsulated, basophilic structures ranging from 30 to 3,000 µm in length and 20 to 1,000 µm wide were observed within the skeletal muscle fibers. Multiple bradyzoites were bounded by a capsule of varying thickness from one cyst to another (less than 1 µm to 2 µm thick) (Fig. 2B). The center of some of these cysts were degenerated and characterized by a pale area. Multifocal areas of mixed inflammatory infiltrates (containing mild neutrophils, histiocytes, lymphocytes, and plasma cells) were observed. No inflammation was severe enough to be observed macroscopically. No fibrosis was associated with lesions.

For cattle, all cardiac muscle fragments analyzed contained circular to elongated basophilic structures inside cardiomyocytes (Figure 2C) and some Purkinje fibers. These structures were identical to those described for sheep but reached a maximum of 60 µm in length. Multifocal deposition of mature fibrous connective tissue was observed between the heart fibers (Figure 2D). This fibrosis was sometimes associated with the presence of parasitic cysts, and in some cases also with mild multifocal lymphoplasmic infiltration (Figure 2E). Further, fibrosis was associated with the presence of parasitic cysts.

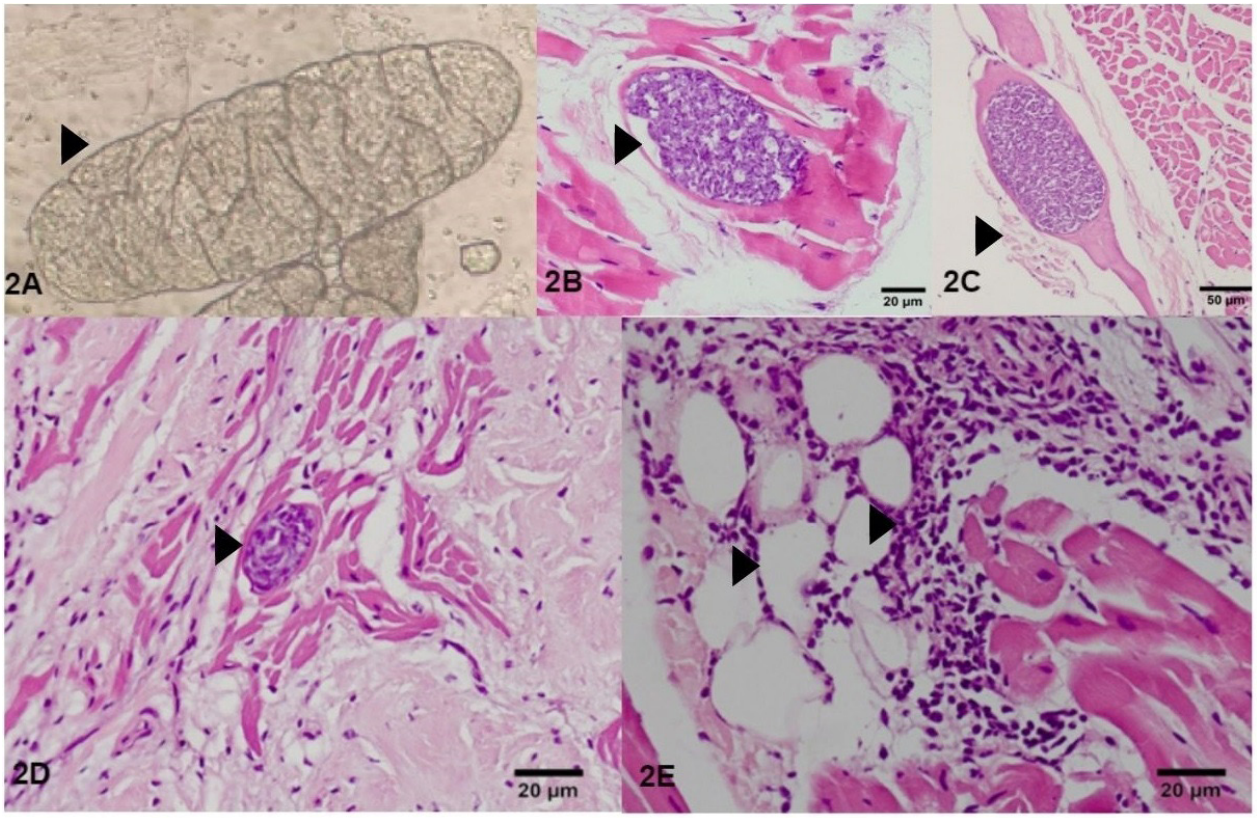

Figure 2. (2A) presence of microscopic cysts upon direct examination in sample of hearth; (2B) *Sarcocystis* spp. in sheep, in skeletal muscle, encapsulated basophilic elongated cyst containing multiple bradyzoites is observed within the muscle fiber, objective lens magnification 40×; (2C) *Sarcocystis* spp. in cattle. Encapsulated basophilic elongated cyst containing multiple bradyzoites is observed within a Purkinje fiber in the heart tissue, objective leans magnification 20×; (2D) *Sarcocystis* spp. in cattle. A small parasitic cyst inside a cardiomyocyte in heart tissue is shown. Cardiomyocytes from the affected area are surrounded by mature fibrous tissue (fibrosis), objective lens magnification 40×; (2E) *Sarcocystis* spp. in cattle. Discrete mixed inflammatory infiltrate containing neutrophils, histiocytes, lymphocytes and plasmocytes is observed in heart tissue along with cardiomyocytes and adipocytes, indicating myocardial sarcocystosis. objective magnification 40×. Hematoxylin and eosin.

DNA specific for *Sarcocystis* spp. was detected in all samples using PCR. Genetic sequencing and evaluation of these consensus sequences identified *S. gigantea* in the tissues of sheep and *S. cruzi* in the tissues of cattle.

Discussion

Sarcocystis spp. were detected by direct microscopic examination in all tissues of sheep and cattle. *Sarcocystis* infections are highly prevalent in many countries globally (Dubey et al., 2016; Hornok et al., 2015; Latif et al., 2015), including Brazil (Ruas et al., 2000; Ferreira et al., 2018; Minuzzi et al., 2019). Identification of *Sarcocystis* spp. is routinely performed by observing the cyst wall via light and electron microscopy (Dubey et al., 2016; Gjerde, 2016). However, some species of *Sarcocystis* are morphologically indistinguishable (Gjerde, 2016) and therefore can only be identified by molecular methods (Stojecki et al., 2012). Therefore, molecular studies are important for the identification of zoonotic species in samples.

In the present study, the presence of *S. gigantea* macroscopic cysts in sheep tissue samples was confirmed by sequencing. Histopathological examination identified multiple bradyzoites bounded by a capsule surrounded by inflammatory cells. In a previous study in Spain, during a 1-year period, macroscopic cysts were found in 12% (712/5720) of slaughterhouse sheep carcasses, where narrow and/or wide cysts were found in striated muscles and oval cysts in the esophagus muscle. Of the 712 animals presenting with cysts, 564 (79%) had total carcass condemnation (Martínez-Navalón et al., 2012). A study of 1,479 sheep (Panziera et al., 2018) of different categories found macroscopic cysts in 31 slaughtered animals. In Brazil, 10 of 130 animals slaughtered for consumption were infected with *Sarcocystis* spp. (Minuzzi et al., 2019). The situation in sheep is complex. No species that infect small ruminants have a zoonotic character, and carcass condemnation is based only on the negative visual impact

that cysts may have on the consumer, which could be resolved by removing the parts presenting with the cysts (Martínez-Navalón et al., 2012).

Sarcocystis cruzi was identified in heart samples from cattle after genetic sequencing. Upon histopathological examination, cysts were found in all cardiac muscle fragments with multifocal deposition of mature fibrous connective tissue (fibrosis) in the middle of the heart fibers and was sometimes associated with mild multifocal lymphoplasmic infiltration. In southern Brazil, *S. cruzi* is the main species involved and may be related to the habitual coexistence of definitive (dogs) and intermediary hosts (cattle) in Brazilian farms, favoring the parasite life cycle (Ferreira et al., 2018).

The slaughterhouse is an important place for diagnosing animal diseases, especially zoonotic diseases (Ungar et al., 1990). By inspecting slaughterhouses, it is possible to observe and examine carcasses and viscera for abnormal conditions, thus preventing their consumption by humans (Prata & Fukuda, 2001). However, a major difficulty faced by official inspectors in slaughterhouses has been related to a lack of certainty in diagnosing various diseases and establishing an appropriate and reliable destination for the carcasses and offal of these animals (Freitas, 1999). The new regulation (RIISPOA) requires the total condemnation of carcasses that present intense Sarcocystis infection. For carcasses with mild lesions the affected area should be removed, and the carcass heat treated, conditional use of the carcass, decreasing disposal rates.

However, total condemnation of carcasses with *Sarcocystis* infection does not solve this problem. Most of the infected cattle have no macroscopic lesions, and more than 90% of cattle are infected by *Sarcocystis* spp. without zoonotic potential (Ruas et al., 2000; Ferreira et al., 2018). Public health measures applicable to the control of *Sarcocystis* spp. and other protozoa with zoonotic potential, such as *Toxoplasma gondii*, involve raising awareness among the population not to consume raw or rare meat tissues and indicating freezing of pre-consumed tissues.

In summary, all tissues examined were infected with *Sarcocystis* spp. Molecular analysis confirmed the presence of *S. gigantea* in sheep and *S. cruzi* in cattle. Given the concern for public health, further studies and new techniques should be used to minimize losses to producers and industry due to the possibility of a high prevalence of this protozoan in Brazilian herds.

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