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ORIGINAL PAPER

Sarcocystosis in wild red deer (*Cervus elaphus*) in Patagonia, Argentina

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Abstract *Sarcocystis* spp. are protozoan parasites with a heteroxenous life cycle, which produce cysts in the muscle of herbivorous animals. In these animal species, sarcocystosis is frequently asymptomatic, although it may occur with high prevalence. Seven *Sarcocystis* spp. have been described in red deer (*Cervus elephus*). The aim of this study was to determine the prevalence of sarcocystosis, and to perform the morphological and molecular characterization of *Sarcocystis* spp. found in wild red deer of the Nahuel Huapi National Park (NHNP), Patagonia, Argentina. Full necropsies of 62 red deer killed by hunters in the NHNP and neighboring areas were performed. Samples of heart and skeletal muscle were examined histologically and selected samples were also examined by transmission electron microscopy (TEM), PCR and sequencing. *Sarcocystis* spp. thin walled cysts were detected in 62 % (38/62) of heart, and in 22 % (3/14) of skeletal muscle samples examined histologically. TEM revealed a smooth and thin cyst wall (≤1 μm), with scarce and separated ribbon-like protrusions. A total of three partial and one full 18S ribosomal RNA (rRNA) gene sequences were obtained, and showed the highest identity (≥99 %) with *Sarcocystis taeniata*, a species described in moose (*Alces alces*). The morphological and molecular results indicate that red deer in Argentina are frequently infected with *S. taeniata*, a species for which the definitive host is unknown. The present results also confirm that *Sarcocystis* spp. using cervids as intermediate host are not host-specific. Further studies are needed to improve the epidemiological knowledge of Sarcocystosis in red deer.

Keywords *Sarcocystis taeniata* · Red deer · Argentinean nonnative ungulate · TEM · Sequencing

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Introduction

Red deer (*Cervus elaphus*) have been introduced in several countries and have become a serious threat to wildlife and agriculture (Novillo and Ojeda 2008). These ungulates can carry and transmit pathogens of concern to livestock and wildlife species (Haigh et al. 2002; Mackintosh et al. 2002; Gortázar et al. 2006; Böhm et al. 2007). In addition, red deer can damage the local flora. In Argentina, environmental damage by introduced and invasive European red deer has been documented (Novillo and Ojeda 2008). However, information on diseases carried and transmitted by this introduced species is scant.

Sarcocystis spp. are intracellular protozoa with an heteroxenous life cycle, that produce muscle cysts in the intermediate hosts, such as domestic cattle, sheep, pigs, wild cervids, and also humans, and reproduce sexually in the

intestine of definitive hosts (DH; several carnivorous or omnivorous animal species) (Dubey et al. 2015). Most *Sarcocystis* spp. infections (sarcocystosis) are asymptomatic, although disease may occur in intermediate hosts (Dubey et al. 2015; Avapal et al. 2004). *Sarcocystis suihominis* and *Sarcocystis hominis*, which infect pigs and cattle, respectively, have public health importance because humans are their definitive hosts in which nausea, abdominal pain and diarrhea have been reported (Tenter 1995; Fayer 2004; Solaymani-Mohammadi and Petri 2006).

Cervid animals are intermediate hosts of several *Sarcocystis* spp., some of which have unknown definitive hosts and other uses canids or felids as DH (Dubey et al. 2015). In North America and Europe, sarcocystosis has been reported in elk (*Cervus canadensis*), white-tailed deer (*Odocoileus virginianus*), mule deer (*Odocoileus hemionus*), roe deer (*Capreolus capreolus*), reindeer (*Rangifer tarandus tarandus*), moose (*Alces alces*) and red deer (Pond and Speer 1979; Mahrt and Colwell 1980; Crum et al. 1981; Dahlgren and Gjerde 2007; Dahlgren et al. 2008; Gjerde 2013; Dubey et al. 2015). Although no zoonotic *Sarcocystis* spp. has been reported in cervids the impact of sarcocystosis in these species has not been thoroughly investigated (Pond and Speer 1979; Mahrt and Colwell 1980; Crum et al. 1981; Dubey et al. 2015) and it is not known if *Sarcocystis* spp. is host-specific for cervid species. Thorough morphological descriptions, cross-infection studies, and/or DNA sequence data are necessary to achieve proper identification of *Sarcocystis* spp. (Dubey et al. 2015). Several *Sarcocystis* spp. are reported to affect European red deer. Among these are *Sarcocystis cervicanis*, *Sarcocystis hjorti*, *Sarcocystis hardangeri*, *Sarcocystis ovalis*, *Sarcocystis elongata*, *Sarcocystis tarandi*, and *Sarcocystis truncata*, most of which have unknown DH (Dahlgren and Gjerde 2010; Gjerde 2014a; Dubey et al. 2015).

In the United States, Pond and Speer (1979) have reported prevalences of sarcocystosis of 80 % (57/72) for mule deer, 50 % (12/24) for white-tailed deer, 50 % (12/24) for elk, and 13 % (2/15) for bison. In Canada, a health surveillance found a prevalence of the infestation of 100 % (2/2) for elk, 96 % (196/205) for moose, 94 % (17/18) for bison, 75 % (27–36) for mule deer, 75 % (3/4) for bighorn sheep (*Ovis canadensis*), 73 % (11/15) for mountain goats (*Oreamnos americanus*), and 49 % (137/277) for white-tailed deer (Mahrt and Colwell 1980). In Argentina, a prevalence of *Sarcocystis cruzi* cysts of 71.5 % (372/380), and thick-walled *Sarcocystis* spp. of 23.1 % (88/380) were reported in loin samples from cattle (Moré et al. 2011). Further molecular differentiation of *Sarcocystis* spp. in the same samples revealed 313, 29, 14, and 2 positive samples for *S. cruzi*, *S. sinensis*, *S. hirsuta* and *S. hominis*, respectively (Moré et al. 2013). In the same country, *Sarcocystis* spp. (species not identified) was found in guanacos (*Lama guanicoe*) with a prevalence of 67 % (8/12) (Beldomenico et al. 2003). However, to the best of our knowledge, the prevalence of sarcocystosis and the identification of the *Sarcocystis* spp. infecting wild deer has not been reported in Argentina.

The aim of this study was to determine the prevalence of sarcocystosis, and to perform morphological and molecular identification of the *Sarcocystis* spp. in wild red deer (*C. elaphus*) in Patagonia, Argentina.

Material and methods

Samples

Field work was conducted during the red deer hunting season in the Nahuel Huapi National Park (NHNP) and in livestock farms located close to this National Park, in the area of San Carlos de Bariloche and Junín de los Andes, Río Negro and Neuquén provinces, respectively, all in Northern Patagonia, Argentina.

Full necropsies were performed on 62 red deer, and samples of heart ($n = 62$) and skeletal muscle ($n = 14$) were collected and used for this study.

Histological examination

Sixty two hearts (apical area) and 14 skeletal muscle (gluteal muscles) samples were fixed by immersion in 10 % buffered formalin, pH 7.2, for 24 to 60 h before being embedded in paraffin wax, sectioned at 4 μm, and processed routinely for the production of hematoxylin and eosin stained sections.

Direct microscopic examination

Additional heart and skeletal muscle samples from red deer were collected and frozen at −20 °C. After histologic examination of all the samples was complete, only the heart samples from those animals in which *Sarcocystis* spp. were detected histologically, were thawed and processed for direct microscopic examination as previously described (Moré et al. 2011). Briefly, 5 to 10 g of each sample were ground in a tissue homogenizer with the addition of 50 ml phosphate buffered saline (PBS), pH 7.2. Two to three milliliter-aliquots of the homogenate were placed in a Petri dish, diluted with 10 ml of PBS and observed in an inverted microscope. Sarcocysts or cysts parts observed were counted, and each sample was categorized according to the presence of low (1–2 cysts/plate), moderate (3–5 cysts/plate), or abundant (≥6 cysts/plate) numbers of cysts.

To collect sarcocysts for further analyzes, five fresh samples of skeletal muscle (gluteal muscles) and the same numbers of samples from heart were refrigerate (4–8 °C), processed as sample of pooled muscles for each animal within

1 week, and then used for direct microscopic examination following the methodology mention above.

Transmission electron microscopy

Ten sarcocysts detected during direct examination of refrigerated sample of pooled muscles homogenates from 3 different animals were collected using micropipettes, fixed in 2 % glutaraldehyde, processed and analyzed by transmission electron microscopy (TEM) as previously described (Moré et al. 2011).

PCR and sequencing

DNA extraction was performed from 10 individual sarcocysts collected during direct microscopic examination of the refrigerated samples, using a commercial kit (Wizard Genomic, Promega, USA) according manufacturer's instruction. A fragment of the *Sarcocystis* spp. 18S ribosomal RNA (rRNA) gene was amplified by polymerase chain reaction (PCR) using the primers SarcoFext and SarcoRext previously described (Moré et al. 2013). Amplification products (with an estimated concentration of at least 40 ng/μl) were purified using the QIAQUICK purification kit (QIAGEN, Germany) and submitted for sequencing to the Lightrun service of GATC Biotech with both primers mentioned above. Additionally, two positive samples in the mentioned PCR were amplified with primers ERIB1 and PrimerB in order to amplify the full-length sequence of the 18S rDNA of most *Sarcocystis* spp. (~1850 bp). These products were purified, cloned into plasmids and sequenced as previously described (Moré et al. 2013). Sequences obtained were aligned and analyzed using the Geneious software. Consensus sequences obtained were compared with others reported in GenBank by BLAST analysis.

Results

Histology and direct microscopic examination

Sarcocystis spp. cysts were observed in HE sections of 38/62 (62 %) of heart (Fig. 1) and in 3/14 (22 %) of skeletal muscle samples. The sarcocysts were always seen within skeletal or cardiac muscle fibers which in most cases showed no muscle fiber degeneration, although segmental hyaline change was occasionally seen in infected muscular fibers. All cysts contained numerous mature banana-shape bradyzoites. Three out of 38 heart samples containing sarcocysts showed mild, interstitial, focal, lymphoplasmacytic myocarditis, while one of those 38 samples showed steatosis. Hyalin degeneration of fibers was observed in the three skeletal muscle samples containing sarcocysts but not in any of the infected myocardial fibers. Very few lymphocytes were occasionally present surrounding the infected muscle cells.

By direct microscopic examination of 34 heart samples (four samples were not analyzed due to insufficient amount of tissue), *Sarcocystis* spp. infection was categorized as low in 44 % (15/34), moderate in 27 % (9/34), and abundant in 29 % (10/34). All five sample of pooled muscles evidenced microscopical sarcocysts. All sarcocysts observed were fusiform, clearly divided by septa and measured up to 700 μm long and 40–90 μm wide. The sarcocyst wall was thin (≤1 μm) and had scarce and hair-like villar protrusions (Fig. 2).

All the cysts processed by TEM had a smooth and thin wall (≤1 μm), with scarce and separated ribbon-like protrusions (vp). The vp were 0.5–1 μm long and 0.1–0.2 μm thick (Fig. 3). The ground substance layer measured 0.5 μm and lacked microtubules. Bradyzoites contained numerous micronemes located in the anterior half and amylopectin granules located in the posterior half (Fig. 3).

PCR and sequencing

A total of three consensus sequences (857, 845 and 765 bp) were obtained from different samples amplified with SarcoFext/SarcoRext and deposited in the GenBank (accession numbers KT626599, KT626600, and KT626601). The three sequences showed the highest identity (≥99 %) with *Sarcocystis taeniata* sequences (KF831279, KF831292, among others) and also 98 % with a *Sarcocystis grueneri* sequence (KC556825). Two cloned amplification products were obtained and identical consensus sequences of 1886 bp were obtained; BLASTn comparison revealed a 99 % sequence identity with *S. taeniata* sequences (KF8312895, KF8312896, KF831289, among others). The full-length 18S rRNA gene sequence obtained in the present study was deposited in the GenBank with the accession number KT626602.

Discussion

The results of morphological and molecular studies conducted on sarcocysts obtained in the present study lead to the identification of *S. taeniata*, a species previously described in moose (*A. alces*) from Canada (Gjerde 2014b), but none of the *Sarcocystis* spp. previously described in red deer was identified. *S. taeniata* was recently described in moose, cyst wall morphology was based only in scanning electron microscopy (SEM), which did not allow to differentiate it from *S. grueneri* (Gjerde 2014b). Differentiation between these two species was achieved by 18S rRNA and *cox*1 gene sequence comparison (Gjerde 2014b). In the present study, the scarce and thin ribbon-like villar protrusions detected in the TEM observation are in agreement with the previous *S. taeniata* SEM images

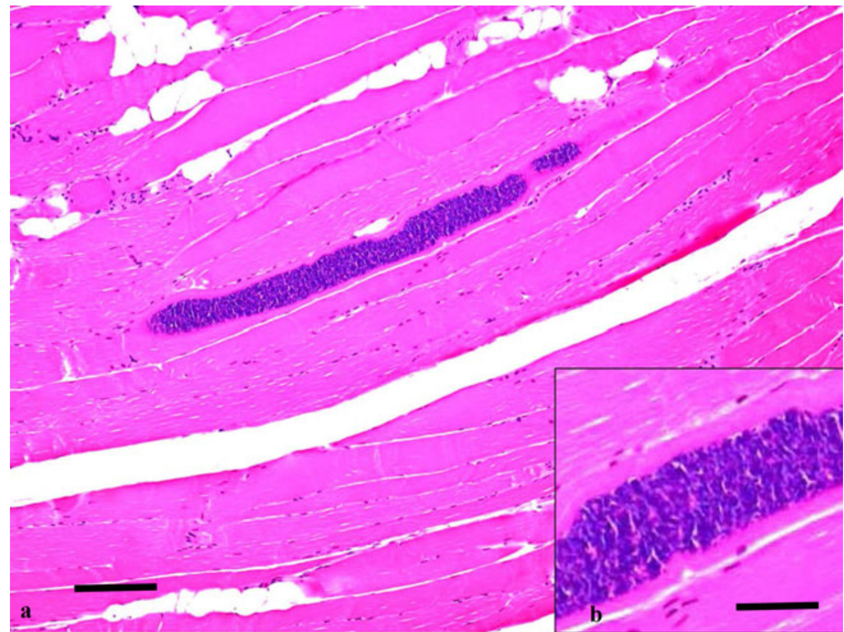

Fig. 1 Histopathological identification of sarcocysts in heart tissue from a red deer (hematoxylin and eosin stain). Magnification, *scale bar*: **a** ×100, 50 μm and **b** ×400, 12 μm

from Gjerde in 2014. Additionally, the 18S rRNA sequences obtained in the present study showed the highest identity (≥99 %) with *S. taeniata* sequences in GenBank. Phylogenetic analysis of the mentioned sequences evidenced a close relation with *S. grueneri* sequences from reindeer (*R. t. tarandus*) (Gjerde 2014b). This species of *Sarcocystis* uses reindeer as intermediate host, and fox (*Vulpes vulpes* and *Vulpes lagopus*), raccoon-dog (*Nyctereutes procyonides*) and dog as DH (Dubey et al. 2015). The DH of *S. taeniata* remains unknown. Based on these similarities, it is possible to suggest that *S. taeniata* uses canids as DH. Experimental infection and molecular studies are required to confirm/rule out this hypothesis.

In the UK, an increase in the prevalence of *Sarcocystis* spp. was reported in free-range deer (Böhm et al. 2006) over a 7-year period. It has been suggested that this condition may reflect the high animal concentration and frequent contact of affected animals with the DH. In Patagonia, although the evolution of the prevalence of sarcocystosis is not known, the high prevalence of *Sarcocystis* spp. infection in heart (62 %) identified in red deer in this study, suggests that there

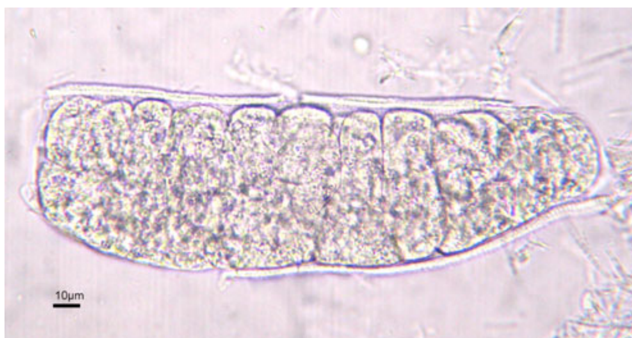

Fig. 2 Direct microscopical observation of a sarcocyst portion from a red deer sample of pooled muscles. Note the thin wall and undulating cyst surface

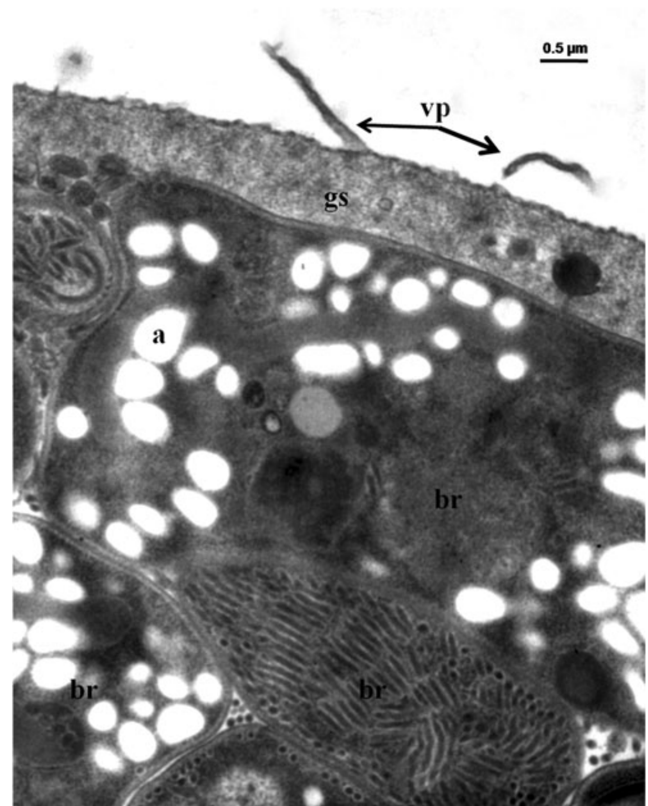

Fig. 3 Transmission electron microscopical (TEM) examination of the cyst wall from a microscopical sarcocyst in reed deer (*C. elaphus*). Note the scarce ribbon-like villar protrusions (*vp*), a thin ground substance layer (*gs*), and sectioned bradyzoites (*br*) containing several electron lucid amylopectin granules (*a*)

is also a frequent contact between the DH and deer carcasses. Additionally, the selection of hearth tissues sampled in the present study could have an influence in the outcome of *S. taeniata*, which may have a "predilection" of myocardium in red deers.

It is known that a major cause of death of European red deer in Patagonia is hunting and predation by pumas (*Felis concolor*) (Flueck et al. 2005; Novillo and Ojeda 2008). It is therefore possible that pumas play a role as definitive host of *Sarcocystis* spp., and also that hunting activity (which usually involves abandoning significant parts of the deer carcasses in the field) could increase access to those carcasses by other potential DH, such as feral dogs (*Canis lupus familiaris*), Andean fox (*Lycalopex culpaeus*), Patagonian weasel (*Lyncodon patagonicus*), or scavenger birds such as the Andean condor (*Vultur gryphus*) and Southern crested caracara (*Caracara plancus*). More studies are needed in order to identify properly other *Sarcocystis* spp. affecting wild red deers in Argentina.

Most samples analyzed in the present study showed cysts in the muscle which were not associated with gross or microscopic lesions; however, a few samples showed focal lymphoplasmacytic myocarditis and hyaline degeneration of skeletal muscle. Similar lesions have been reported in experimental infections with *S. cruzi* in calves and with *Sarcocystis tenella* in lambs (Dubey et al. 2015)as well as with *Sarcocystis* spp. in rocky montain elk calves (Foreyt et al. 1995) .

The result of the present study indicates that wild European reed deer in Argentina are frequently infected with *S. taeniata*, a species which affects also moose. The present study represents the first report of *S. taeniata* in the Southern Hemisphere and in a different cervid species than moose. Moreover, the present results support the hypothesis that *Sarcocystis* spp. from wild cervids could affect more than one cervid animal species as intermediate host, as *S. taeniata*, which was thought to infect only moose, has now been demonstrated to infect also red deer.

The potential for *Sarcocystis taeniata* of deer to be transmitted to native wildlife and livestock, as well as its potential zoonotic potential requires further investigation.

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Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

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