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Pampas fox (*Lycalopex gymnocercus*) new intermediate host of *Sarcocystis svanai* (Apicomplexa: Sarcocystidae)

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ABSTRACT

Several *Sarcocystis* spp. have carnivores as definitive host and sarcocysts are common in muscles of herbivores (intermediate host). However, sarcocysts have been found in muscles of wild and domestic carnivores suggesting they are intermediate host for some *Sarcocystis* spp. Here, we report mature sarcocysts in the muscles of Pampas fox (*Lycalopex gymnocercus*). A total of 36 free-living foxes were analyzed. Different skeletal muscles were assessed by microscopic and molecular methods. Cysts and/or DNA of *Sarcocystis* sp. were detected in 61.1% (22/36) foxes. Histopathology revealed the presence of sarcocysts in 52.8% (19/36) foxes. The tongue and masseter were the muscles more frequently infected. Of all the samples processed by homogenization of pooled muscles of each animal, 45.4% (10/22) evidenced muscle cysts and 68.2% (15/22) resulted positives by PCR. Individual cysts obtained from the ten positive samples in direct microscopic examination were all positive by PCR. Five amplicons from individual cysts from different samples were selected for sequencing together with four PCR products obtained from the pooled muscles. All nine sequences shared a high identity among them (99.8–100%) and showed the highest identity by BLAST (99%) with a *S. svanai* sequence (KM362428) from a North American dog. By transmission electron microscopy, the sarcocyst wall was thin (<1 μm), had minute undulations, with tiny evaginations and without evident villar protrusions. The cyst wall type is referred as "type 1". *Sarcocystis svanai* infects *L. gymnocercus* with a high prevalence and the presence of mature sarcocysts suggests the role of the Pampas fox as natural intermediate host. The definitive host of *S. svanai* remains unknown.

1. Introduction

Protozoans of the genus *Sarcocystis* (phylum Apicomplexa) are intracellular parasites infecting a high proportion of animals worldwide, including man. Generally, these protozoans have an obligatory two-host and a prey-predator life cycle. Rarely some *Sarcocystis* spp. are transmitted through scavenger-carrion or cannibalism relationships. Sexual development of *Sarcocystis* species occurs in the intestinal mucosa of predator or scavenger definitive hosts (DH), usually carnivores or omnivores, which excreted sporulated oocyst or sporocysts in feces. Intermediate hosts (IH), typically herbivores or omnivores; become infected by ingesting sporocysts via food or water, and parasites reproduce asexually culminating with intracellular cysts (sarcocysts) formation in muscular or neural tissues. The DH becomes infected by ingesting IH tissues with sarcocysts [1].

Several species of *Sarcocystis* have been described in domesticated and wild animals. Altogether, these species have considerable veterinary, economic, and public health importance. However, few of these species have a complete description of their life cycle, while in many cases the information about life cycles is still incomplete [2].

Carnivores typically act as DH of several *Sarcocystis* spp. Nevertheless, during the last years, sarcocysts have been found in muscles of wild and domestic carnivores (including canids, felids and mustelids) acting as IH in different parts of the world [3,4,5,6], but there are no reports in South American carnivores. Some authors considered that muscular sarcocystosis found in carnivores were incidental or unusual infections which resulted from immunosuppression [7,8,9,10]. However, this hypothesis is doubtful because even clinically healthy carnivores have sarcocysts [5,11]. Moreover, it is thought that *Sarcocystis* species

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generally have relatively high host specificity on their IH [1]. Recently, a variety of *Sarcocystis* spp. have been described for canids as IH (e.g. *S. arctica* in *Canis lupus* [6]; and in *Vulpes lagopus* [4]; and *S. caninum* and *S. svanai* in domestic dog [5]).

The Pampas fox, *Lycalopex gymnocercus*, is the most abundant of the wild canids from South America and inhabits grasslands and open woodlands [12]. It is a generalist and opportunistic mesopredator, thus it is able to adapt to large environmental modifications and variations in its prey population dynamics [12,13]. The dietary items vary according to seasonal availability and geographic location [14,15]. Pampas foxes are described as DH for *Sarcocystis* spp. [16]; although they have not been previously shown to be IH for parasites of the genus [2].

This paper is the first report in South America of natural infection in muscle of *Lycalopex gymnocercus* with *Sarcocystis svanai*, a species recently described in domestic dogs in North America [5].

2. Materials and methods

2.1. Samples

The study was conducted in rural areas located in five departments (Azul, Mar Chiquita, Balcarce and General Madariaga) from Buenos Aires province, Argentina. This area is currently dominated by cattle farming and agricultural activities and it is home to high densities of *Lycalopex gymnocercus* [12].

In this study a total of 36 free-living *L. gymnocercus* were analyzed. Most of these animals were recovered during the commercial hunting season authorized in Buenos Aires (n = 26) and others (n = 10) were found dead along road ways animals. The sample's collection and transport to the laboratory was allowed by the *Ministerio de Asuntos Agrarios* and *Dirección de Flora y Fauna* of Buenos Aires. Samples of the tongue, diaphragm, masseter, triceps, semitendinosus and heart were obtained at necropsy and an aliquot was fixed in buffered formalin 4% for 48 h for histopathological analysis (n = 36). Additionally, samples of the same muscles above mentioned were separated from each animal (n = 22) and were kept at −20 °C (n = 11) or refrigerated at 2–8 °C (n = 11) for further processing by homogenization, for microscopic and molecular studies.

2.2. Histopathological analysis and direct microscopic observation

Formalin-fixed muscular samples were dehydrated in serial alcohol solutions, embedded in paraffin, and sectioned (5 μm). Sections were deparaffinized, hydrated, stained with hematoxylin and eosin (H&E) and mounted with balsam for additional light microscopic examination. Digital images of sarcocysts in histological sections were recorded with a camera (Leica DFC295).

Ten grams of pooled muscles of each animal (n = 22) were minced in a meat grinder adding 50 ml of PBS. The homogenate was filtered using a strainer with gauze, collected in a 50 ml tube, and centrifuged at 600 ×*g* for 5 min. The supernatant was discarded and a sample (1 ml) of the pellet was kept in 1.5 ml eppendorf for DNA extraction. The remaining was resuspended in 20 ml of PBS and put in a Petri dish for observation in an inverted microscope (Nikon). Sarcocysts observed were purified and preserved essentially as described previously for molecular studies and *Sarcocystis* spp. identification [17]. Additionally, sarcocysts observed in refrigerated samples with no evident autolysis were purified and several cysts from each animal sample were fixed in 2.5% glutaraldehyde for Transmission electron microscopy (TEM).

2.3. Molecular analysis

DNA was extracted from all the homogenates of pooled muscle samples (n = 22) and from individual cysts using a commercial kit (Wizard genomic DNA purification, Promega) according to manufacturer's instructions. A fragment of the *Sarcocystis* spp. 18S ribosomal RNA (rRNA) gene was amplified by polymerase chain reaction (PCR) using the primers SarcoFext and SarcoRext as previously described [17]. Amplification products (with an estimated concentration of at least 40 ng/μl) were purified using a commercial kit (Wizard SV clean upsystem, Promega) according to manufacturer's instructions, and submitted for sequencing to the Genomic Unit, in the Biotechnology Institute CICVyA – CNIA –INTA, Argentina, with both primers mentioned above. Sequences obtained were aligned and analyzed using the Geneious software (version R9). Consensus sequences obtained were compared with others reported in GenBank by BLASTn analysis.

2.4. Transmission electron microscopy (TEM)

Fixed sarcocysts observed in the refrigerated muscle samples were submitted to the Electron microscopy Central Service from the Faculty of Veterinary Sciences, National University of La Plata, Argentina. Samples were processed by standard techniques with a vacuum protocol and observed in a JEM 1200 EX II (JEOL) transmission electron microscope as previously described [18].

3. Results

The 36 Pampas foxes (18 females; F and 18 males; M) analyzed in the present work belonged to different departments of Buenos Aires (25 from Azul, 7 from Balcarce, 2 from General Madariaga, and 2 from Mar Chiquita). Twenty nine foxes of the 36 were classified as adults (A) and seven as juveniles (J) according to their size. More than half of the foxes analyzed (22/36 = 61.11%) had at least one cyst and/or DNA of the genus *Sarcocystis* in many of the muscles (10 F/A; 2 F/J and 10 M/A).

3.1. Histopathological analysis and direct microscopic observation

Histopathology revealed the presence of fusiform basophilic bodies with septae consistent with cysts of the genus *Sarcocystis* in 52.8% (19/36) of the Pampas foxes analyzed (10 F/A; 1 F/J and 8 M/A). The cysts were scarce in each sample. The tongue was the muscle more frequently infected (11/19), followed by the masseter (8/19), the triceps (3/19), the semitendinosus (3/19) and the diaphragm (1/19). In four foxes, cysts were found in several muscles (n = 3, semitendinosus, triceps and masseter; and n = 1, tongue and masseter). The cysts appeared to be thin-walled, mature (with banana-shape bradyzoites), and with no visible surrounding inflammatory reactions (Fig. 1). No cysts were found in the heart.

Of all the samples processed by homogenization of pooled muscles and microscopy, only 45.4% (10/22) evidenced muscle cysts (4 F/A; 1

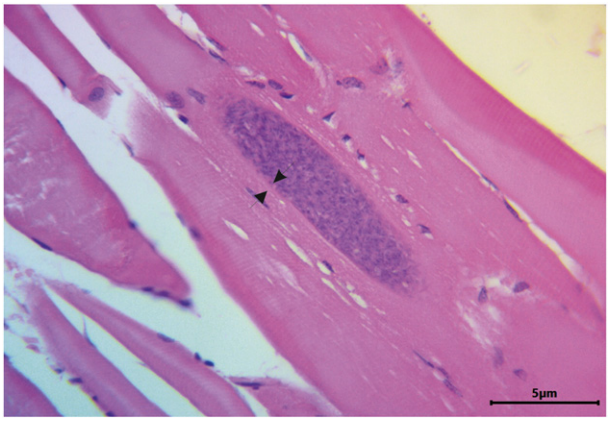

Fig. 1. Histological section of the sarcocyst in skeletal muscle from *Lycalopex gymnocercus*; H&E stain. Longitudinal cut of a sarcocysts on muscle fiber. Note the thin cyst wall (between arrows). Scale bar = 5 μm.

F/J and 5 M/A). Sarcocysts were observed in 6/11 of the frozen samples and in 4/11 of the refrigerated muscles. All cysts observed were microscopic, with septae and measuring 300 μm length and 40 μm width (range 220–310 μm by 28–42 μm; N = 12). The cysts wall was thin (≤1 μm), the maximum thickness was 0.7 μm (range 0.4–0.7 μm, N = 12) (Fig. 2).

3.2. Molecular analysis

Of all the pooled samples, 68.2% (15/22) resulted positive on the genus specific PCR (7 F/A; 1 F/J; 7 M/A). Individual cysts obtained from the ten positive samples in direct microscopic examination were all positive by PCR (4 F/A; 1 F/J; 5 M/A). Five amplicons from individual cysts from different samples together with four PCR products obtained from the pooled muscles were selected for sequencing. When compared through BLAST searches with other (near) complete gene sequences available in GenBank, all nine sequences shared a high identity among them (99.8–100%) and shared the highest identity (99%) with a *S. svanai* sequence (KM362428) with only one single base mismatch. The sequences also showed 99% homology, with lower score and more than two mismatches with various *Sarcocystis* spp. i.e. *Sarcocystis* sp. (JQ733511) from *Phalacrocorax carbo*, *S. columbae* (GU253883) and *S. arctica* (KX022103 = 5 mismatches and 1 gap). A total of five sequences (three from individual cysts and two from pooled muscles) were registered on the GenBank with the accession numbers KY292483 to KY292487.

3.3. Transmission electron microscopy (TEM)

Five mature cysts from refrigerated muscles samples of two Pampas foxes were processed and observed by TEM. Ultrastructurally, the sarcocyst wall was thin (<1 μm), had minute undulations, with tiny blebs (evaginations) and without evident villar protrusions. The ground substance layer (Gs) was homogenous without large granules or microtubules. Maximum thickness of the sarcocyst wall with Gs was 0.8 μm (range 0.4–0.8 μm, N = 6). Bradyzoites with numerous micronemes were found at the apical third (1/3th). Three to four rhoptries with electron dense contents and numerous amylopectin granules at the last third of the zoite (3/3 th) were observed (Fig. 3A and B). The cyst wall type is referred as Type 1 according to Dubey et al. [2].

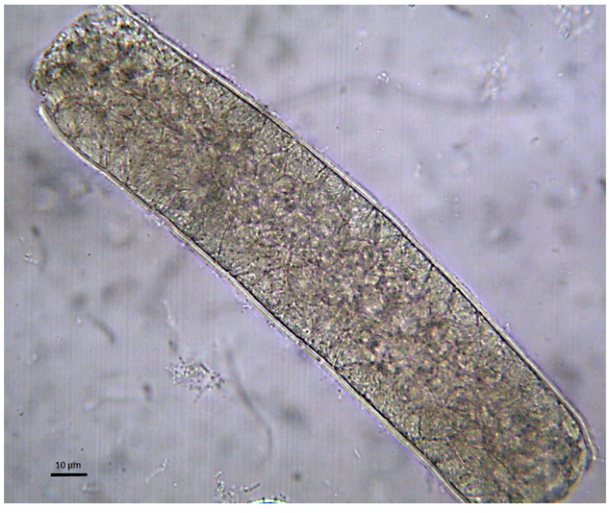

Fig. 2. Sarcocysts of *Sarcocystis svanai* (wet mounts) isolated from the skeletal muscle of *Lycalopex gymnocercus*. Scale bar = 10 μm.

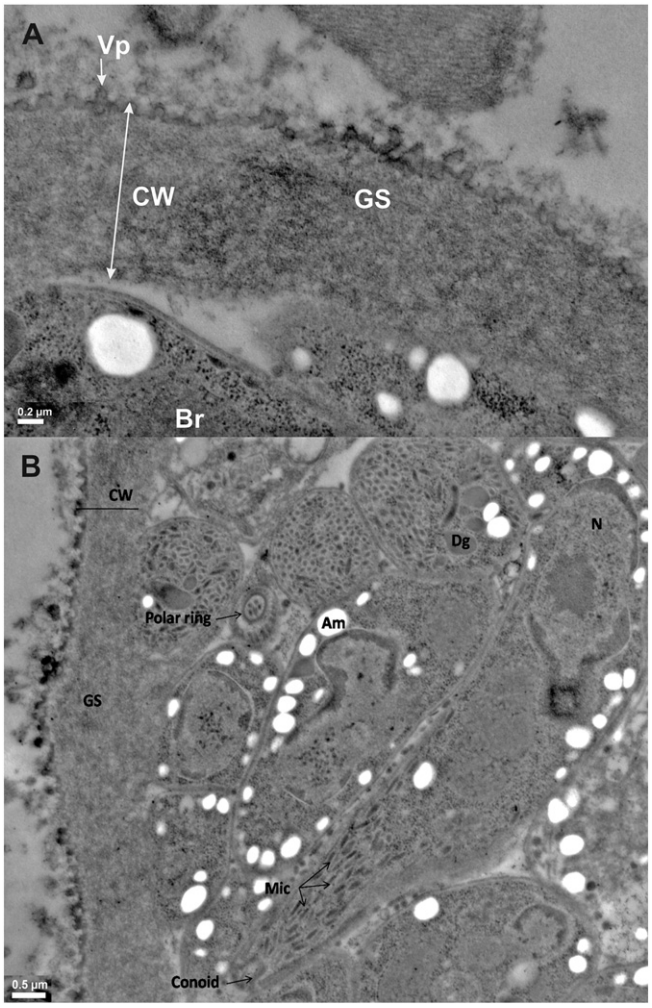

Fig. 3. TEM of two longitudinally cut (A, B) from two sarcocysts of *Sarcocystis svanai* in *Lycalopex gymnocercus*, with different magnification. A: Higher magnification of the sarcocyst wall. Note the thin cyst wall (CW) with a smooth ground substance (GS), wavy parasitophorous vacuolar membrane with blebs or ondulated villar protrusions (Vp) and a bradyzoite (Br). Scale bar = 0.2 μm. B: lower magnification of a sarcocysts. Note thin cyst wall with a smooth ground substance (Gs), and wavy parasitophorous vacuolar membrane with blebs. Note a mature bradyzoite with a conoid, nucleus (N), amylopectin granules (Am), polar ring, dense granules (Dg), and numerous micronemes (Mic). Scale bar = 0.5 μm.

4. Discussion

Although sarcocysts has been found in muscles of various wild and domestic carnivores [3,5], reports of prevalence and species of *Sarcocystis* involved in the infection are scarce. This study is the first report of sarcocysts in *L. gymnocercus* and in a carnivore from South America. Sarcocysts were detected by different techniques in the muscles of *L. gymnocercus* in a striking high prevalence (61.11%), but no inflammatory reactions were observed around the sarcocysts. In Japan, Kubo et al. [3] also found a high infection of sarcocysts in several wild carnivores (30–100%) and no inflammatory reaction was observed around the sarcocysts, although the species of *Sarcocystis* present was not determined. In USA, presence of sarcocysts in skeletal muscle of coyotes was reported but with a lower prevalence (2/52 = 3.85%; [19]). Taking into account the findings of the present study, it is possible to assume that muscular sarcocystosis is a common occurrence in *L. gymnocercus* and is a subclinical disease. Moreover, the presence of mature cysts (containing bradyzoites) is an indicator that *L. gymnocercus* could be acting as a specific IH as previously reported by Dubey et al. [2]. However, it is worth noting that muscular sarcocystosis may become a clinical

disease in wild canids and domestic dogs [2,3]. In fact, there are reports of myositis and hepatitis in carnivores associated with *Sarcocystis* species [4,5,20,21,22]. The tongue and masseter were the muscles more frequently infected in *L. gymnocercus*. Interestingly, no sarcocysts were found in heart samples. This coincides with a report by Calero-Bernal et al. [6] where sarcocysts were obtained from the tongue of the wolf but no cysts were found in the heart. These observations should be taken in account in future studies of *Sarcocystis* spp. identification sampling wild canids muscles.

In the present study, the prevalence obtained by molecular methods was higher (68.2%) than the obtained by homogenization and microscopy (45.4%). Probably, the freezing and autolysis of muscles in some animals could negatively affect the observation of the cyst, and parasites were detected only based on the DNA presence.

The morphology of the sarcocyst wall is an important criterion to distinguish *Sarcocystis* spp. [2]. Based on these criteria, the morphology of the sarcocysts wall observed in this study corresponds to the TEM wall Type 1 and was ultrastructurally similar to the species *S. svanai* described by Dubey et al. [5] in dogs from North America. The cyst wall of this type is thin (<1 μm), devoid of villar protrusions, with tiny blebs giving the appearance of an undulated surface. The observed cyst wall type differs from Types 9 and 9c described for *S. caninum* in dog muscles and *S. arctica* in Alaskan wolf (*Canis lupus*), respectively [5,6]. These species also affect canids; however the cysts wall are ≥2 μm thick and have well defined villar protrusions. Considering the morphological criterion, the species detected in *L. gymnocercus* is more closely related with *S. svanai* than with *S. caninum* or *S. arctica*.

Molecular characteristics are an important taxonomic aid, helping on species differentiation [4]. There are a few cases of muscular sarcocystosis in carnivores where the species involved have been characterized by molecular methods, mainly by sequencing short fragments of the 18S rRNA gene and/or cytochrome *c* oxidase subunit 1 gene (cox1) [4,5,23,24,25,26]. Sequence comparisons reported in these papers have indicated that several *Sarcocystis* spp. may form sarcocysts in carnivores and that these species share a high sequence identity at the 18S rRNA gene with various *Sarcocystis* spp. using birds as IH and less so with *S. neurona* [4]. Our results showed a high proportion of samples and all individual cysts processed positive on a screening PCR. A total of nine sequences from different animals were obtained and all of them were almost identical. This suggests a single infection with one species; especially considering that four sequences were obtained from products from pooled DNA samples muscles. If more than one *Sarcocystis* species is present in the same sample, mixed chromatograms should be expected [27]. The sequences obtained from *L. gymnocercus* muscles/cysts exhibited the higher identity (and higher score) by BLAST (99%) with a *S. svanai* sequence (KM362428), having only one single base mismatch. This result is consistent with the TEM cyst wall type description. The sequences also showed 99% homology, JQ733511 from *Sarcocystis* sp. from *P. carbo* and *S. columbae* (GU253883). Additionally, sequences have 99% identity with *S. arctica* sequences (e.g. KX022103) but showed five mismatches and one gap. As mentioned previously, sequences from *Sarcocystis* sp. in canid muscles shared 18s rRNA gene sequence identities with species using birds as IH. Probably, further characterization of the species affecting canids should include other genes like cox1, rpoB or SAGs [4,5]. Therefore, considering morphological and molecular analysis performed in this study, we conclude that *S. svanai* affects Pampas fox with a high frequency. Moreover, the presence of mature cysts reinforces the role of this South American canid as a natural IH for *S. svanai*. Therefore, we report the sequences with the accession numbers KY292483 to KY292487 as *S. svanai* 18sRNA gene partial sequence from *L. gymnocercus*. Until now, this species was only described in North American's dogs [5] but probably other wild canids from America could act as IH, but such assumption needs further investigation.

The definitive host of *S. svanai* remains unknown; nevertheless, it is possible to suggest that it could be a canids' predator (e.g. *Puma concolor*) distributed at least in North and South America. Some *Sarcocystis* spp. are transmitted by cannibalism, using a single host species to complete the life cycle [2]. This appears not to be the case of *S. svanai* as such species was not detected in Pampas fox neither in European fox intestinal samples [16,18]. Further research should look into the identification of the natural DH so as to improve the understanding of the epidemiology of muscular sarcocystosis in dogs and Pampas foxes. On the other hand, the genus *Sarcocystis* is a good model for ecological interaction studies, allowing us to understand and define trophic levels of the intermediate and definitive hosts, and later to establish the parasite distribution within a host population [28].

Conflict of interests

The authors declare no conflict of interests.

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