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Acute fulminant necrotizing myopathy in a dog caused by co-infection with ultrastructural *Sarcocystis caninum* and *Sarcocystis svanai*-like apicomplexan protozoa

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**Highlights** Canine Muscular Sarcocystosis\_ Sukura et al

- Fatal muscular sarcocystosis in a dog
- *Sarcocystis caninum* and *Sarcocystis svanai* have global distribution
- co-infection with *S. caninum* and *S. svanai* caused fatal myopathy in a dog
- *Sarcocysts* adapted to dog as an intermediate host

Abstract

Typically, carnivores are the definitive and herbivores the intermediate hosts for protozoan *Sarcocystis* spp. In the definitive host, the parasite has sexual multiplication in the intestine. Asexual phases occur in the musculature of different intermediate hosts. Although intestinal sarcocystosis is common in dogs, muscular symptomatic sarcocystosis is rarely reported. Here we report a fatal dual *Sarcocystis* spp. infection in a dog. The dog

had acute onset of non-ambulatory tetraparesis. While neurological findings suggested a generalized neuromuscular disease with peripheral neuropathy concordant with the neurological deficits, the highly elevated muscle enzymes were more suggestive of a myopathy. Despite supportive therapy, the dog died three days after the onset of clinical signs. Necropsy revealed severe monophasic multifocal myodegeneration with severe pyogranulomatous inflammation. Histology revealed multiple sarcocysts in skeletal muscles and a smaller number in the heart. In light microscopy, both thin-walled and very thin-walled sarcocysts were found in skeletal muscles. Transmission electron microscopy confirmed the presence of two types of mature sarcocysts. Morphologically, cysts were indistinguishable from *Sarcocystis caninum* and *Sarcocystis svanai*, which were previously reported in a dog from USA. A region of the 18S rRNA gene sequence confirmed the presence of one species, *S. arctica/caninum*, without evidence for a dual infection. This is the first report of muscular sarcocystosis in a dog in Europe and, intriguingly, revealed morphologically similar species across the Atlantic.

Keywords: *Sarcocystis* spp; canine; muscular sarcocystosis

## 1. Introduction

Although the dog is the major definitive host for *Sarcocystis* (with several species found in feces) (Dubey et al., 2016), muscular sarcocystosis in dogs is rarely reported (Sykes et al., 2011, Chapman et al., 2005, Dubey et al., 2015, Dubey et al., 2016) and is often an incidental finding. Dubey et al. (2016) reviewed eight reported cases of muscular sarcocystosis in dogs, five of which with related clinical signs. Here we report a fatal case of canine muscular sarcocystosis. Rather than primary myositis, pathogenesis in this case

was necrotizing myopathy with secondary pyogranulomatous myositis. Intriguingly, two morphologically distinct *Sarcocystis* species were revealed; these resembled *Sarcocystis caninum* and *Sarcocystis svanai*. DNA sequencing confirmed the presence of *S. arctica/caninum*, but failed to provide evidence for *S. svanai*. A dual infection with these two species has been previously reported in a dog in Canada (Dubey et al., 2015). Previous other reports of canine muscular sarcocystosis were from the USA, Canada, India, and Kenya (reviewed by Dubey et al., 2016).

## 2. Material and methods

Case history: A six-year-old male neutered Bichon Frise was presented to Helsinki University Veterinary Teaching Hospital (HUVTH) in July 2016 with a history of acute weakness in all four limbs. The owners noticed the first clinical signs in the previous evening; no abnormalities were observed before. Otherwise, the dog was healthy and had never traveled abroad. Despite supportive therapy, the dog died on the third day after onset of clinical signs and was sent to necropsy (Faculty of Veterinary Medicine, University of Helsinki).

Routine necropsy was performed. Samples were taken from all parenchymal organs and from the skeletal muscles, peripheral nerves, spinal cord, and brain. Samples were fixed in neutral buffered 10% formalin. Tissues were routinely processed, sectioned at 5- $\mu$ m thickness and stained with hematoxylin and eosin (HE) and later with Periodic acid-Schiff stain (PAS) for light microscopy (LM) investigation.

Skeletal muscle tissue fixed for LM was also used for transmission electron microscopy (TEM). Formalin-fixed skeletal muscle tissue was cut into 1 mm<sup>3</sup> pieces and further fixed with 2% glutaraldehyde in 100 mM phosphate buffer overnight at +10°C. Samples were then post fixed with 1% buffered osmium tetroxide for 1 h. After dehydration, the specimens were epon-embedded into embedding resin (TAAB Ltd., UK). All steps after fixation were performed at room temperature. Semi-thin sections were cut and stained with Toluidine Blue for LM. Areas with identified cysts were selected for ultrathin sectioning. Sections were collected on pioloform-coated single-slot copper grids and post-stained with 1% aqueous uranyl acetate and lead citrate (Leica Ultrastain II). Sections were analyzed by a JEM 1400 transmission electron microscope (Jeol Ltd, Japan) operated at 80 kV. Images were acquired with an Orius SC1000B bottom-mounted CCD camera (Gatan Inc., USA).

For molecular identification, individual sarcocysts were not isolated. Rather, total genomic DNA was extracted from paraffin-embedded skeletal muscle tissue using a DNeasy<sup>®</sup> Blood and Tissue kit (QIAGEN, CA). PCR was performed using nested primers targeted to the partial small subunit 18S rRNA gene. The sequence variation in the target region among species of *Sarcocystis* allows species level identification. For the first (external) PCR reaction, published primers 18S9L and 18S1H (Li et al., 2002) were used, of which the first was slightly modified (by adding G at the position 9: 18S9L<sub>mod</sub>-GGATAACCGTGGTAATTCTATG) to correspond to sequence of *Sarcocystis* spp. For the second (nested) PCR reaction, new *Sarcocystis* specific primers 18Sf1-CAAGTTTCTGACCTATCAGC and 18Sr1-CCTATCTTGTTATTCCATGC were designed. The reaction mixture (50 µl) contained 1 µl of template DNA solution, 20 pmol of each primer (forward and reverse), 0.2 mM of each dNTP, 1 U of DreamTaq<sup>™</sup> DNA polymerase (Thermo Fisher Scientific, MA), and PCR reaction buffer containing 3 mM MgCl<sub>2</sub>. For the

nested PCR amplification, 1 µl of the external PCR product was used as a template. The PCR reaction conditions were as follows: initial denaturation step at 94°C for 10 min, followed by 35 cycles of 94°C for 60 sec, 54°C for 60 sec and 72°C for 120 sec, and a final extension at 72°C for 10 min. The nested PCR product obtained was excised from the agarose gel, purified with QIAquick® Gel Extraction kit (QIAGEN), and sequenced at the Institute for Molecular Medicine Finland SeqLab using the same primers as for nested PCR. The sequences were visualized, aligned, and compared with the previously published sequences using Geneious version R8 (<http://www.geneious.com>).

### 3. Results and discussion

The dog was unable to walk upon admission to HUVTH. At physical examination, the dog exhibited strenuous breathing while excited. Neurological examination by board-certified veterinary neurologists revealed non-ambulatory tetraparesis and absent (hind limbs) and severely decreased postural reactions (front limbs). Spinal reflexes were severely decreased to absent in all four limbs. Menace response and palpebral reflex were bilaterally decreased. There was no pain in palpation of the head, spine, or limbs. On the basis of the neurological examination findings, a generalized neuromuscular or lower motor neuron disease was suspected. Complete blood cell count revealed no abnormalities. A biochemistry panel showed increased serum AST (459 U/l; range 17-54) and CK activity (5018 U/l; range 60-235). Additionally, triglycerides were high (1.93 mmol/l; range 0.1-0.5) but cholesterol was within the reference range. Thoracic radiographs revealed no abnormal findings. The dog was anesthetized for electrodiagnostic investigations. Electromyography showed no changes in insertional activity and no spontaneous activity. Motor nerve conduction velocities from peroneal and ulnar nerves were within normal reference ranges. Compound muscle action potentials showed

temporal dispersion. Repetitive nerve stimulation showed no decrement. IgG and IgM *Toxoplasma gondii* and IgG *Neospora caninum* serology titres measured with indirect immunofluorescence (IDEXX Laboratories, Ludwigsburg, Germany) were negative. Furthermore, serology test for acetylcholine receptor (AChR) antibodies (Comparative Neuromuscular Laboratory, La Jolla, CA, USA) was negative. A suspected diagnosis of acute polyradiculoneuritis was made, although the highly elevated muscle enzymes were not typical for the disease.

The dog was hospitalized and supportive care (paracetamol, ondansetron, metoclopramide and IV fluid therapy) was administered. An indwelling urinary catheter was also placed. The following day, the dog improved slightly and flexor reflexes were stronger than on the day of presentation. The dog was eating and drinking and the owners decided to take the dog home overnight. While at home, his status worsened and according to the owner the urine color changed to brown and the dog was panting. The owners took the dog back to HUVTH. Despite supportive care (IV fluids, oxygen cage, ondansetron, metoclopramide, paracetamol, methadone) in intensive care, the dog died due to respiratory distress.

In a dog with acute lower motor neuron tetraparesis, the most important differential diagnoses include acute polyradiculoneuritis, botulism, acute fulminant myasthenia gravis, and tick paralysis. The presence of severely elevated CK and AST did not support any of these differential diagnoses. Additionally, there was no history of ingestion of suspicious material (such as raw meat or dead animals) that could be a source of botulinum toxin. Furthermore, tick paralysis has not been reported in Finland, and electrodiagnostic testing (RNS) or laboratory results (negative AChR antibodies) did not support a diagnosis of acute fulminant myasthenia gravis. Elevated CK indicates severe muscular injury; elevated

AST may indicate muscle or liver damage, or both. Previously reported clinical signs in canine muscular sarcocystosis include ataxia, stiff gait or inability to walk, generalized pain, anorexia, diarrhea, fever, and panting (Dubey et al., 2015). While our patient showed many similar signs, no pain reaction was detected in palpation of the muscles.

At necropsy, macroscopic findings were almost insignificant. There was acute subcutaneous hemorrhage (5 x 2 cm) in the chest cranial to the right front leg. Acute hemorrhage was observed between muscle membranes under the left shoulder (7 x 3 cm) and under the left bicep femori muscle (5 x 1 cm). Submandibular lymph nodes were slightly enlarged and there were small hemorrhagic areas on cut surface. The liver was swollen, soft, and congested. There was one small (1 cm) nodular lesion in the left medial lobe of the liver. The right kidney (6 cm) was slightly larger than the left (5 cm). Some hyperemia (1 x 0.2 cm) was observed dorsally in the spinal cord at the area of cauda equine.

The main histopathological finding was severe monophasic multifocal acute muscular necrosis with pyogranulomatous inflammation (Fig. 1). Skeletal muscle cells were multifocal rounded and hyper-eosinophilic; cross striation was absent in affected cells. The sarcoplasm was vacuolated and the sarcolemma was discontinuous. Neutrophils, lymphocytes, and macrophages were multifocal and surrounded and infiltrated necrotic muscle cells. Macrophages were actively phagocytosing necrotic sarcoplasm. Although numerous sarcocysts were detected in myofibers, fulminant inflammation was not focused in sarcocysts but in degenerate myocytes (Fig. 1 A). In immune-mediated myositis, mononuclear inflammatory reactions invade intact myofibers. In this case, inflammation



was clearly secondary and focused on already necrotized myofibers, which excluded immune-mediated disorders but suggested myopathies.

Cut surfaces of sarcocysts were round or oval, measuring 25 to 30  $\mu\text{m}$ . Length was up to 500  $\mu\text{m}$ , depending on the section panel. Thin and very thin cyst walls were identified (Fig. 1 B, C). Approximately 80% of the cysts showed thin appearance; a minority of the cysts had very thin cyst walls. Inside the cysts, numerous small (1-2  $\mu\text{m}$ ) and round basophilic structures with a surrounding clear halo were observed (Fig. 1 B, C). These structures had PAS-positive granules. One sarcocyst was also observed in the myocardium without any inflammatory reaction. Cysts were negative for *Toxoplasma* immunostaining. Endothelium of different organs were carefully investigated for intravascular schizonts; none were discovered.

The histopathological pattern indicates that sarcocysts in the dog did not initiate a fulminant inflammatory reaction but may have been directly toxic to myocytes. Myositis was secondary reaction due to removal of debris from necrotized myocytes. The observed monophasic reaction may be related to the rapid onset of the episode; if the animal lived longer there could have been muscle fibers in different phases that also showed fibrosis and regeneration. It is not possible to differentiate between monophasic and early polyphasic reaction types.

There was acute severe diffuse congestion with some acute hemorrhages surrounding veins in the liver. Hepatocytes were diffusely slightly vacuolated and some apoptotic hepatocytes were observed. Focal nodular hyperplasia of hepatocytes was also observed.

Sarcocyst walls were slightly different at LM level and showed two distinct types in TEM evaluation. Sarcocyst wall structures have been used for specific diagnosis. Dubey et al (2016) reviewed at least 82 distinct types of cyst walls; these were grouped into Types 1 to 42 with subtypes. Ultrastructurally, very thin cyst walls mimic type 1 (Fig 2 A-C) and the thin wall cysts mimic type 9 (Fig 2 D-F) (Dubey et al, 2016). Near the sarcocyst host cell, severe necrotizing and autolytic changes were observed (Fig 2 A). On the basis of ultra-morphology, the type 9 and type 1 cell wall sarcocysts were very similar to *Sarcocystis caninum* and *Sarcocystis svanai*, respectively. These pathogens have been previously reported to cause a symptomatic disease in a dog in USA and Canada (Dubey et al, 2015).

A region of 18S rRNA gene, 461 bp in length, was successfully sequenced. Double peaks, which could support the presence of more than one species, were not detected in the sequencing chromatograms. The DNA sequence has been deposited in GenBank under the accession number MG851712. A comparison with the previously published sequences in GenBank showed 100% identity with *S. caninum* (GenBank accession no. KM362427) and *S. arctica*. (eg, KF601301). Dubey et al. (2015) noted that the 18S rRNA gene sequence is identical in *S. caninum* and *S. arctica*. These two species might be conspecific (*S. caninum* as a junior synonym of *S. arctica*). Sequence identity of the present specimen with *S. svanai* (eg. KM362428) was 99% (difference of 5 bp, including 4 substitutions and 1 insertion). In the sections used for DNA extraction, the number of *S. svanai*-like organisms may have been insufficient for DNA amplification, and thus the unambiguous sequencing result does not exclude a dual infection with *S. arctica/caninum* and *S. svanai*. Histology revealed 4 times more *S. arctica/caninum* like cysts compared to

*S. svanai*-type; the ratio may likely have influence also DNA amplification from the paraffin sections.

A similar combination of parasites that causes similar disease in USA and Europe indicates that *Sarcocystis* are not accidentally in canine musculature but have adapted to the dog also as an intermediate host. Similar clinical signs in reports of all clinically affected dogs have shown that these parasites alone, or in a coinfection, are pathogenic to dogs. The total life cycle of these parasites with the definitive host is still unknown.

*Sarcocystis arctica*, with similarities to *S. caninum*, has been detected in the muscles of arctic foxes (*Vulpes lagopus*) in Norway (Gjerde et Schulze, 2014) and Alaska (Cerqueira-Cézar, 2017) and in wolves (*Canis lupus*) in Alaska (Calero-Berna, 2016). These observations demonstrate that canines globally can act as an intermediate host for the species. *Sarcocystis svanai*, which has morphological similarities to our type 1 sarcocysts, has been reported not only in a dog in USA (Dubey et al., 2015) but also in Pampas foxes (*Lycalopex gymnocercus*) in Argentina with high prevalence (Scioscia et al., 2017).

Our results confirmed clinical canine sarcocystis myopathy with global reach and identified two ultrastructurally similar *Sarcocystis* species that use the dog as an intermediate host. Clinically, sarcocystosis should be considered as a differential diagnosis in dogs with acute onset of flaccid nonambulatory tetraparesis.

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Figure text

Fig. 1. Skeletal muscle with thin (A, B) and very thin walled (C) sarcocysts. A, Note that sarcocysts (SC) do not evoke fulminate inflammation. Rather, secondary inflammation is seen in necrotized tissue. Degenerate muscle cells (asterisk), hemorrhage, and macrophages clearing necrotized material (arrow). Sarcocysts had thin wall (B) but some had very thin appearance (C). B, C, Inside the sarcocysts were multiple merozoites/bradyzoites showing basophilic structures (LM, HE staining).

Fig. 2 A-C. A, Type 1 sarcocyst wall (arrow). The host cell near the sarcocyst showed pronounced necrotizing/autolytic changes (asterisk). B, Sarcocyst wall type 1 without villous projections, inside maturing bradyzoites with typical structures like amylopectin granules (apg), dense granules (dg), nucleus (n), and micronemes (Mn). Slight septa-like structures divide the sarcocyst in lobules (B). Details of sarcocyst wall that are less than 0.5  $\mu\text{m}$  thick, the parasitophorous membrane (pvm) has an electron-dense inner layer, which is absent in invaginate areas (arrow, C). D-F Type 9-like cyst type showed villar pleomorphic protrusions (arrow, D). The cyst wall was almost four times thicker, reaching 1.5-2  $\mu\text{m}$  width. (E, F). Detail of the cyst wall, invaginations in deeper section of villous bottom in which the electron-dense layer of pvm was absent (arrow, F), host myofiber (mf).



