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Sarcocystis caninum and *Sarcocystis svanai* n. spp. (Apicomplexa: Sarcocystidae) Associated with Severe Myositis and Hepatitis in the Domestic Dog (*Canis familiaris*)

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Abstract

There are several reports of *Sarcocystis* sarcocysts in muscles of dogs but these species have not been named. Additionally, there are 2 reports of *Sarcocystis neurona* in dogs. Here, we propose 2 new names, *Sarcocystis caninum*, and *Sarcocystis svanai* for sarcocysts associated with clinical muscular sarcocystosis in 4 domestic dogs (*Canis familiaris*), 1 each from Montana and Colorado in the USA, and 2 from British Columbia, Canada. Only the sarcocyst stage was identified. Most of the sarcocysts identified were *S. caninum*. Sarcocysts were studied using light microscopy, transmission electron microscopy, and PCR. Based on collective results 2 new species, *Sarcocystis caninum* and *Sarcocystis svanai* were designated. *Sarcocystis caninum* and *Sarcocystis svanai* were structurally distinct. *Sarcocystis caninum* sarcocysts were up to 1.2 mm long and up to 75 μm wide. By light microscopy, the sarcocyst wall was relatively thin and smooth. By transmission electron microscopy (TEM), the sarcocyst wall "type 9", 1–2 μm thick, and contained villar protrusions that lacked microtubules. Bradyzoites in sections were 7–9 μm long. Sarcocysts of *S. svanai* were few and were identified by TEM. *Sarcocystis svanai* sarcocysts were "type 1", thin walled (< 0.5 μm), and the wall lacked villar protrusions but had tiny blebs that did not invaginate. DNA was extracted either from infected frozen muscle biopsies or formalin-fixed paraffin-embedded sections. Dogs were either singly infected with *S. caninum* or multiply co-infected with

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S. caninum and *S. svanai* (the result of a mixed infection) based on multi-locus DNA sequencing and morphology. BLASTn analysis established that the sarcocysts identified in these dogs were similar to, but not identical to *S. canis* or *S. arctosi*, parasites found to infect polar bears (*Ursus maritimus*) or brown bears (*Ursus arctosi*), respectively. However, the *S. caninum* sequence showed 100% identify over the 18S rRNA region sequenced to that of *S. arctica*, a parasite known to infect Arctic foxes (*Vulpes lagopus*).

Keywords

Dog; *Canis familiaris*; *Sarcocystis*; hepatitis; USA; Canada

SPECIES of *Sarcocystis* usually have an obligatory 2-host life cycle, with herbivores as intermediate hosts and carnivores as definitive hosts (Dubey et al. 1989). The intermediate host becomes infected with *Sarcocystis* species by ingesting sporulated sporocysts excreted in the feces of the definitive host. The parasite multiplies asexually in the intermediate host and persists as sarcocysts. After consumption of infected tissues containing sarcocysts the parasite undergoes sexual cycle in intestine of the definitive host resulting in the excretion of sporulated sporocysts in feces (Dubey et al. 1989).

The domestic dog (*Canis familiaris*) is a definitive host for numerous species of *Sarcocystis* (Dubey et al. 1989). Additionally, dogs are intermediate for *Sarcocystis* species. Sarcocysts of unknown species have been reported in the muscles of a few dogs including 1 dog from India (Sahasrabudhe and Shah 1966), 1 dog from Georgia, USA. (Hill et al. 1988), 1 dog from Alabama, USA (Blagburn et al. 1989), and 1 dog from Kenya (Bwangamoi et al. 1993); these reports were not associated with clinical disease. Clinical myositis associated with numerous sarcocysts has been reported in a dog from Canada (Chapman et al. 2005) and 2 dogs from the USA (Sykes et al. 2011). The *Sarcocystis* species found in these latter reports were not rigorously characterized using both multi-locus DNA sequencing and morphology, including high resolution transmission electron microscopy (TEM) of their sarcocysts, so they have not been named.

In addition to the reports of these muscular sarcocysts, there are several reports of 2 other species of *Sarcocystis*, *S. canis*, and *S. neurona* with unusual life cycles (Vashisht et al. 2005; Dubey et al. 2006). *Sarcocystis canis* infections have been reported in several hosts, including dogs (Dubey et al. 2006). Immature and mature schizonts and merozoites are the only stages known for *S. canis* (Dubey and Speer 1991) and the parasite has not yet been cultivated in cell culture or in any animal. Owing to these limitations, there are no specific diagnostic tests or reagents. Its limited genetic characterization has been based on DNA extracted from the liver of a polar bear that died of hepatitis (Garner et al. 1997; Dubey et al. 2006); no DNA has yet been derived from infected dogs.

Sarcocystis neurona is an another unusual species with dogs and several other species as intermediate or aberrant hosts and the opossum (*Didelphis virginiana*) as definitive host, (Dubey et al. 2001; Dubey et al. 2006; Britton et al. 2010); sarcocysts are not formed in aberrant hosts. Immature *S. neurona*–like sarcocysts were reported in the muscle from a dog with chronic myositis (Vashisht et al. 2005). Recently, mature *S. neurona* sarcocysts were

found in muscles of a 9 week-old dog, believed to be congenitally infected; this dog had generalized schizont-associated sarcocystosis, including eyes and turbinates (Dubey et al. 2014a). Thus, there is uncertainty concerning the asexual cycle of *Sarcocystis* species in dogs. Part of the difficulty has been that the diagnosis of sarcocystosis in dogs was made at necropsy, only few organisms were seen, in vitro cultures have not been attempted, and a full molecular characterization on extracted DNA has not been undertaken. As stated earlier, clinical sarcocystosis associated with the presence of numerous sarcocysts was reported in 2 dogs from USA (Sykes et al. 2011). Fortunately, muscle biopsy tissue from these dogs was frozen permitting a thorough DNA and morphologic characterization. Additionally, 2 new cases of muscular sarcocystosis are also reported here. The molecular and morphologic characterization supports the classification of 2 new *Sarcocystis* species. To facilitate further investigations of the life cycles of these new species of *Sarcocystis* in dogs, we propose naming the species of *Sarcocystis* in skeletal muscles of dogs as *Sarcocystis caninum* and *Sarcocystis svanai.*

MATERIALS AND METHODS

Naturally infected dogs

Information regarding the 4 dogs (A–D) investigated with myositis is summarized in Table 1. Detailed clinical and laboratory tests performed on Dog A were stated by Chapman et al. (2005) and on Dogs B and C by Sykes et al. (2011). Case history and sections for Dog A were reviewed again but no additional information could be obtained. Paraffin block of infected muscle tissue was used to extract DNA for the present study. A detailed history of the fourth dog is as follows.

Dog D was an 18-month old female spayed Rhodesian Ridgeback, from Chilliwack, in the Fraser River delta of British Columbia, Canada, developed anorexia and diarrhea with transient head tremors. On examination (14 December 2012) by a local veterinarian the dog had pyrexia (40.7 °C; normal < 39.1 °C), she was slightly jaundiced, her liver was palpably enlarged and her urine was also bright yellow. Serum biochemistry tests identified a severe liver disease, with an alanine transaminase (ALT) of 1,147 U/L (normal < 100 U/L), an alkaline phosphatase (ALP) of 285 U/L (normal < 212), and a total bilirubin of 170 micro-mol/L (normal <15). She was treated symptomatically and started to eat within 48 h. One month later the dog had profound weakness in all 4 legs with muscle pain that developed over 1 week. She was referred for neurologic evaluation; the weakness was most pronounced in the quadricep muscle groups and her patellar reflexes were absent bilaterally but conscious proprioception was normal. Provisional diagnosis was of generalized neuromuscular disease. Hematologic testing was normal but serum biochemistry confirmed continued, though less severe liver disease (the ALT was now 664 U/L with a normal < 151 U/L, while the total bilirubin and the ALP were normal); urinalysis was normal. The serum creatinine kinase (CK) was over 7,568 U/L (normal < 554 U/L), consistent with a severe myopathy. The iliac lymph nodes (draining the quadriceps muscles) were enlarged on abdominal ultrasound; aspirates of liver, spleen and iliac lymph nodes did not reveal any abnormality. Muscle biopsy was performed. The *Toxoplasma gondii* IgM and IgG antibody titers were negative (<1:25) and the dog had a low (1:50) *Neospora caninum* antibody titer.

Clindamycin (21.9 mg/kg, oral, twice daily, Apo-Clindamycin 300 mg tablets, Apotex, Inc., Toronto, Canada) was initiated and the dog showed marked improvement within 24 h. The muscle biopsy (22 January 2013) revealed a severe, inflammatory and necrotizing myositis with intramyofiber sarcocysts. The dog was markedly better within a week and after 4 weeks was able to hike 8 km; a follow-up neurologic examination was normal after 6 wk and the serum chemistry tests were normal at that stage, other than mildly elevated CK at 734 U/L (normal < 554 U/L). Clindamycin was continued for 3 months and the dog has been normal for more than 1 year.

Frozen muscle biopsy tissue, formalin fixed muscle tissue, and paraffin embedded tissues were shipped from the Comparative Neuromuscular Laboratory, University of California San Diego, California to the Animal Parasitic Diseases Laboratory, United States Department of Agriculture, Beltsville, Maryland for the present report.

Histological and transmission electron microscopy (TEM) examination

For the present study, histological sections and TEM preparations of dog A (Chapman et al. 2005) were evaluated again. For dogs B–D, muscle biopsy specimens fixed in formalin were post-fixed in osmium tetroxide, dehydrated in serial alcohol solutions and proplene oxide before being embedded in araldite resin. Thick sections (1–5 μm) were stained with hematoxylin and eosin (H and E) or toluidine blue for light microscopy. For TEM, ultrathin sections (60–90 nm) were stained with uranyl acetate and lead citrate then examined in a JEOL JEM 1400 electron microscope.

Molecular characterization

DNA was extracted from a paraffin block of dog A, and from frozen muscle samples of dogs B–D using DNeasy Blood and Tissue kit (QIAGEN, Germantown, Maryland). Genomic DNA was amplified using primers that amplify a 1669 nucleotide fragment within a conserved 3′ terminal portion of the *18S rRNA* gene to delineate the phyla of the organisms involved (Carlson-Bremer et al. 2012) as well as primers that amplify across a 1593 nucleotide length of the *18S rRNA* gene as follows: Fext-GGTTGATCCTGCCAGTAGTCA; Fint-TAAAGATTAAGCCATGCATGTC; Rext-CCTCTAAGTGTTAAGGTTCAC; and Rint-TACAAAGGGCAGGGACGTAA. The following primers that amplify the *rpoB* gene, a multi-copy polymorphic marker in the organellar apicoplast genome, were used to further discriminate between taxa and permit multilocus molecular genotyping (Wendte et al. 2010): F-TAGTACATTAGAAATCCCTAAAC; Rext- TCWGTATAAGGTCCTGTAGTTC; and Rint-GAATAYCTWGMWACTCCACA. For comparative purposes, DNA from *S. canis* (Dubey et al. 2006) was amplified with the above markers, because of its close relationship with sarcocysts in dogs (Sykes et al. 2011). The PCR amplification and DNA sequencing were conducted as per established protocols (Wendte et al. 2010). Some samples possessed heterozygous positions upon DNA sequencing of the PCR population. To determine unequivocally the number of haplotypes present, the PCR population was TOPO-cloned and 10 clones sequenced. The resulting sequences were compared with GenBank sequences after aligning and visualizing within the Seqman program of the Lasergene software.

For the phylogenetic analyses, DNA sequences encoding the *rpoB* gene and the small subunit *18S rRNA* within the nuclear ribosomal gene array were downloaded from a wide-spectrum of apicomplexan parasites deposited in GenBank and aligned against the DNA sequences obtained from the dog samples. The *18S rRNA* sequence from *Eimeria tenella* [U67121] and *rpoB* sequence from *S. neurona* [GQ851961] were selected as appropriate outgroups for the respective phylogenetic tree construction. All subsequent alignments were manually edited using the MEGA5 program.

Phylogenetic tree reconstruction and the evolutionary history for these 2 loci were inferred using 2 methods independently, maximum likelihood and neighbor joining. Phylogenetic trees shown in Figures 1 and 2 were derived using the maximum likelihood method and constructed using the MEGA5 program after deletion of gapped positions (Tamura et al. 2011). Each phylogeny was tested using 10,000 bootstrap replicates.

RESULTS

PCR amplification of the small subunit *18S rRNA* gene array within the nuclear ribosomal gene complex yielded a single, homogenous band for the sarcocysts from each of the dog specimens. Full *18S rRNA* sequences were obtained for dogs B and D, respectively whereas only partial *18S rRNA* sequences were successfully amplified and sequenced for dogs A and C, respectively. The full length *18S rRNA* sequences from Dog B possessed heterozygous SNP positions at 10 sites when compared to Dog D, which possessed only 1 homozygous DNA sequence type across the 1669 nucleotides sequenced. To determine the number of sequence haplotypes present in Dog B, the PCR population was TOPO-cloned and 10 clones DNA sequenced. Two haplotypes were resolved, the majority of sequence traces (7/10 sequences) were identical to Dog D, whereas the minority (3/10 sequences) possessed an identical sequence trace to each other, but had 10 different nucleotide positions and 2 single nucleotide INDELS variant from Dog D, consistent with co-infection of Dog B with two distinct *Sarcocystis* types.

An alignment was constructed encompassing 721 nucleotides of the *18S rRNA* gene array for all dog sarcocysts sequenced at the *18S rRNA* gene array. Dogs C and D had the same *18S rRNA* sequence across 721 nucleotides, which was identical to GenBank sequence KF601305 (annotated as *S. arctica* from an arctic fox). Across the 721 nucleotide alignment, Dog B either possessed the identical sequence type to that of Dogs C and D, or the other sequence type that encompassed 4 variant nucleotide positions (SNPs). The sequence obtained for Dog A was only 247 nucleotides, and it was within a non-polymorphic region of the *18S rRNA*, consequently, it was indistinguishable from the other 3 dogs. Maximum likelihood and neighbor-joining trees were constructed (MEGA5 program) for the 721 nucleotide alignment and each possessed the same branching topology (data not shown). Figure 1 shows the maximum likelihood tree, which establishes that the DNA sequence obtained from the dog sarcocysts represent 2 taxa within the family Sarcocystidae. Dogs B to D were infected with parasites that were indistinguishable from *S. arctica* at the portion of the *18S rRNA* gene array sequenced. Dog B was also infected with another species of *Sarcocystis* that had higher similarity with *S. canis* (DQ146148), but differed at 2 nucleotide positions (Fig. 1).

PCR amplification at the *rpoB* locus for Dogs B–D, followed by BLASTn analysis, established that the coccidian parasite shared by these 3 dogs was new. Trees constructed by maximum likelihood methodology resolved the dog sarcocyst sequence type into an individual taxon that was most similar to *S. canis* at *rpoB* (Fig. 2). However, as for the *18S rRNA* gene array, a second unambiguous molecular sequence type was resolved by TOPO cloning for Dog B, confirming that Dog B was co-infected with another closely related coccidian parasite that was more similar to *S. campestris* (Fig. 2)

Of the 47 cysts examined by TEM from all dogs in the present study, 44 (27 from dog B, 13 from dog C, 4 from dog D) had a type 9 sarcocyst wall using the classification of Dubey et al. (1989). Three sarcocysts were identified as type 1 (Dubey et al., 1989) with a thin wall, 1 in Dog B was mature with numerous bradyzoites; because these samples were only investigated by TEM, their light microscopic appearance is uncertain.

Description of *Sarcocystis caninum*, n. sp. (Fig. 3–7)

Sarcocysts up to 1.2 mm long and up to 75 μm wide. In 5 μm thick H and E stained sections, the sarcocyst wall without striations (Fig. 3A, B). In 1 μm thick sections stained with toluidine blue, the sarcocyst wall approximately 1 μm thick with villar protrusions (Fig. 3C). Bradyzoites in 1 μm sections 6.0–7.5 × 2.0–3.0 μm in size (n=13) (Fig. 3C). By TEM the parasitophorous vacuolar membrane (Pvm) of the sarcocyst wall undulated, wavy, with elongated villar protrusions (Vp) on the entire wall (Fig. 4). The Vp up to 1 μm long and up to 0.8 μm wide, depending on the plane of section, narrow at the base and expanded laterally (Fig. 4–6). The Pvm lined by approximately 60 nm thick electron dense layer (Edl) except at invaginations, interrupted at irregular distances. Interior of the Vp homogenous, without microtubules (Fig. 6). The Pvm invaginated slightly at the base of Vp. Underneath the Vp was the homogenous granular substance layer (Gs), without large granules or micotubules. The total sarcocyst wall, including the Vp and the Gs layer up to 2 μm thick (Fig. 6). The Gs continued into the interior of the sarcocyst as septa. Individual or groups of metrocytes and bradyzoites separated by septa. Metrocytes ovoid, with few organelles (Fig. 4). Bradyzoite elongated, 7.5–9.0 μm long (n=6). Bradyzoites with double layered pellicle with electron dense thickening at the conoidal end with organelles found in other *Sarcocystis* species bradyzoites, including rhoptries with electron dense contents, mironemes, mitochondriion, amylopectin granules, and micropore (Fig. 7). Micronemes numerous often arranged in rows, towards 1/4 –1/5 th of conoidal part. Rhoptries few (maximum 3 in any section) with narrow neck, and bulbous blind end, sometimes oriented towards conoid. Amylopectin granules numerous, absent or rare at the location of micronemes. Large mitochondrion. Nucleus terminal to subterminal (Fig. 7).

TAXONOMIC SUMMARY—Type host: Canis familiaris.

Other hosts: Unknown.

Type locality: North America.

Etymology: Named after the host genus.

Specimens deposited: Specimens deposited in the United States National Parasite Collection in the Division of Invertebrate Zoology and National Museum of Natural History, Smithsonian Institution, Washington, D.C under (USNM-) include histological sections stained with H and E and toluidine blue from Dog B from Colorado (Syntype specimens for *S. caninum* .no -------), voucher specimens of H and E-stained sections from Dog C from Montana (.no -------), H and E and toluidine blue from Dog D (.no -------) from Canada, and H and E and Toluidine blue sections and paraffin from dog A (.no -------).

The DNA sequences were deposited in GenBank (Accession numbers--)

Description of *Sarcocystis svanai*, n. sp. (Fig. 8–10)

Diagnosis: Only 3 sarcocysts identified by TEM of 2 dogs, 1 immature in Dog C, and 1 maturing, and 1 mature in Dog B. Sarcocysts discovered during TEM examination, light microscopic length unknown. Sarcocysts thin walled (0.5 μm). The Pvm undulated, with tiny blebs (evaginations), without villar protrusions (Fig. 8–10). Pvm lined by Edl except at evaginations, evaginations including Edl 50–100 nm thick (Fig. 10). Maximum thickness of the sarcocyst wall with Gs < 1 μm. The Gs homogenous without large granules or microtubules (Fig. 8, 9). Bradyzoites with numerous micronemes, towards 1\4 th of the conoidal end (Fig. 8). Up to 4 rhoptries per section with electron dense contents. Numerous amylopectin towards the 3/4th of the bradyzoite (Fig. 8).

TAXONOMIC SUMMARY—Type host: Canis familiaris.

Other hosts: Unknown.

Type locality: North America.

Etymology: Named after the host (dog in Sanskrit is called *svana*).

Specimens deposited: Specimens deposited in the United States National Parasite Collection in the Division of Invertebrate Zoology and National Museum of Natural History, Smithsonian Institution, Washington, D.C under (USNM-) include histological sections stained with H and E and toluidine blue from Dog B from Colorado (Syntype specimens for *S. caninum* .no -------), voucher specimens of H and E-stained sections from Dog C from Montana (.no -------), H and E and toluidine blue from Dog D (.no -------) from Canada, and H and E and Toluidine blue sections and paraffin from dog A (.no -------).

The DNA sequences were deposited in GenBank (Accession numbers--)

DISCUSSION

As stated earlier, sarcocysts in muscles of domestic dogs and cats, and wild felids and canids have been rarely reported. Therefore, it is unlikely that their life cycles will be completed experimentally. There was a heavy infection of *Sarcocystis* in all 4 dogs in the present study and fortunately, muscles were preserved well so we obtained good quality DNA, and highly resolved morphology by TEM. The dogs were from geographically separate areas, ruling out a common meal as a potential source of infection. The intensity of *Sarcocystis* infection was

similar in all 4 dogs, with most sarcocysts being *S. caninum*. This replication indicates that this is not a spurious occurrence. Here, we propose new names, *Sarcocystis caninum* and *S. svanai* for the species in muscles of dogs. We are aware that only the sarcocyst stage is known and the life cycle will be difficult to complete. The parasites are named mainly based on their structurally distinct sarcocyst wall, which is also supported by their phylogenetic resolution into 2 taxa using the *18S rRNA* and *rpoB* genetic markers. The differences in structure of the sarcocyst walls were not due to different stages of development because bradyzoites were seen in the sarcocysts of both species. All available evidence indicates that the basic structure of the sarcocyst wall is stable after bradyzoites are formed (Sheffield et al. 1977; Dubey et al. 1989).

The structure of the sarcocyst wall is considered one of the most useful criteria for distinguishing *Sarcocystis* species within a given host. Dubey et al. (1989), and Dubey and Odening (2001) divided the structure of the cyst wall into 37 types based on their ultrastructure. All sarcocysts have invaginations or evaginations on their cyst wall. The simplest of the sarcocyst wall (Type 1) is present in the *Sarcocystis* species in rodents but has also been found in higher mammals, including primates (Dubey et al. 1989), and also in *S. svanai* in the present study. The structure of the sarcocyst wall as a taxonomic criterion is only useful for *Sarcocystis* species within a given host. For example, there are 4 morphologically distinct species of *Sarcocystis* in sheep: *S. tenella*, and *S. arieticanis* with canids as definitive hosts and *S. gigantea* and *S. medusiformis* with felids as definitive hosts. Three distinct species of *Sarcocystis* (*S. capracanis*, and *S. hircicanis* with canids as definitive hosts, and *S. moulei* with felids as definitive hosts) are described in goats, but these species are morphologically but biologically different from species in sheep (Dubey et al. 1989).

The sarcocyst wall of *S. caninum* is unique with respect to villar protrusions, and distinct from the sarcocyst wall in *S. svanai*. Thus, *S. caninum* and *S. svanai* are easily distinguished by their sarcocyst wall (Fig. 10). The structure of *S. caninum* and *S. svanai* (Fig. 10) can be distinguished structurally from *S. neurona* sarcocysts found in 2 dogs from Illinois and Mississippi (Vashisht et al. 2005; Dubey et al. 2014a). The villar protrusions in *S. neurona* sarcocysts have tubules that extend into the ground substance whereas villar tubules are absent in *S. caninum* and *S. svanai*. As stated earlier, there are other reports of sarcocysts in dogs. The sarcocyst wall was not studied ultrastructurally for the 2 dogs from India and Kenya (Sahasrabudhe and Shah 1966; Bwangamoi et al. 1993), and only few details were provided for the sarcocysts in the 2 dogs from Georgia, and Alabama to determine species present (Hill et al. 1988; Blagburn et al. 1989). Hill et al. (1988) found 3 sarcocysts in a 2 year old dog. The sarcocyst wall was 1.5–2.0 μm thick and enclosed bradyzoites that were 11–12 μm long; there is no mention of ultrastructural studies on these cysts. Blagburn et al. (1989) found a single sarcocyst in a histological section of a frozen muscle biopsy later fixed in formalin; the sarcocyst was 47 × 52 μm in size, and the cyst wall had 1.5 × 0.9 μm sized villar protrusions. Thus, from the available information it is evident that sarcocysts in the present report are distinct from those described from dogs previously.

Recently, Gjerde and Schulze (2014) described the infection of 2 foxes in Norway with *Sarcocystis* parasites. Their sarcocysts were up to 12 mm long, and had short knob-like

protrusions on the cyst wall, but they did not provide a detailed description of the ultrastructure of the parasite. At the level of light microscopy, Gjerde and Schulze (2014) described the sarcocysts present in the Arctic foxes to be morphologically similar to the sarcocysts found in dogs B and C described by us (Sykes et al. 2011), and which we now refer to as *S. caninum* and *S. svanai*. In the absence of any ultrastructural characterization of the parasites found in the Arctic foxes, it is impossible to know whether the parasites found in the foxes are in fact the same or different species as *S. caninum.* We would like to emphasize that unless cross transmission experiments are performed under controlled conditions, it should not be assumed that *Sarcocystis* species identified from 2 different hosts are the same, although they may appear genetically and morphologically similar (Dubey and Rosenthal 2013; Dubey et al. 2014b). It is also incumbent on investigators to only name a new species when they have appropriate resolution at both the molecular and ultrastructural level.

Sarcocystis svanai in the present study has a sarcocyst wall structurally similar to *S. arctosi* sarcocysts from the brown bear (*Ursus arctos*) from Alaska, USA but very distinct from *S. caninum* (Dubey et al. 2007). However, the comparison stops there; their bradyzoites are structurally distinct. Dogs infected with *S. caninum* and *S. svanai* also experienced hepatitis, but these 2 parasites possess distinct sequence types and only share sequence similarity at the *18S rRNA* and *rpoB* loci with *S. canis*, a parasite known to cause severe hepatitis in dogs and other animals, but whose life cycle and sarcocyst are unknown.

Molecular characteristics are now an added taxonomic aid (Gjerde and Schulze 2014). However, the findings need to be cautiously interpreted because it is often not possible to know if only 1 species is present in the host investigated, unless sarcocysts can be separated from host tissue with certainty. In the present study, the 2 *rpoB* sequence types were unique from all published sequences publically available in GenBank. The closest relationship of the *S. svanai rpoB* sequence was to *S. campestris*, which was first named for the parasite found in the badger (Cawthorn et al. 1983). The *rpoB* sequence for *S. caninum* was most similar to *S. canis* but its sarcocysts are unknown. Genetic evidence indicates that *S. caninum* is different molecularly from *S. canis* at both the *18S rRNA* as well as *rpoB* loci. (Fig. 1, 2). The DNA sequences from *S. caninum* and *S. svanai* were similarly resolved from each other at the *18S rRNA* locus. Indeed, 10 SNPs and 2 INDELS separated the 2 species at the *18S rRNA* locus versus 11 SNPs at the *rpoB* locus, respectively, between *S. svanai* and *S. caninum* (Fig. 1, 2). In contrast, only 2 SNPs separate *S. falcatula* from *S. neurona* at *rpoB*, 2 closely related species infecting the Virginia opossum. Hence, the extent of sequence polymorphism detected supports the separate species designation. Furthermore, the 2 sequence types identified at the *rpoB* locus were further substantiated by the detection of 2 morphologically different sarcocysts in the Dog B tissues by TEM. This latter observation supports infection by 2 different, but related species of *Sarcocystis*, rather than co-infection by 2 different strains of *S. caninum* that each possessed a different, maternally inherited apicoplast organellar genome that was polymorphic at *rpoB.*

The 4 dogs in this study were from geographically separated areas of North America. All 4 dogs had severe myositis, such severe muscular myositis and ataxia are rare in any host affected by sarcocystosis (Dubey et al. 1989). All 4 dogs had evidence of liver injury as

revealed by elevated serum enzymes and hyperbilirubinemia. Unfortunately liver biopsy was not made in any dog because neosporosis, and not sarcocystosis, was suspected when dogs were first examined by veterinarians. It is speculative that the schizogonic multiplication of the parasite in liver before sarcocyst development might have contributed to hepatitis. Defining and naming the 2 species of *Sarcocystis* in dogs with myositis may draw attention of clinicians and eventually lead to completion of their life cycles.

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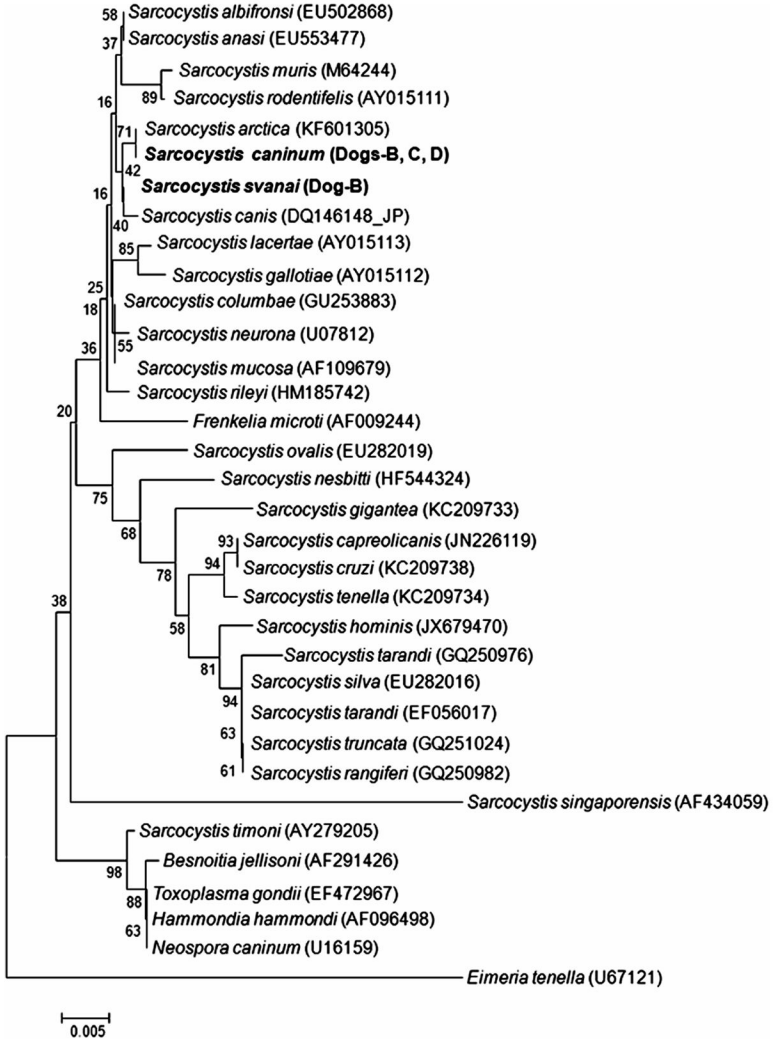

Figure 1.

Maximum likelihood SSU *18S rRNA* sequence phylogenetic tree. An alignment of partial SSU *18S rRNA* sequences were used to construct a phylogenetic tree to root the dog *Sarcocsytis* within the coccidia, and more specifically, within the Family Sarcocystidae. Reference *18S rRNA* gene sequences were retrieved from the NCBI Genbank database and aligned using ClustalX. To construct the maximum likelihood phylogenetic tree, aligned sequences were directly incorporated into MEGA. The percentage of replicate trees in which the various taxa clustered together in the bootstrap test (10,000 replicates) is shown next to the branches.

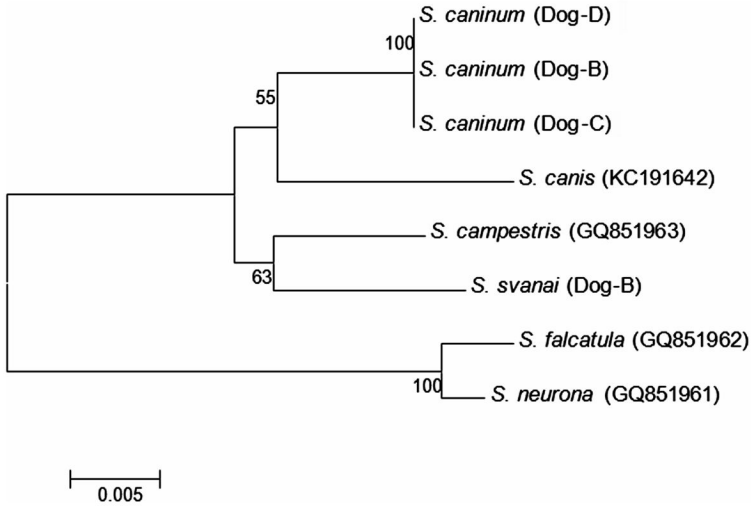

Figure 2.

Maximum likelihood phylogenetic tree obtained using the *rpoB* genetic marker. An alignment of the *rpoB* DNA sequences was used to construct phylogenetic trees to root the *Sarcocystis caninum* and *Sarcocystis svanai* sequences within the Sarcocystidae. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) is shown next to the branches. Only nodes with greater than 50% support are resolved. The *rpoB* locus differentiated *S. caninum* from *S. svanai* into 2 different species that are closely related to *S. canis* and *S. campestris*, respectively.

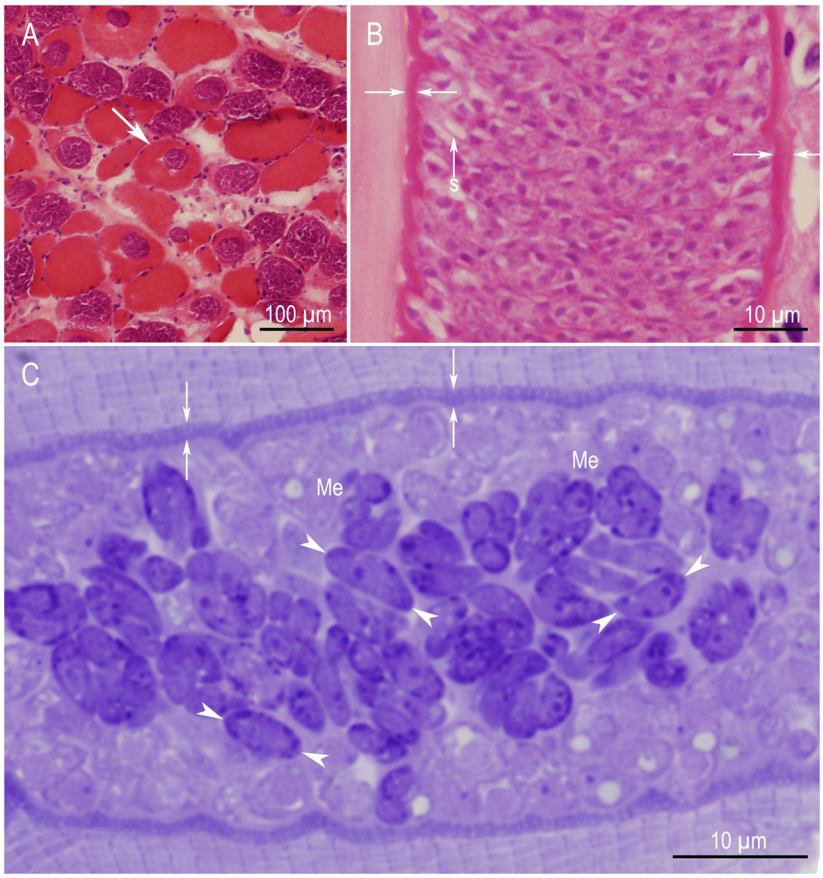

Figure 3.

Light microscopic structure of sarcocysts of *Sarcocystis caninum*, n.sp. from the naturally–infected dog B. **A** Low power view of sarcocysts (arrow) in triceps. Hematoxylin and eosin stain. **B** Higher magnification of the sarcocyst wall (indicated by opposing arrowheads) continued in to the interior of the sarcocyst as septa (S). The cyst wall appears without projections. Hematoxylin and eosin stain. **C** Higher magnification of the cyst wall (indicated by opposing arrowheads) in 1 µm section stained with toluidine blue. Note minute protrusions on the cyst wall, faintly stained metrocytes (Me) and darkly stained bradyzoites in longitudinal section (marked by opposing arrowheads).

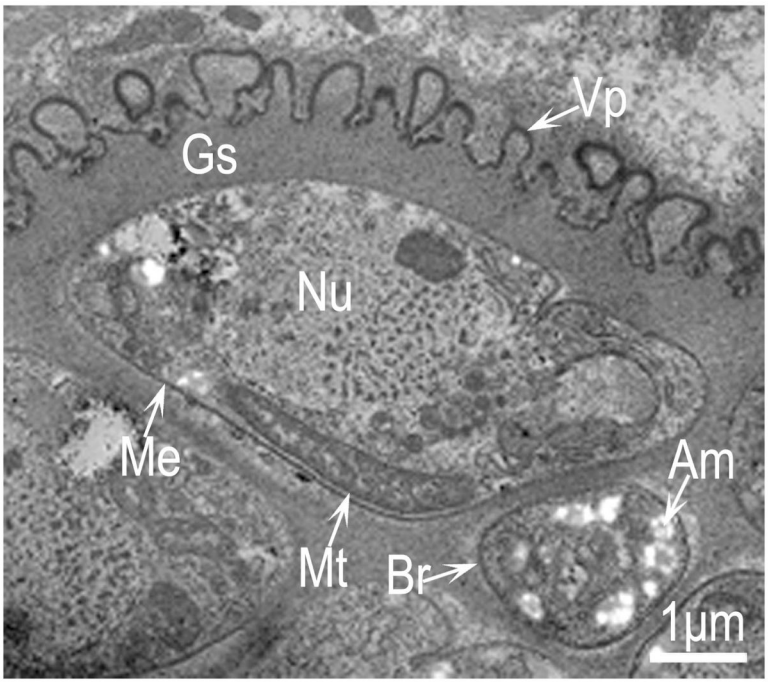

Figure 4.

TEM of an immature sarcocyst of *Sarcocystis caninum*, n. sp. Dog D. Note pleomorphic villar protrusion (Vp), smooth ground substance (Gs), a metrocyte (Me) with a nucleus (Nu), and cross section of a bradyzoite (Br) with numerous amylopectin granules (Am).

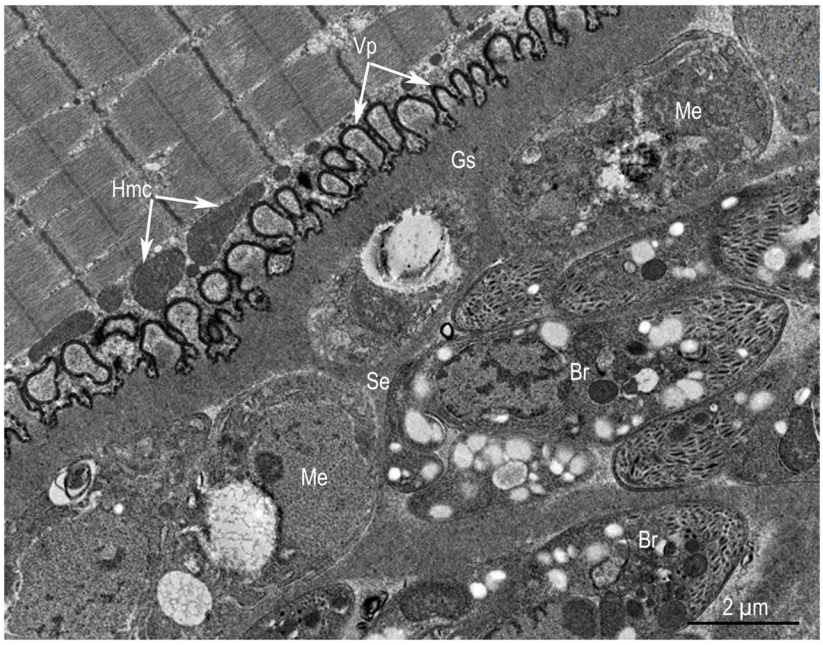

Figure 5.

TEM of *Sarcocystis caninum*, n. sp. sarcocyst in dog B. Note cytolysis of the host cell and the presence of numerous host cell mitochondriion (Hmc) surrounding the cyst wall. The villar protrusions (Vp) are pleomorphic. The ground substance (Gs) is smooth and continued in the interior as septa (Se). Also note metrocytes (Me) with few organelles without amylopectin granules and bradyzoites (Br) with numerous micronemes, and amylopectin granules.

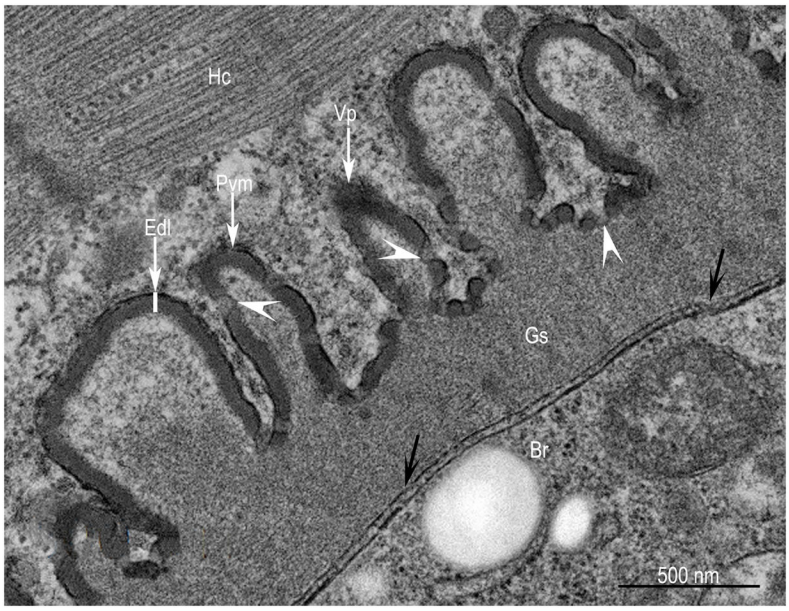

Figure 6.

Higher magnification of the cyst wall of *Sarcocystis caninum*, n. sp. sarcocyst in dog B. Note convoluted parasitophorous vacuolar membrane (Pvm) with prominent villar protusions (Vp) that are lined by an electron dense layer (Edl), except at invaginations (arrowheads) at irregular distances. The ground substance (Gs) is smooth and juxtaposed with double layered pellicle (black arrows) of the bradyzoite.

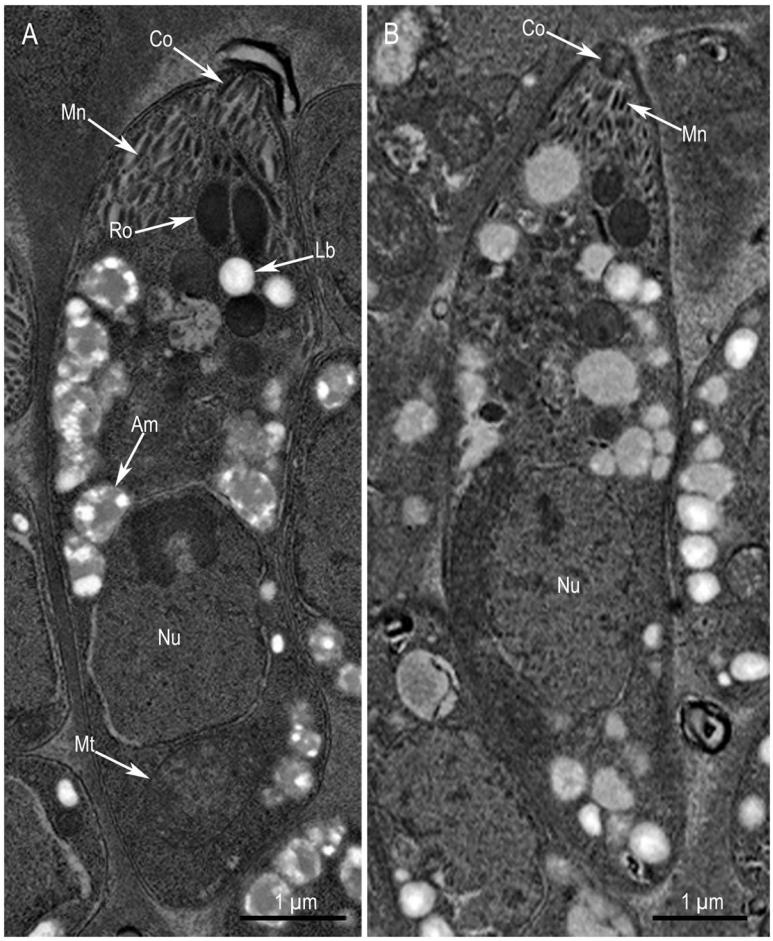

Figure 7.

TEM of 2 longitudinally cut bradyzoites (**A, B)** from 2 sarcocysts of *Sarcocystis caninum*, n. sp. in dog B. Note double layered pellicle, slightly thickened at the conoidal end, conoid (Co), few rhoptries (Ro), a mitrochondriion (Mt), numerous micronemes (Mn), subterminal nucleus (Nu), and numerous amylopectin granules (Am).

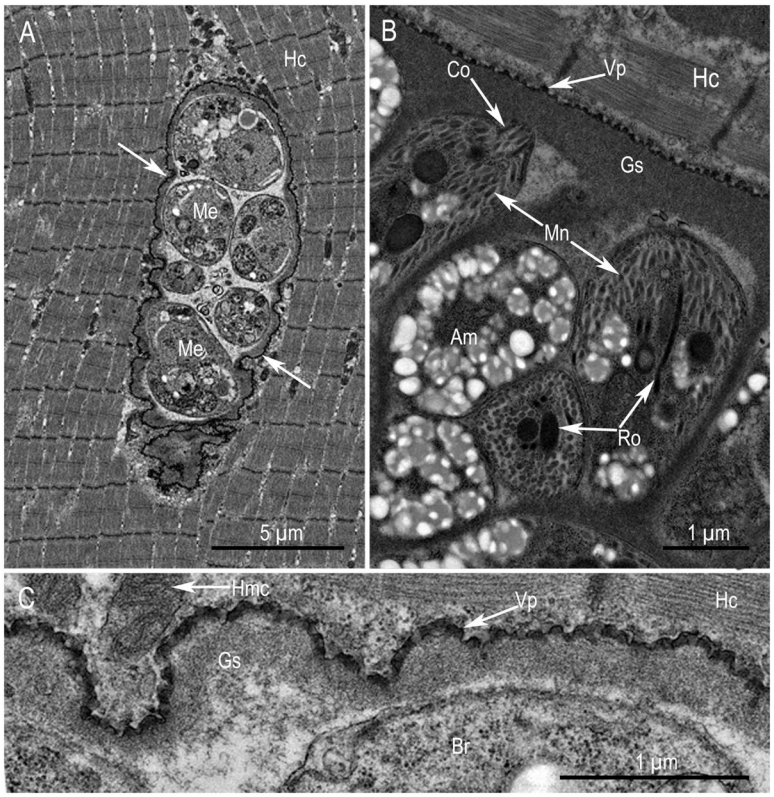

Figure 8.

TEM of *Sarcocystis svanai*, n. sp. from dogs. (**A**) Note an immature sarcocyst with few metrocytes (Me), thin cyst wall with blebs (arrows), and host cell (Hc). Dog C. (**B**) Nearly mature sarcocyst from dog B. Note 2 bradyzoites juxtaposed with the cyst wall. The parasitophorous vacuolar membrane (Pvm) has tiny blebs on the cyst wall. The ground substance (Gs) is smooth. Note conoid (Co), rhoptries (Ro), numerous micronemes (Mi), and amylopectin granules (Am). (**C**) Details of the sarcocyst wall shown in 8A. Note wavy parasitophorous vacuolar membrane (Pvm) with blebs, and smooth ground substance (Gs). Also note pellicle (black arrows) of the bradyzoite (Br) juxtaposed with Gs. Also note host cell mitochondrion (Hmc).

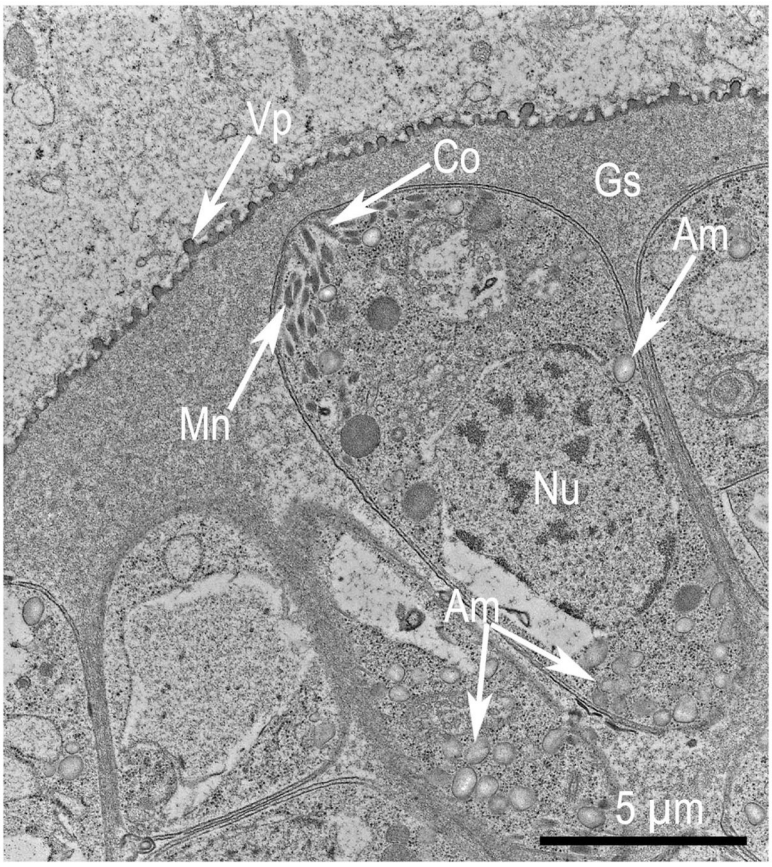

Figure 9.

TEM of a sarcocyst of *Sarcocystis svanai*, n. sp. from dog B. Note thin cyst wall with a smooth ground substance (Gs), and wavy parasitophorous vacuolar membrane (Pvm). Note a maturing bradyzoite (Br) with a conoid (Co), nucleus (Nu), and amylopectin granules (Am).

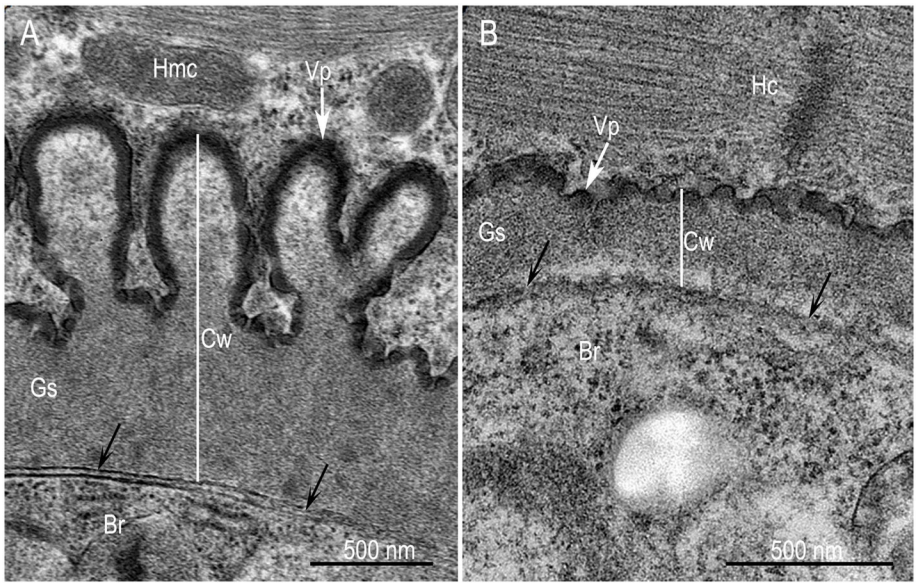

Figure 10.

Comparison of the sarcocyst walls of 2 species of *Sarcocystis*. **A** *Sarcocystis caninum*, n. sp. **B** *Sarcocystis svanai*, n. sp. The cyst wall (Cw) of *S. caninum* is more than twice thicker than that of *S. svanai*. Also note, for orientation, the host cell (Hc), host cell mitrochondriion (Hmc), villar protrusions (Vp), ground substance layer (Gs) juxtaposed to bradyzoite (Br), and double layered pellicle (black arrows).

Table 1

Details of dogs infected with proposed new species of *Sarcocystis.*

Dog ID	USNM	Location	Breed	Age	Sex	Date ill	Main signs	Liver	Muscles examined	Treatment	Biopsy date	TEM	Reference
A (D1174, AFIP 2944647**)**	HE Toluidine blue	British Columbia, Canada	Labrador	5 yr	Male	May, 2003	*	High AST, ALT, AP	Shoulder, biceps	CL, 9 wk, improved	May, 2003	Yes, *S. caninum*	Chapman et al. (2005)
B (case1, 148946)	HE Toluidine blue	Cañon City, Colorado	Golden Retriever	11 yr	Male	Jan, 2010	†	High ALT and ALP at onset	Digital flexor	Cl, died, not necropsied	April 8, 2010	Yes, *S. caninum* + *S. svanai*	Sykes et al. (2011); present study
C (case 2, 150825) D6145	HE Toluidine blue	Rural Montana	Rottweiler	4 yr	Female	March, 2010	‡	Increased ALT and ALP, hyperbilirubinemia at onset	Biceps, triceps	CL, decoquinate, improved	May 17, 2010		
D (190499, AFIP 4027107) D6779	HE Toluidine blue	British Columbia, Canada	Rhodesian Ridgeback	1.5 yr	Female	Jan, 2013	§	Hepatic enlargement, High CK- 7000	Quadriceps	Cl	22 January 2013	Yes, *S. caninum*	Present study

*Lethargy, anorexia, fever, ataxia, enability to walk, dysphagia.

†Lethargy, ptyalism, panting, anorexia, ataxia, diarrhea then stiff gait and generalized muscle pain 28 days after the onset of illness.

‡Lethargy, ptyalism, panting, anorexia, diarrhea then stiff gait and generalized muscle pain 28 days after the onset of illness.

§Lethargy, fever, muscle weakness, ataxia-see text).

Cl=clindamycin.