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Sarcocystis inghami n. sp. (Sporozoa: Sarcocystidae) from the skeletal muscles of the Virginia opossum *Didelphis virginiana* in Michigan

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Abstract

This report describes the newly identified *Sarcocystis inghami* n. sp. from the skeletal muscles of opossums (Mammalia: Didelphidae) that were collected from south central Michigan (42°43′-42°79′N, 84°18′-84°86′W), USA. The new species is distinguished from all species described from North and South American opossums by the distinctive morphology of the villar protrusions on the cyst wall. Sarcocysts of *S. inghami* are microscopic, up to 700 μm long and 110 μm wide. The sarcocyst wall is up to 7 μm thick, with long, stalked protrusions which average 5.5 × 1.2 μm. These are constricted at the base, expanded laterally, rounded off distally and occasionally bifid. The villar protrusions have numerous microtubules without electron-dense bodies that extend from the tips into the granular layer. Bradyzoites are 10.7 × 4.3 (8–12 × 4–5) μm. This is the second species of *Sarcocystis* sarcocyst described from the Virginia opossum in North America.

Introduction

Species of *Sarcocystis* Lankester, 1882 are one of the most widespread protozoan parasites both in terms of their host range and geographical distribution. *Sarcocystis* spp. have predator-prey life cycles, with carnivores as definitive hosts and other vertebrates as intermediate hosts (reviewed by Odening, 1998). Intermediate hosts become infected by ingesting sporocysts and/or oöcysts excreted in the faeces of the definitive host. After a short period of schizogony, the parasite forms sarcocysts in tissues especially muscles. The definitive host becomes infected by ingesting mature sarcocysts in infected tissues of intermediate hosts. Sexual reproduction of the parasite occurs in the intestinal mucosa of the definitive host. Some animals can act as both intermediate and definitive hosts, but usually not for the same species of *Sarcocystis* (see Dubey et al., 1989).

The North American opossum *Didelphis virginiana* Kerr has been known as the definitive host for at least three pathogenic species of *Sarcocystis*, *S. falcatula* Stiles, 1893, *S. neurona* Dubey, Davis, Speer, Bowman, de Lahunta, Granstrom, Topper, Hamir, Cummings & Suter, 1991 and *S. speeri* Dubey & Lindsay, 1999. Sarcocysts have also been found in the skeletal muscles of North American opossums, i.e. *Sarcocystis* spp. of Scholtyseck, Entzeroth & Chobotar (1982) and *S. greineri* Cheadle, 2001. In South American opossums *D. marsupialis* Linnaeus, *S. garnhami* Mandour, 1965 (host given as *Philander* sp.) and *S. didelphidis* Scorza, Torrealba & Dagert, 1957 have been reported, and *S. marmosae* Shaw & Lainson, 1969 occurs in *Marmosa murina* Linnaeus (reviewed in Cheadle, 2001).

As a part of a general study of the systematics, phylogeny and host-parasite associations of *Sarcocystis* spp. infecting opossums and horses, we describe a new species of *Sarcocystis* found in the skeletal muscles and tongue of two Virginia opossums from south central Michigan, USA, during the summer of 2002.

Materials and methods

During the period from June, 2002 to September, 2002, 17 adult Virginia opossums from central and south Michigan (42°43′-42°79′ N, 84°18′-84°86′W) were examined for the presence of *Sarcocystis* spp.

Virginia opossums that were road-kills, and those live-trapped and humanely killed, were collected. Each animal was assigned an identification number and the locality data were recorded. Samples were removed from tongue, abdominal and other skeletal muscles.

For light microscopy and histopathology, specimens were fixed in 10% neutral buffered formalin for a period of two weeks. The tissue was processed, embedded in paraffin wax, sectioned at 5 μm, and stained with H&E. Sections of tongue and other skeletal muscles were scanned for the presence of sarcocysts using a dissecting microscope at $\times$20 and $\times$40 magnifications. When a sarcocyst was detected, the cyst-containing section was observed at higher magnification for verification of the sarcocyst's wall. Sarcocysts, and the bradyzoites within, were measured, photographed and their sizes recorded. Measurements were taken using a calibrated ocular micrometer. Maximum and minimum values are given, followed in parentheses by the arithmetic mean and standard deviation. Measurements are in micrometres, unless otherwise stated.

The specimens for transmission electron microscopy (TEM) consisted of 5 μm paraffin sections of a cyst in the muscle. The glass slides were soaked in xylene to remove the coverglass, transferred to 100% ethanol and rehydrated in a graded series of ethanol solutions. The slides were transferred from the final, 30% ethanol solution to 0.1 M phosphate buffer and then placed in 1% osmium tetraoxide in 0.1 M phosphate buffer for two hours. The slides were dehydrated in a graded ethanol solutions, transferred from 100% ethanol to propylene oxide, then to 50% propylene oxide resin (30 min) and finally to 100% resin (4 h). The hardened resin, containing the section, was then peeled off the slide with a razor blade, mounted on a resin stub, and sectioned. Sections were stained with uranyl acetate and lead citrate and examined with a Phillips 301 transmission electron microscope.

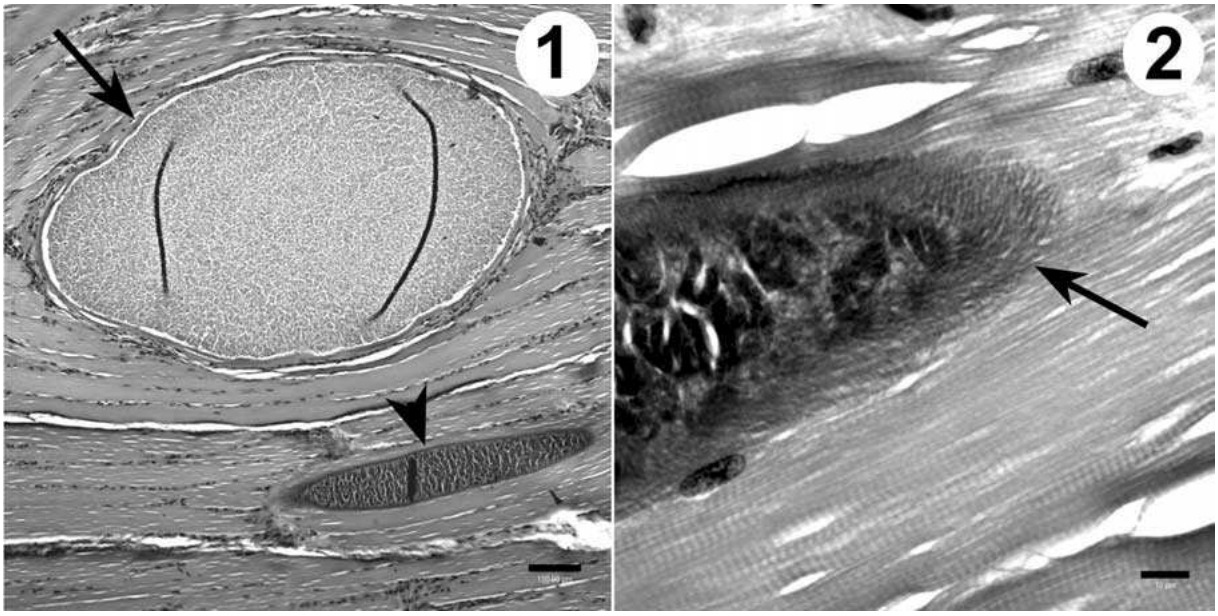
For isolation of *Sarcocystis* spp. oöcysts/sporocysts, faecal specimens were collected from the small intestine of opossums and initially examined for the presence of *Sarcocystis* sporocysts using saturated NaCl (360 g^{-1}l, sp. gr. 1.21) for faecal floatation (Ewa & Daniel, 1999). The mucosal scrapings from the small intestine were examined using potassium bromide (KBr) discontinuous density gradient centrifugation method as described (Elsheikha et al., 2003). The genotype of the recovered sporocysts was determined by Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP) methods as described (Tanhauser et al., 1999). Genotyping of the isolate was carried out using DNA extracted from sporocysts that had been collected and purified from opossum small intestine using the Dneasy Tissue kit (QIAGEN).

Sarcocystis inghami **n. sp.**

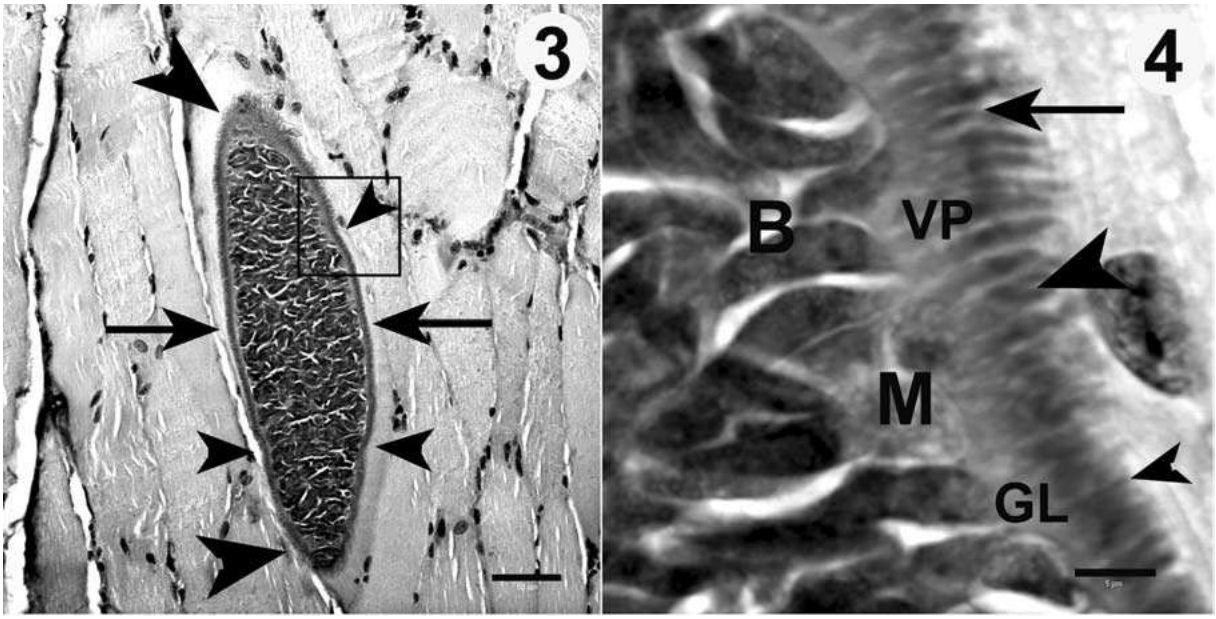
Type-intermediate host: Virginia opossum *Didelphis virginiana* Kerr.
Natural definitive host: Unknown.
Type-locality: South-Central Michigan (42°43′–42°79′N, 84°18′–84°86′W), USA.
Site of infection: Skeletal muscles.
Type-specimen: Syntype (H&E-stained tissue sections) of sarcocysts have been deposited in the United States National Parasite Collection (USNPC), Beltsville, Maryland, USA. USNPC no. 092429 and The Natural History Museum, London, UK. Reg. no. 2003:31:3:1.
Prevalence: 11.8% (2/17 opossums examined). *Sarcocystis-infected* opossums has concurrent infections of *Besnoitia darlingi* Brumpt, 1913 cysts in the muscles (Figure 1) and *Sarcocystis neurona* oöcysts and sporocysts in the intestinal tissue.
Etymology: The specific appellation *inghami* is refers to Ingham County of Lansing, Michigan, USA, the locality from which the opossums were collected.

Description (Figures 1–7)

Light microscopy
[Based on 20 fixed and stained sections]. Fusiform sarcocysts in skeletal muscles and tongue. Sarcocysts are microscopic, 335-700 (493.4 $\pm$ 154.7) long by 79-110 (91.4 $\pm$ 8.7) wide. Cyst wall is of variable thickness due to different lengths of villar protrusions (VP), depending on region of cyst; wall is thick at anterior and posterior ends and relatively thin in middle. In the terminal 2/3 of sarcocyst, VP are hair-like, unevenly arranged, of unequal length and longer than those in middle. VP are of equal length and evenly spaced in middle of cyst (Figures 2–4). Slight indentation in outer cyst wall occurs near terminal 2/3 of cyst (Figures 3–4). However, this indentation is not observed in all the examined sections. In middle of cyst, sarcocyst wall reaches 7 thick and VP measure 5-6 (5.5 $\pm$ 0.5) long by 1-1.5 (1.21 $\pm$ 0.2) wide and are *c.* 0.5 apart, mostly with rounded off tips. Granular layer (GL) *c.* 1 thick, located immediately



Figures 1-2. Differential interference contrast (DIC) photomicrographs of longitudinal sections of a sarcocyst of *Sarcocystis inghami* n. sp. from the tongue of the Virginia opossum (*Didelphis virginiana*). H&E. 1. Note the co-infection of *S. inghami* sarcocyst (arrowhead) with *Besnoitia darlingi* cyst (arrow). 2. High magnification of *S. inghami* sarcocyst anterior end. Note the long hair-like villi at the end of the sarcocyst (arrow). *Scale-bars*: 1, 100 μm; 2, 20 μm.



Figures 3-4. Longitudinal sections of a sarcocyst of *Sarcocystis inghami* n. sp. from the abdominal muscles. H&E. 3. Note the difference in the length and distribution of villar protrusions in the middle region of the sarcocyst (arrows) compared to VP towards the ends of the sarcocyst (large arrowheads). Also note slight indentations (small arrowheads) in the sarcocyst wall at beginning of the outer two-thirds of the sarcocyst. 4. Close-up of the indentation in the sarcocyst wall. Note the clear difference in length of VP in the region towards the middle (small arrowhead) compared to that distally (arrow). Also note that the change in the length of the VP starts at the indentation in the terminal third of the sarcocyst (large arrowhead). *Abbreviations*: B, bradyzoite; M, Metrocyte; VP, villar protrusions; GL, granular layer. *Scale-bars*: 3, 50 μm; 4, 5 μm.

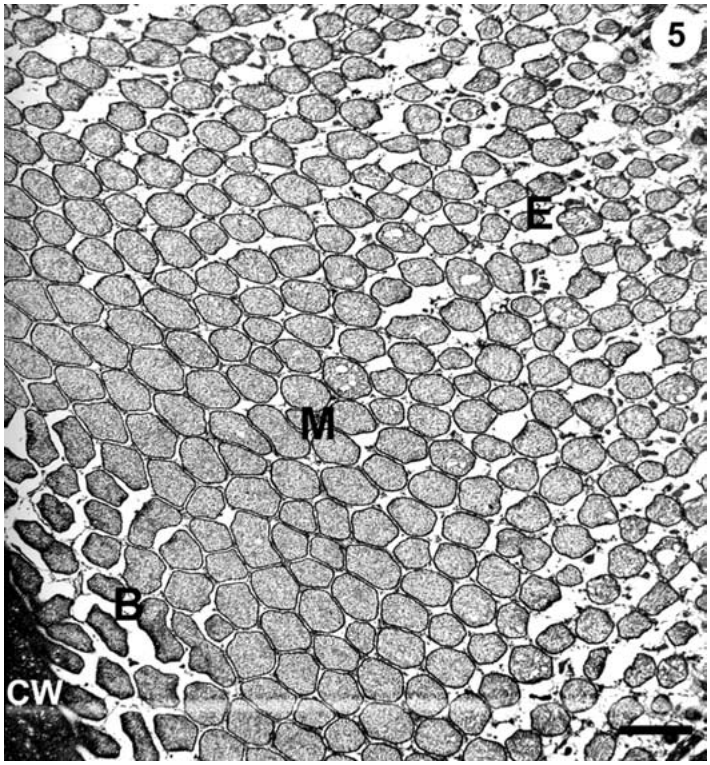


Figure 5. Cross- and tangential sections of *Sarcocystis inghami* n. sp. sarcocyst villar protrusions (VP) at different levels at the end of the cyst. Note the difference in size and the spacing between the VP at each level of sectioning. At the base, VP are medium-sized with wide spaces and larger with narrow spaces in between in the mid-region, while at distally they are smaller-sized with wider spaces in between. *Abbreviations*: CW, cyst wall; B, base of villi; M, middle part of the villi; E, terminal end of the villi. *Scale-bar*: 0.2 μm.

beneath primary sarcocyst wall, gives rise to ground substance and septa (Figure 4). Interior portion of sarcocyst divided with thin septa separating pockets of bradyzoites. Several metrocytes are visible at periphery of sarcocyst. Bradyzoites and metrocytes butted against granular layer. Longitudinally cut bradyzoites measure 10-12 (10.7 $\pm$ 0.9) by 4-5 (4.4 $\pm$ 0.36), with structures typical of Apicomplexa (Dubey et al., 1989), banana-shaped, posteriorly arranged nucleus and occur as single unit and not in pairs.

Transmission electron microscopy

VP have numerous, prominent, longitudinally arranged microfilaments (slender microtubules), which run length of villi, extending from tips to granular layer (ground substance). In cross-section, each VP has hundreds of these microtubules scattered throughout entire filament core (Figure 5). VP often appear constricted at base, expanded in middle and rounded off toward distal end (Figures 5–7). Few VP with bifurcate distal end are observed in middle and terminal parts of cyst. Parasitophorous vacuolar (PV) membrane and its underlying electron-dense layer have total thickness of 40 nm. PV membrane is ornamented with bump-like or knob-like structures that are interrupted at almost regular intervals, giving appearance of bead-like inpocketings on periphery of VP (Figure 7). PV membrane ornamentation with bump-like or knob-like structures are electron dense in basal regions of VP, but composed only of small portion of PV membrane terminally.

Granular layer (GL) gives rise to ground substance and septa that subdivide sarcocyst (Figure 6). GL is *c.* 1 thick. Bradyzoites with organelles typically found in *Sarcocystis* spp. bradyzoites (Dubey et al., 1989). Numerous micronemes scattered in anterior third of conoidal end of bradyzoites, with dense bodies, rhoptries and many amylopectin granules.

Sporulated oöcysts/sporocysts

No sporocysts were detected in faeces from the small intestine of opossums by NaCl faecal flotation. However, with centrifugation of a mucosal homogenate containing specimens using a discontinuous KBr-

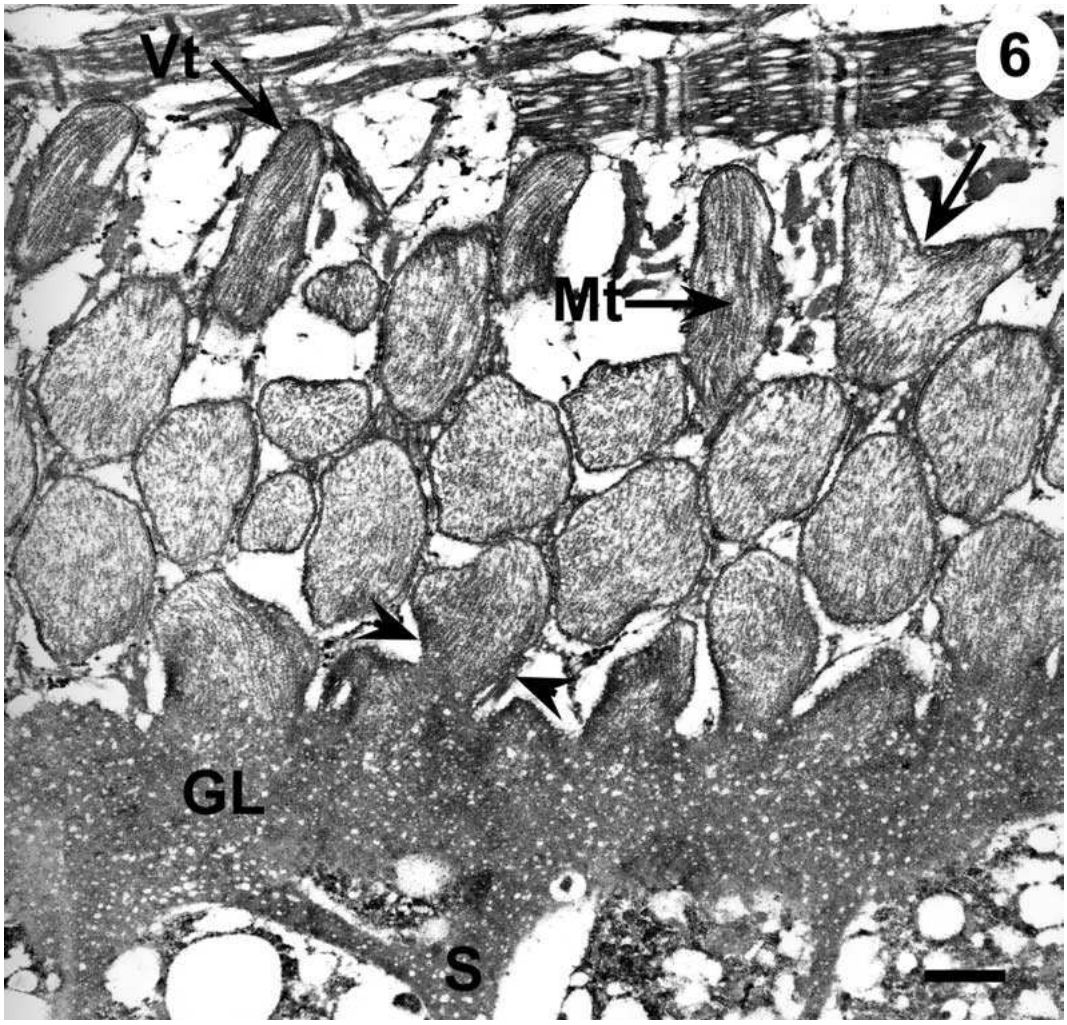


Figure 6. TEM micrograph of *Sarcocystis inghami* n. sp. sarcocyst from the tongue of the Virginia opossum. Note the villar protrusions (VP) in the mid-region of the cyst are constricted, with a narrow stalk (opposing arrowheads), and the villar tips (Vt) are rounded off. The VP contain numerous rows of microtubules (Mt). Note the granular layer (GL) continuing into the sarcocyst as septa (S). Also, note the characteristic bifurcation of one VP (arrow). *Scale-bar*: 0.5 μm.

gradient, it was possible to recover and purify numerous sporulated oöcysts/sporocysts. It is conceivable that the use of NaCl instead of Sheather's sugar floatation for the detection of *Sarcocystis* spp. sporocysts in opossum faeces could have hampered the effective isolation of sporocysts.

RFLP analysis of the recovered sporocysts showed banding patterns characteristic of *S. neurona* for the two opossum isolates, with bands at 180 and 154 bp with *Hin*fI digestion, and a single band at 334 bp with *Hin*dIII digestion.

Discussion

Measurements of *S. inghami* n. sp. were compared to those of other *Sarcocystis* species reported from opossums to determine similarities and/or differences (Table 1). The sarcocysts of the presented material had well-developed villar protrusions and a scant distribution of metrocytes in comparison to the large number of mature bradyzoites that filled the entire sarcocysts. Therefore, the sarcocysts in the present study were almost totally mature. Sarcocysts of *S. inghami* were significantly smaller in size than those of the other valid species described from opossums, but larger than those given by Scholtyseck et al. (1982) for ma-

Table 1. A comparison of structural measurements of sarcocysts of *Sarcocystis inghami* n. sp. found in the muscle of Virginia opossums with those of closely related *Sarcocystis* spp.

Parasite	*S. didelphidis*	*S. garnhami*	*S. marmosae*	*S. greineri*	*Sarcocystis* spp.	*S. inghami* n. sp.
Host	*Didelphis marsupialis*	*Didelphis marsupialis*	*Marmosa murina*	*Didelphis virginiana*	*Didelphis virginiana*	*Didelphis virginiana*
Locality	Venezuela	Belize	Brazil	Florida, USA	Michigan, USA	Michigan, USA
Source of data	Scorza et al. (1957)	Mandour (1965)	Shaw & Lainson (1969)	Cheadle (2001)	Scholtyseck et al. (1982)	Presented material
Sarcocyst	Macroscopic	Macroscopic	Macroscopic	Macroscopic	Microscopic	Microscopic
length* (mm)	0.9	0.31-3.3	2	2-8	0.140	0.335-0.700
width	345	110-250	800	108-189	70	79-110
Bradyzoite:						
length	6.5	5.3-6.9	6.2-9.0	11	7-10	9.7-12
width	1.5	1.3-1.9	1.8-3.0	4.4	2.5-3	3.9-5
Specimen	Fixed	Fixed	Dried smear	Live	Fixed	Fixed
Protrusions (VP):						
length	5.2	8-6	11.5-13.0	2.8-4.0	3.4-7	4.9-6.2
width		1.5-2.0	2.6	1.3-2.0	0.8-1.4	0.9-1.54
Morphology		Acidophilic, sharply pointed spines	Finger-like, with rounded tips	Stumpy, digitiform with some pedunculation	Finger-like, with serrate surface	Stalked, expanded in middle, rounded off and often bifid distally

*Measurements are in micrometres except where indicated.

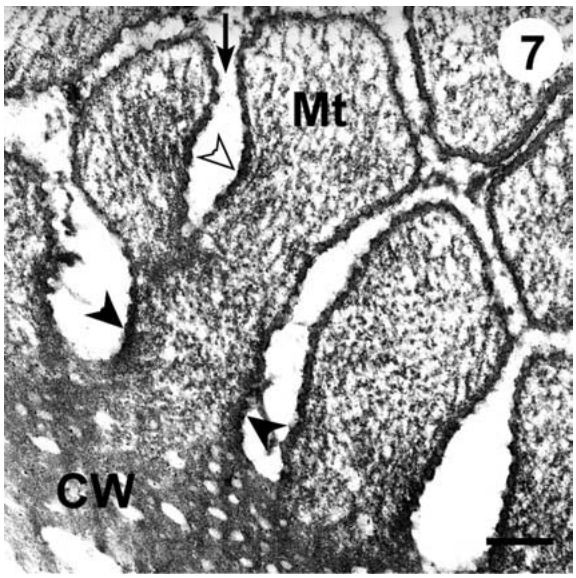


Figure 7. TEM micrograph of *Sarcocystis inghami* n. sp. sarcocyst from the tongue of the Virginia opossum. Close-up of a villar protrusion (VP) at the end of the cyst. The VP is constricted with a narrow stalk (opposing arrowheads) and bifurcates distally (arrow). Note the ornamentations and bead-like inpocketings (empty arrowhead) on the primary cyst wall (CW) and the presence of microtubules (Mt). *Scale-bar*: 0.4 μm.

terial from the same host species and neighbouring geographical locality.

Sarcocysts can be divided into 36 types based on the structure of the cyst wall (Dubey et al., 1989; Dubey & Odening, 2001). Microscopically, the cyst wall protrusions/villi of *S. inghami* are distinct from all 36 types of sarcocyst wall previously reported.

S. inghami VP are relatively similar to those of *S. greineri* (see Cheadle, 2001) and those described by Scholtyseck et al. (1982). However, the VP of *S. greineri* are generally more slender, stumpy and digitiform with some pedunculation basally, whereas the villar protrusions described by Scholtyseck et al. (1982) are digitiform and have a serrate surface. Moreover, the cyst wall protrusions for *S. inghami* are longer than those from *S. greineri* sarcocysts and contain a larger number of fibrillar elements. The bradyzoites are much more similar in size to those described by Cheadle (2001), who found that live bradyzoites within the cyst measured *c.* 11 $\times$ 4.4 μm. *S. garnhami* sarcocysts, described by Mandour (1965), have sharply pointed spines on the cyst wall, and *S. marmosae* sarcocysts, described by Shaw & Lainson (1969), have finger-like projections on the cyst wall which are rounded off distally.

S. inghami n. sp. can be readily differentiated from the other species by the characteristic morphology of the VP, which have a narrow base, are wider in the middle and rounded off distally. Additionally, the cyst wall of the *S. inghami* is variable in thickness, and has protrusions of variable length and distribution depending on the region of the sarcocyst but independent of the plane of sectioning and the folding of the villi. Most importantly, the bifurcation of some VP in the primary cyst wall was a unique feature of this new taxon.

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