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Sarcocysts of an Unidentified Species of *Sarcocystis* in the Sea Otter (*Enhydra lutris*)

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ABSTRACT: The number of *Sarcocystis* species that infect sea otters (*Enhydra lutris*) is unknown. Sea otter tissues were recently shown to harbor sarcocysts of *S. neurona* and of unidentified species of *Sarcocystis*. Whereas sarcocysts of *S. neurona* have walls 1–3 μm thick with type 9 villar protrusions, ultrastructure of a distinct thin-walled sarcocyst (0.5–0.7 μm thick) lacking villar protrusions, but instead exhibiting minute type 1 undulations on the sarcocyst wall, is described in this report. Parasites characterized from a sea otter infection were inferred to be related to, but distinct from, other species belonging to *Sarcocystis*, based on sequencing and phylogenetic analysis of a portion of the beta subunit of the plastid-encoded RNA polymerase gene.

Disease in a group of sea otters (*Enhydra lutris*) was recently attributed to infection with *Sarcocystis neurona*, a parasite that causes fatal neurologic disease in horses and other mammals (Dubey, Lindsay, Saville et al., 2001). Sea otters from the coast of California and Washington died of encephalitis associated with *S. neurona* schizonts (Rosenke et al., 1999; Lindsay et al., 2000, 2001; Miller et al., 2001). In addition, *S. neurona* sarcocysts were found in 2 other sea otters (Rosenke et al., 1999; Dubey, Rosypal et al., 2001). Parasites lacking the thick sarcocyst walls and elongated villar protrusions characteristic of *S. neurona* also have been observed in sea otters (in sea otter no. 2 of Dubey, Rosypal et al., 2001). To characterize this unidentified parasite better, transmission electron microscopy (TEM) was used to define its ultrastructure. To further aid future efforts to identify and diagnose sea otter infections, the plastid-encoded beta subunit of RNA polymerase (*rpoB*) was amplified from infected sea otter tissue and compared with homologous sequences from *S. neurona*, *S. falcatula*, and *S. lindsayi* (Table I).

Two sarcocysts from a paraffin section of the skeletal muscle of sea otter were deparaffinized, postfixed in osmium tetroxide, and processed for TEM examination. In 1- μm toluidine blue-stained sections, the sarcocysts measured 95×60 and $110 \times 65 \mu\text{m}$. The sarcocyst wall was $<1 \mu\text{m}$ thick without visible villar protrusions (Fig. 1). Septa were indistinct.

Under TEM the sarcocyst wall was found to be 0.5–0.7 μm thick and bore minute, electron-dense undulations located at irregular intervals (Fig. 2A, B). The maximum observed width of the sarcocyst wall at the point of infolding and beginning of septa was 1.0 μm . Only bradyzoites were seen, and 3 longitudinally cut bradyzoites were $5.0\text{--}5.7 \times 1.6\text{--}1.9 \mu\text{m}$ in size. Rhoptries were prominent, and their bulbous blind end was sometimes turned toward the conoidal end (Fig. 2A). The micronemes were located in the anterior half of the bradyzoite. Thus, the thin-walled sarcocysts in the present report were ultrastructurally distinct from those of *S. neurona*, which typically bear walls 1–3 μm

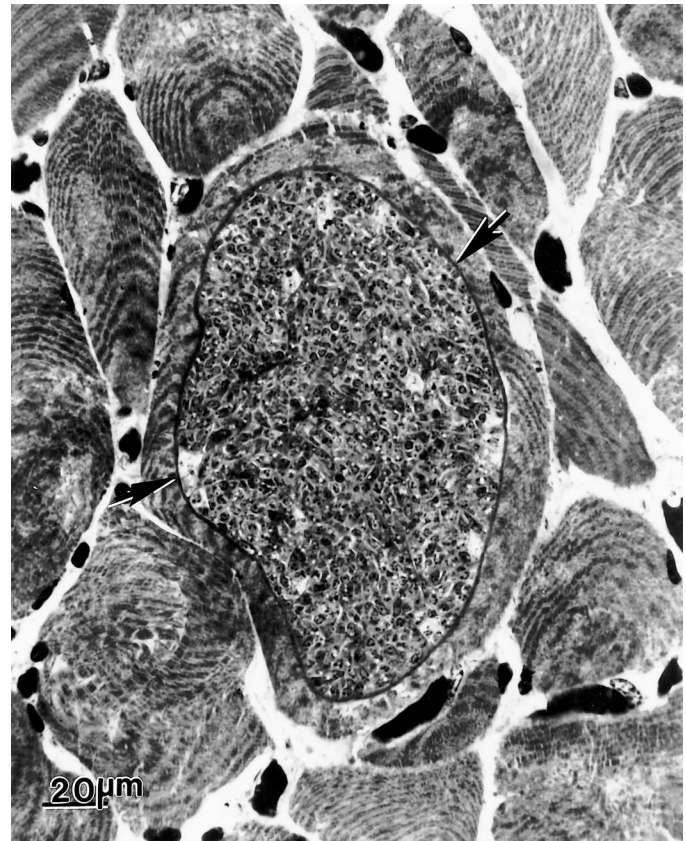


FIGURE 1. Section of sea otter skeletal muscles showing a *Sarcocystis* sp. sarcocyst. Note the thin sarcocyst wall (arrows). Toluidine blue stain.

in thickness featuring prominent villar type 9 protrusions (Dubey et al., 1989; Dubey, Lindsay, Fritz et al., 2001).

In the initial report of an unidentified sarcocyst in the musculature of an encephalitic sea otter, villi were present on the sarcocyst walls,

TABLE I. Sources of parasite isolates.

Name	Host species, locale, and reference	GenBank accession
<i>Sarcocystis lindsayi</i>	<i>Didelphis albiventris</i> ; Brazil; Dubey, Rosenthal, and Speer (2001).	AY164997
<i>Sarcocystis</i> sp.	<i>Enhydra lutris</i> ; Washington; Dubey, Rosypal, Rosenthal et al. (2001).	AY164998
<i>S. falcatula</i>	<i>Didelphis albiventris</i> ; Argentina; Dubey, Rosenthal, and Speer (2001).	AY164999
<i>S. neurona</i>	<i>Didelphis albiventris</i> ; Brazil, SN 35-OP; Dubey Lindsay, Kerber et al. (2001).	AY165000
<i>S. falcatula</i> -like	<i>Didelphis marsupialis</i> ; Argentina; Dubey, Lindsay, Rosenthal et al. (2001).	AY165001

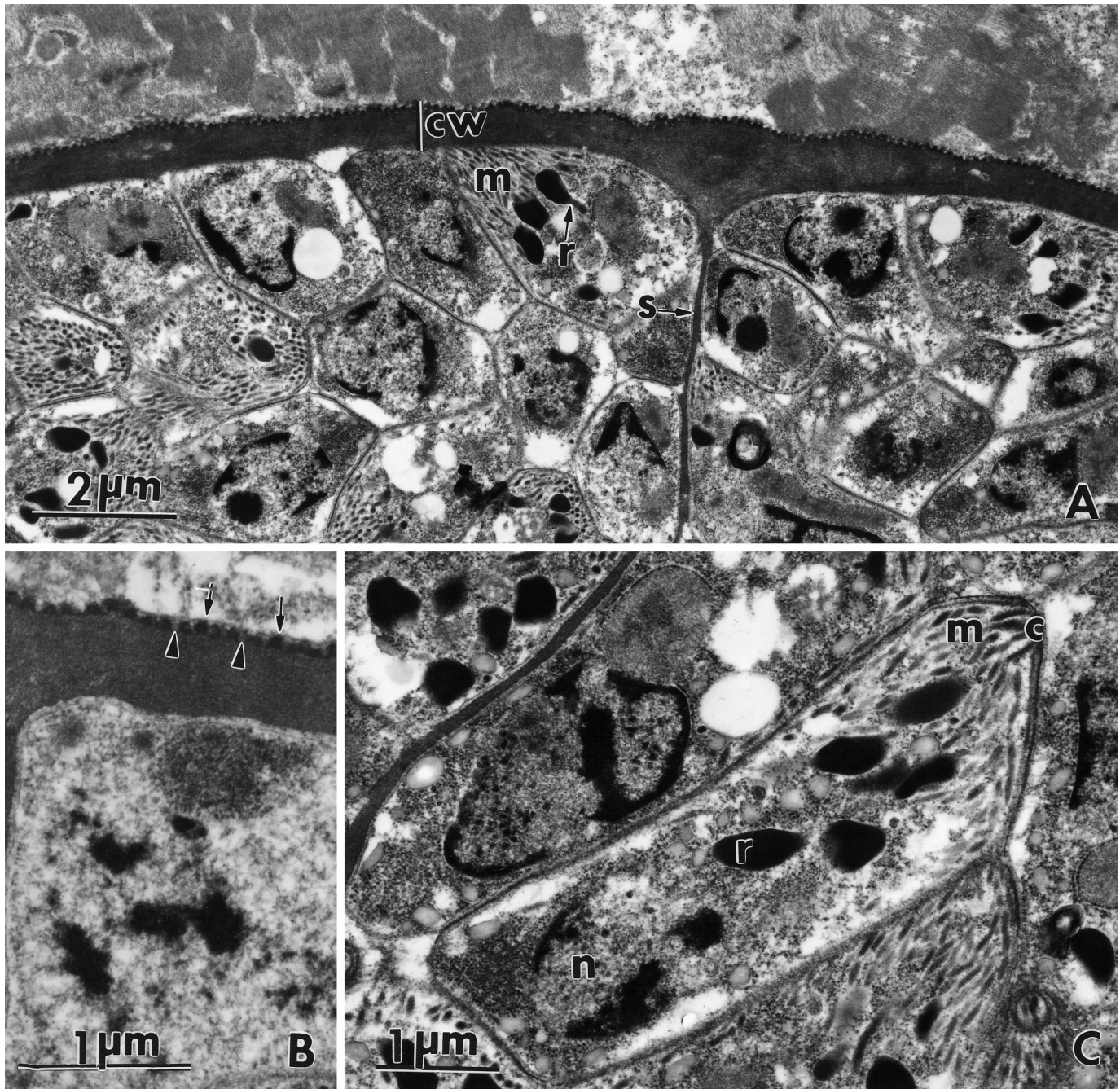


FIGURE 2. Transmission electron micrograph of a mature sarcocyst from the skeletal muscle of the sea otter. **A.** Note the thin cyst wall (cw) with minute protrusions. The ground substance is homogenous without microtubules and continues into the sarcocyst interior as septa (s). All organisms present are bradyzoites. A rhoptry (r) in 1 bradyzoite has its bulbous end turned toward the conoidal end. Also note numerous micronemes (m) toward the conoidal end. **B.** Higher magnification of the sarcocyst wall. Note the minute protrusions on the sarcocyst wall, interrupted at irregular intervals (arrow heads). **C.** Longitudinal section of a bradyzoite showing the conoid (c), micronemes (m), rhoptries (r), and terminal nucleus (n).

but autolysis obscured additional ultrastructural details (Rosonke et al., 1999). Dubey, Rosypal et al. (2001) described the ultrastructure of *S. neurona* sarcocysts in skeletal muscle of a sea otter that had died of *S. neurona*-associated encephalitis. Only sarcocysts resembling those of immature *S. neurona* were found in the encephalitic sea otter (sea otter no. 1 of Dubey, Rosypal et al., 2001). However, in the musculature of a second sea otter described by Dubey, Rosypal et al. (2001), light microscopy indicated that there were at least 2 additional types of sarcocysts; thin-walled sarcocysts, possessing septa but lacking villi, were

distinct from the third type of sarcocyst. The third type of sarcocysts were thick-walled, with 7- μ m villar protrusions, and were found in the tongue and not in the skeletal muscle. In the present report, using TEM, the presence of a structurally distinct, thin-walled sarcocyst was confirmed. Whether these sarcocysts correspond to those illustrated previously by Dubey, Rosypal et al. (2001) cannot be known with certainty because only 2 sarcocysts were examined ultrastructurally and because the true diversity of this mixed, natural infection is undefined.

DNA was extracted from sea otter isolate and used as a template in

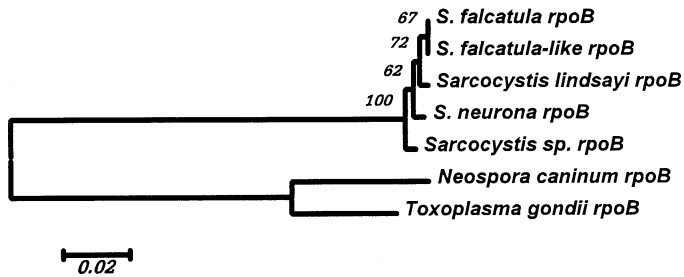


FIGURE 3. Midpoint-rooted neighbor-joining tree reconstructed from variation in the *rpoB* B gene. Kimura 2-parameter distances were calculated for each pair of sequences. The percentage of bootstrap replicates ($n = 1,000$) in which a given node was recovered is indicated. Five hundred base pairs of the *rpoB* gene were sequenced from *Sarcocystis* sp. and from representatives of *S. neurona*, *S. falcatula*, and *S. lindsayi* in the sea otter (see Table I for details on isolates). The *Neospora caninum* and *Toxoplasma gondii* homologs were obtained from GenBank (accession nos. AF095904 and AF138960, respectively).

a polymerase chain reaction (PCR) by using degenerate primers designed to amplify the *rpoB* gene, encoded by the plastid genome of apicomplexans—primers F1 (5'-gcg gtc cca aaa ggg tca gtg gat atg atw twt gaa gat gc) and R3 (5'-gcg gtc cca aaa ggg tca gtc ctt tat ktc cat rtc t). The resulting 504-bp PCR products were directly sequenced using BigDye chemistries and an ABI 3100 automated fluorescent sequencer. Homologous sequences were characterized from isolates of *S. neurona*, *S. falcatula*, and *S. lindsayi*, the origins of which are summarized in Table I. These were aligned to each other and to homologs from *Neospora caninum* and *Toxoplasma gondii* by using CLUSTAL W 1.8 (Thompson et al., 1994), available on the bioinformatics server of the Baylor College of Medicine. Relationships of these sequences were investigated by constructing a gene genealogy by calculating Kimura 2-parameter distances from 1,000 bootstrap replicates of the alignment and using the Neighbor-Joining algorithm using MEGA 2.1 (Kumar et al., 2001).

The *rpoB* sequence obtained from the otter isolate was placed as a basal member of a clade that also contained the other examined isolates belonging to *Sarcocystis* but that included neither *N. caninum* nor *T. gondii* (Fig. 3). Concordant topologies were obtained when the minimum evolution and maximum parsimony criteria were used (data not shown). Several nucleotide substitutions distinguish this otter specimen from the isolates representing other species of *Sarcocystis*. In contrast, the *rpoB* of isolates representing *S. falcatula* are comparatively homogeneous. Thus, morphological and genetic evidence indicates that sea otters, in addition to being at the risk of exposure to *S. neurona* parasites, serve as host to at least 1 other species of parasites belonging to the genus *Sarcocystis*.

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Morphology Is Not a Reliable Tool for Delineating Species Within *Cryptosporidium*

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ABSTRACT: Within the coccidia, morphological features of the oocyst stage at the light microscope level have been used more than any other single characteristic to designate genus and species. The aim of this study was to conduct morphometric analysis on a range of *Cryptosporidium*

ridium spp. isolates and to compare morphological data between several genotypes of *C. parvum* and a second species *C. canis*, as well as a variation within a specific genotype (the human genotype), with genetic data at 2 unlinked loci (18S ribonucleic deoxyribonucleic acid and HSP