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Ultrastructural and molecular confirmation of the development of *Sarcocystis neurona* tissue cysts in the central nervous system of southern sea otters (*Enhydra lutris nereis*)

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Abstract

In 2004, three wild sea otters were diagnosed with putative *Sarcocystis neurona*-associated meningoencephalitis by histopathology and immunohistochemistry. Schizonts, free merozoites and tissue cysts were observed in the brains of all three infected animals. Tissue cysts from sea otter 1 (SO1) stained positively using anti-*S. neurona* polyclonal antiserum. However, positive staining does not preclude infection by closely related or cross-reactive tissue cyst-forming coccidian parasites. Two immature tissue cysts in the brain of SO1 were examined using transmission electron microscopy. Ultrastructural features included cyst walls with thin villous projections up to 1 μm long with tapered ends and a distinctive, electron-dense outer lining layer composed of linearly-arranged, semi-circular structures with a "hobnailed" surface contour. Small numbers of microtubules extended down through the villi into the underlying granular layer. Metrocytes were short and plump with an anterior apical complex, 22 subpellicular microtubules, numerous free ribosomes and no rhoptries. Some metrocytes appeared to be dividing, with two adjacent nuclear profiles. Collectively these ultrastructural features were compatible with developing protozoal cysts and were similar to prior descriptions of *S. neurona* tissue cysts. Panspecific *18S rDNA* primers were utilized to identify protozoa infecting the brains of these otters and DNA amplification and additional sequencing at the *ITS1* locus confirmed that all three otters were infected with *S. neurona*. No other *Sarcocystis* spp. were detected in the brains or skeletal muscles of these animals by immunohistochemistry or PCR.

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We believe this is the first ultrastructural and molecular confirmation of the development of *S. neurona* tissue cysts in the CNS of any animal.

Keywords

Sea otter; *Sarcocystis neurona*; Tissue cyst; Central nervous system; Brain; Ultrastructure; *18S*; *rDNA*; *ITS1*

1. Introduction

Sarcocystis neurona is a single-celled apicomplexan parasite that causes severe, often fatal systemic disease in a wide range of animals including horses, harbor seals and sea otters (Dubey et al., 1991; LaPointe et al., 1998; Miller et al., 2001a; 2001b; Kreuder et al., 2003). The definitive hosts for *S. neurona* are new world opossums, specifically *Didelphis virginiana* and *Didelphis albiventris* (Dubey et al., 2001c, 2001d). These animals may shed infective sporocysts in their feces for prolonged periods without showing clinical signs (Porter et al., 2001). Several animals, including raccoons, (Dubey et al., 2001g; Stanek et al., 2002) skunks (Cheadle et al., 2001b; Dubey and Hamir, 2000), fisher (Gerhold et al., 2005), armadillos (Cheadle et al., 2001a), cowbirds (Mansfield et al., 2008), cats (Dubey and Hamir, 2000; Dubey et al., 2003a), dogs (Vashist et al., 2005), harbor seals (LaPointe et al., 1998) and sea otters (Rosonke et al., 1999; Lindsay et al., 2000; Dubey et al., 2001f; Thomas et al., 2007) serve as intermediate hosts for *S. neurona*, supporting the development of tissue cysts (or sarcocysts) in skeletal muscle and myocardium. Tissue cysts from skeletal muscle of a sea otter with *S. neurona*-associated meningoencephalitis were fed to an opossum, resulting in fecal shedding of sporocysts (Dubey et al., 2001f). Horses were considered by some researchers to be aberrant hosts for *S. neurona* that were unable to support the development of tissue cysts (Dubey et al., 1991, 1993, 2001e). However, Mullaney et al. (2005) described putative *S. neurona* tissue cysts from the tongue of a naturally infected horse, leaving the role of the horse as a true or aberrant intermediate host unresolved.

For nearly all reports of *S. neurona* infection in naturally or experimentally infected animals, the only parasite stages described in the brain and spinal cord are merozoites and schizonts (Dubey et al., 1991,2001d,2001e,2001f;Dubey and Hedstrom, 1993;Lapointe et al., 1998;Rosonke et al., 1999;Lindsay et al., 2001; Miller et al., 2001a,2001b;Kreuder et al., 2003). *Sarcocystis neurona* tissue cysts have only been described from tissues located outside of the CNS (Dubey, 1993;Dubey and Hedstrom, 1993;LaPointe et al., 1998;Rosonke et al., 1999; Cheadle et al, 2001a,2001b; Dubey et al. 2001a,2001b,2001c,2001d,2001e,2001f, 2002;Stanek et al., 2002) with three notable exceptions. The first two reports note the presence of *S. neurona*-like tissue cysts in brain tissue from a bird and a domestic cat (Dubey and Hamir, 2000;Dubey et al., 2003a). The third case described putative *S. neurona* tissue cysts from the brains of five sea otters, based on microscopic examination and immunohistochemistry (Thomas et al., 2007). However, *S. neurona* tissue cysts can react inconsistently with polyclonal antisera raised against *S. neurona* and *Sarcocystis falcatula* merozoites (Stanek et al., 2002). Without molecular or ultrastructural confirmation, no bona fide evidence currently exists to establish whether *S. neurona* tissue cysts are capable of developing in the CNS of any animal host.

Here we present necropsy findings from three wild sea otters with protozoal meningoencephalitis. In all three cases tissue cysts, schizonts and merozoites were visualized in the neuropil. Due to cross-reactivity and variable staining of monoclonal and polyclonal antisera directed against *S. neurona* and *S. falcatula*, molecular and ultrastructural confirmation was required to confirm that the tissue cysts were *S. neurona*. This report establishes that *S.*

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neurona tissue cysts are capable of developing in the CNS of naturally infected intermediate hosts.

2. Materials and methods

2.1. Necropsy

During April and May of 2004, three sea otters (SO1, SO2 and SO3) were submitted to the Marine Wildlife Veterinary Care and Research Center (MWVCRC) in Santa Cruz, California, USA for necropsy. Necropsy procedures were as previously described (Kreuder et al., 2003).

SO1 was an adult male that stranded live near Morro Bay, California on April 22, 2004. At the time of stranding the otter was hyperthermic (40.5° C), unresponsive to external stimuli and quadriparetic. Severe head and front paw tremors were noted, especially when the animal was handled or was trying to eat or swim. During the next 5 weeks of hospitalization, SO1 was administered oral antiprotozoal therapy (Ponazuril, 5 mg/kg orally, once daily; Bayer Corporation, Westhaven, Connecticut, USA). Due to continuing head and forelimb tremors, caudal paraparesis and inability to self-groom or forage, the otter was euthanized in May, 2004.

SO2 was an adult male that was recovered freshly dead (≤ 3 days post-mortem with refrigeration) on April 19, 2004 in Pismo Beach, California and submitted for necropsy.

SO3 was a sub-adult male recovered freshly dead near Morro Bay, California on April 10, 2004 and submitted for necropsy.

2.2. Serology and parasite isolation

Pre- and post-mortem serum was tested for reactivity to *Toxoplasma gondii* and *S. neurona* by indirect fluorescent antibody testing (IFAT), as previously described (Miller et al., 2002a). The established cut-offs for IFAT seropositivity to *T. gondii* is ≥ 320 serum dilution. Cut-offs have not yet been established through test validation for *S. neurona* in sea otters, so the same cut-off was selected. Fresh, aseptically-collected cerebrum and cerebellum were processed for parasite isolation in cell culture using rhesus monkey kidney (MA104) cells, as previously described (Miller et al 2001a). Cerebrum, cerebellum and skeletal muscle were also cryopreserved at -80° C.

2.3. Histopathology and immunohistochemistry

All major tissues were fixed in 10% neutral buffered formalin, trimmed, paraffin-embedded and 5 μm sections were cut and stained with H&E. Immunohistochemistry to screen for reactivity to antibodies against *S. neurona*, *S. falcatula*, *T. gondii* and *Neospora caninum* was performed on formalin fixed, paraffin-embedded tissues as previously described (Miller et al., 2002; Cooley et al., 2007). For detection of *S. neurona* antigens, both polyclonal antiserum raised in rabbits (Dubey et al., 2001a) and a monoclonal antibody (Marsh et al., 2002) were used to screen brain and muscle tissue from suspect animals. For all other protozoan species, polyclonal antisera raised in laboratory animals infected with well-characterized strains were used for antigen screening.

2.4. Transmission electron microscopy

A portion of formalin-fixed hippocampus from SO1 was also processed for transmission electron microscopy (TEM), as previously described (Gozalo et al., 2007). Thin sections were examined using a Zeiss 906E or Philips 400 transmission electron microscope at 60 kv accelerating voltage.

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2.5. Molecular characterisation

DNA was extracted from cryopreserved brain, heart and skeletal muscle from SO1, SO2 and SO3 using the DNeasy Tissue Kit (Qiagen). Genomic DNA preparations were screened for the presence of *T. gondii*, *S. neurona*, and/or *N. caninum* DNA using *18S rDNA* pan-specific hemi-nested primers that were developed to facilitate this study (all primer sequences in the 5′-3′ orientation): *18S* Forward External, GCAAGGAAGTTTGAGGCAAT, *18S* Reverse External, TGCAGGTTCACCTACGGAAA, *18S* Reverse Internal, TCCTTCCTCTAAGTGTTAAGGTTCA. PCR amplicons were sequenced to identify the parasite(s) present in the tissue samples. Positive controls consisted of genomic DNA preparations from well-characterized isolates of *T. gondii* (RH [Type I], 76K [Type II] and CEP [Type III]), *S. neurona* (SN1 and SN3 [Marsh et al, 1999]) and *N. caninum* (NC-1 [ATCC No. 50843]). Negative controls consisted of deionised water and purified genomic DNA from non-infected sea otter brain tissue.

To distinguish among *Sarcocystis* spp. infecting sea otters, nested primers were developed within the *ITS1* locus that amplify an ~500 nucleotide fragment from *S. neurona* and *S. falcatula*, but not other *Sarcocystis* spp., *T. gondii* or *N. caninum*. DNA sequencing of the resulting PCR amplicon differentiates between *S. neurona* and *S. falcatula*. To confirm specificity, the $ITS1_{500}$ primers were tested against DNA extracts from the related apicomplexa Hammondia hammondi, N. caninum, T. gondii, S. falcatula, Sarcocystis campestris, Sarcocystis cruzi and *S. neurona*. The primers were as follows (all sequences in 5′-3′ orientation): $ITS1_{500}$ Forward External, TTCTCTTGTGTGTGCCCCTAC, $ITS1_{500}$ Forward Internal, CAAAATGAACGTGTCTATGTGTGA, $ITS1_{500}$ Revrse External, TGCGTCCTTCATCGTTGCGC $ITS1_{500}$ Reverse Internal, GAGCCAAGACATCCATTGCT. For each PCR reaction, 2-5 ul of genomic DNA were used as template and reaction conditions were as previously described (Miller et al., 2008). Amplification products were visualized using ethidium bromide staining in 1% agarose gels. DNA sequencing was carried out by the Rocky Mountain Lab Core Genome Sequencing Center, Division of Intramural Research (Hamilton, Montana, USA).

3. Results

3.1. Necropsy

Gross necropsy of SO1 revealed diffuse lymphadenopathy, splenomegaly, patchy orange-white discoloration of the ventricular myocardium and mild serous pericardial effusion.

SO2 also had diffuse lymphadenopathy, with orange-white mottling and pallor of the ventricular myocardium, marked hepatosplenomegaly, mild pulmonary edema and multi-organ congestion. This animal was thin, with serous pericardial and pleural effusion. Moderate pulmonary hyperinflation was accompanied by septal emphysema and pneumomediastinum (perimortem dyspnea, presumptive). Small numbers of large (*Corynosoma enhydri*) and small (*Profilicollis* spp.) acanthocephalan parasites were attached throughout the small and large intestinal mucosa. At least 20 acanthocephalans (*Profilicollis* spp.) had migrated through the intestinal wall and were scattered throughout the peritoneum. *Streptococcus phocae* was isolated from heart blood and spleen, suggestive of perimortem bacteremia or sepsis secondary to the acanthocephalan peritonitis.

Gross necropsy of SO3 revealed diffuse lymphadenopathy, pallor and orange-white mottling of the ventricular myocardium, serous pericardial effusion, pulmonary edema and multi-organ vascular congestion. This otter was thin, with moderate intestinal melena. Feces and heart blood were negative for bacterial pathogens. For all three otters, urine was below minimum detection limits for domoic acid (< 10 parts per billion) by HPLC/mass spectrophotometry (MS) analysis.

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3.2. Serology and parasite isolation

Ante-mortem serum collected when SO1 was first found alive on the beach was strongly positive for *S. neurona* and *T. gondii* via IFAT (Table 1). Repeat testing using serum collected after 5 weeks of hospitalization with antiprotozoal therapy revealed a significant reduction in the *S. neurona* titer, but no change in the *T. gondii* titer. Parasite isolation in cell culture from brain collected aseptically at necropsy revealed growth of intracytoplasmic parasites with morphology consistent with *S. neurona*. However, these parasites disappeared after a few days in culture and further attempts at cell passage were unsuccessful.

Post-mortem serum from SO2 and SO3 was strongly seropositive for *S. neurona* (Table 1). SO2 was also strongly seropositive for *T. gondii*, while SO3 was weakly seropositive. *Toxoplasma gondii* was isolated from brain tissue from SO2 and *S. neurona* was isolated from the brain tissue of SO3 in cell culture. All three otters were seronegative for *N. caninum* (< 320 serum dilution) and no parasites consistent with *N. caninum* were isolated.

3.3. Histopathology and immunohistochemistry

Based on histopathology, multiple lymph nodes and the splenic periarteriolar lymphoid sheaths from SO1, SO2 and SO3 exhibited marked lymphoid hyperplasia and variable lymphoplasmacytic and neutrophilic inflammation. Small aggregates of lymphocytes, plasma cells, macrophages, rare neutrophils and microglia (glial nodules) were also visualized in the cerebrum, cerebellum and brainstem in H&E-stained tissue sections. Small foci of necrosis were present within or adjacent to the inflammatory lesions and nearby blood vessels were sometimes ectatic and lined by plump, hyperplastic endothelium. Some white matter tracts contained irregular clear spaces with a foamy appearance (edema).

H&E-stained tissue sections of brain from SO1 contained rare schizonts and free merozoites, plus numerous round to elliptical, 20 to 40 μm diameter, thick-walled tissue cysts containing tiny, (3 to 5 μm diameter) basophilic bradyzoites (Fig. 1A and B). At low power the cyst walls appeared smooth, but at 600 x magnification fine villous protrusions were discernable on the surface of some, but not all tissue cysts. There was no evidence of septation within the cysts and the bradyzoites were somewhat loosely arranged, with prominent nuclei and pale, basophilic cytoplasm. Larger, (100-200 μm long × 20-50 μm wide) tissue cysts, characterized by thick cyst walls with prominent surface projections, were also present in the myocardium (Fig. 1C), tongue, tunica muscularis of the gastric cardia and various skeletal muscles, accompanied by mild lymphoplasmacytic inflammation.

Results for immunohistochemical staining of brains from SO1, SO2 and SO3 for *S. neurona*, *S. falcatula*, *T. gondii* and *N. caninum* are summarized in Table 2. For SO1, all tissue sections were negative for staining by *T. gondii* and *N. caninum*, but numerous merozoites in the neuropil reacted positively to both monoclonal and polyclonal antibodies directed against *S. neurona* (Fig. 2A and C), as well as polyclonal antiserum directed against *S. falcatula* (Fig. 2E). This variable and cross-reactive staining pattern was also observed for bradyzoites and the tissue cyst wall (Fig. 2B, D and F). Similar staining characteristics have been observed for merozoites in tissues of laboratory animals infected with well-characterized *S. neurona* and *S. falcatula* strains (Table 2).

A total of 47 tissue cysts were measured from serial sections of brain from SO1. These cysts stained positively when treated with anti-*S. neurona* polyclonal antiserum. The maximum tissue cyst diameter and the minimum and maximum wall thickness were measured at 600 x using a light microscope with a calibrated ocular micrometer. Tissue cysts ranged from 18 to 46 μm diameter (mean = 30 μm), with wall thicknesses ranging from just below 1 μm to 6 μm (average = 1 to 2 μm).

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The pyriform lobe from SO2 possessed a single, thick-walled, 38 μm diameter tissue cyst containing 3-5 μm long, loosely arranged bradyzoites with prominent nuclei and uniformly pale, basophilic cytoplasm. A thick (2 to 2.5 μm wide), pink to amphophilic cyst wall contained prominent surface projections that were barely discernable at 600 x magnification (Fig. 3A). This tissue cyst was absent from tissue recuts stained using antibodies to *S. neurona*, *T. gondii* or *N. caninum*, although rare *S. neurona*-immunopositive merozoites and schizonts were identified. A second cluster of three tissue cysts with distinct morphology were observed in another region of the cerebrum of SO2 via immunohistochemistry, but were not visible in H&E-stained tissue sections. In contrast to the tissue cyst from the pyriform lobe, the tissue cysts in the cerebrum exhibited a thin (essentially invisible), smooth outer wall with no discernable villous protrusions. Both the cyst wall and the enclosed bradyzoites stained strongly positive for *T. gondii* using immunohistochemistry (Fig. 3B). *Toxoplasma gondii* was also isolated from the brain of SO2 in tissue culture (Table 1), suggesting that SO2 was concurrently infected with *T. gondii* and *S. neurona*.

H&E sections of SO3 identified a single small tissue cyst in the pyriform lobe adjacent to the left posterior hippocampus (Fig. 3C). Similar to the unique tissue cysts described from the brains of SO1 and SO2, this cyst exhibited a distinct pink to amphophilic cyst wall enclosing numerous discrete, 3 to 5 μm diameter bradyzoites with prominent nuclei and pale, basophilic cytoplasm. However, this latter cyst was smaller (21 μm), with a thinner cyst wall (0.75 to 1.5 μm) and no discernable surface villi. This latter cyst closely resembled structures described as immature *S. neurona* tissue cysts by Thomas et al. (2007), but due to its small size and the lack of definitive structural features, additional tests were required to rule out the possibility of co-infection by *T. gondii* or *N. caninum*. The tissue cyst was not apparent in subsequent recuts for immunohistochemistry, but low numbers of merozoites and schizonts were observed that stained positive using polyclonal antiserum to *S. neurona*. Brain tissue from SO3 was negative using polyclonal antiserum to *T. gondii* and no parasites in brain or muscle reacted to polyclonal antiserum raised against *N. caninum* for any of the three otters.

3.4. TEM

Two small (20 μm diameter) tissue cysts from the hippocampus of SO1 were examined using TEM. In both cases there was no clear delineation between the outer granular layer and the ground substance separating the centrally-located zoites. These zoites were compatible with metrocytes (bradyzoites were not present) (Fig. 4A). There were no visible internal septations and the metrocytes were loosely and randomly arranged, with features suggestive of immaturity and rapid division; they were short and plump, with an anterior apical complex, 22 sub-pellicular microtubules and small numbers of anterior micronemes. Anterior rhoptries were absent and centrally-placed nuclei were large and round with uniform, finely granular, dispersed, electron-lucent chromatin. Small numbers of electron-lucent, membrane-bound granules, a golgi apparatus, mitochondria and numerous free ribosomes were present in the cytoplasm. Some metrocytes were dividing, with two visible nuclear profiles (Fig. 4A). The tissue cyst wall was characterized by thin villous projections up to 1 μm long by 0.16 to 0.25 μm wide with tapered ends, a hobnailed surface and a fine, granular, electron-dense peripheral layer (Fig. 4A and B). Low numbers of fine microtubules extended from the villous tips down through the villi. There was mild constriction at the base of some villi with slight expansion of the villous body, forming a pennate shape (Fig. 4B).

3.5. Molecular analysis

To help confirm that these thick-walled tissue cysts were from *S. neurona*, molecular analysis was performed using pan-specific primers that bracket a variable region of the highly conserved *18S rDNA* gene. These primers amplify DNA from most tissue-cyst forming coccidian parasite species and DNA sequencing of the PCR products can be used to discern the presence of mixed

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infections in tissue specimens. Over the region amplified, a single polymorphism distinguishes *S. neurona* from *S. falcatula*, and four polymorphisms differentiate between *S. neurona* and either *N. caninum* or *T. gondii* (Fig. 5A). DNA extracts from the related tissue-cyst forming coccidia H. hammondi, N. caninum, T. gondii, S. falcatula, S. campestris, S. cruzi and *S. neurona* were used to confirm that the *18S rDNA* primers amplified all of these species, when present in tissue matrices (Fig. 5B).

DNA extracted from skeletal muscle and brain of SO1, SO2 and SO3 were tested using the *18S rDNA* primers (Fig. 5B). DNA sequencing of amplified PCR products derived from brain tissue from SO1 and SO3 (Fig. 5B) yielded only one homogenous sequence that was identical to *S. neurona*. However, DNA sequencing of PCR products from SO2 revealed a sequence possessing four heterozygous dinucleotide sites that corresponded to polymorphisms shared between *S. neurona* and either *T. gondii* or *N. caninum*. Next, *T. gondii*-specific *B1* primers (Grigg and Boothroyd, 2001) were applied to brain tissue from SO2 and *T. gondii* DNA was detected, confirming that SO2 was co-infected by *S. neurona* and *T. gondii*, but not *N. caninum* (data not shown). No otters possessed *18S rDNA* sequences consistent with co-infection by *S. falcatula* or any other *Sarcocystis* spp. except *S. neurona*.

To help confirm that the brain infections of SO1, SO2 and SO3 were due to *S. neurona* and not another closely related *Sarcocystis* spp., nested $ITS1_{500}$ primers were applied that amplified only *ITS1* DNA from *S. neurona* and *S. falcatula* (Fig. 5B). Over the region amplified, at least 16 polymorphisms exist between *S. falcatula* and *S. neurona* (data not shown). Sequencing of the PCR products amplified using the $ITS1_{500}$ locus unequivocally identified *S. neurona* as the pathogen infecting the brains of SO1, SO2 and SO3 (Table 1).

4. Discussion

Here we present, to our knowledge, the first ultrastructural and molecular confirmation of the development of *S. neurona* tissue cysts in the CNS of any animal. Tissue cysts were observed in the brains of three wild sea otters at necropsy and *S. neurona*-associated meningoencephalitis was confirmed via serology, histopathology, immunohistochemistry, parasite isolation and DNA sequencing and PCR. Although co-infection by *T. gondii* was confirmed in one otter, concurrent infection by additional *Sarcocystis* spp. or *N. caninum* was not detected in any of the three animals via immunohistochemistry or DNA amplification and sequencing of the *18S rDNA* and ITS_{500} loci. Brain tissue cysts from SO1 were also examined using TEM; ultrastructural features were consistent with prior descriptions of *S. neurona* tissue cysts from naturally or experimentally-infected intermediate hosts (Table 3), but distinct from those described from sea and river otters with PCR-confirmed, *Sarcocystis* spp. infections (Dubey et al., 2003b; Walstrom et al., 1999).

The large number of *S. neurona* tissue cysts found within the brain of SO1 provided a unique opportunity for comparison with prior descriptions of *S. neurona* tissue cysts from muscles of cats, birds, armadillos, raccoons, birds, a horse, a dog, a fisher, skunks, sea otters and harbor seals (Table 3). In these other species, tissue cysts ranged from 15 to 700 μm diameter, with smaller tissue cysts often reported as immature stages. Fine surface villi were reported in nearly all cases, except for paraffin sections where villi can be difficult to visualize. Tissue cysts from the brain of SO1 were smaller than those in muscle, with no visible septae, thick cyst walls and fine peripheral villous protrusions that were often difficult to appreciate by light microscopy. In the absence of additional tests, it would be difficult to determine whether these tissue cysts were those of *Sarcocystis* spp. or *N. caninum* by light microscopy alone (Barr et al., 1991). However, ultrastructural features were compatible with descriptions of *S. neurona* tissue cysts from experimentally infected hosts (Dubey et al., 2000,2001c;Cheadle et al., 2001a) and muscles of naturally infected sea otters (Dubey et al., 2001f), including long, narrow

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surface villi that taper towards the ends, a distinct “hobnailed” peripheral membrane immediately overlying a uniform electron dense layer, and microtubules that extend from the villous tips to the underlying granular layer (Table 3). An indistinct or poorly developed granular layer encompassed low numbers of plump, loosely arranged, dividing metrocytes, a lack of mature bradyzoites or internal septation and the presence of small, short villi on the outer cyst wall (Fig. 4A and B), suggesting that some tissue cysts were immature.

Ultrastructural features that distinguish *S. neurona* tissue cysts from those of related *Sarcocystis* spp. include differences in the electron-dense layer, microtubules and the shape of surface villi (Dubey and Lindsay, 1999; Dubey et al., 1999; Saville et al., 2004). However, ultrastructural features of *S. neurona* sarcocysts resemble those of *S. facatula* (Dubey et al., 2001c). Because many *Sarcocystis* spp. produce tissue cysts with surface villi, ultrastructural features should be interpreted in the context of PCR amplification and sequencing of appropriate genetic loci, using primers capable of identifying concurrent infection with > 1 *Sarcocystis* spp., as was performed in the current study.

Sea otters, a bird and a domestic cat have all been reported with putative *S. neurona* tissue cysts in the brain on histopathology (Dubey and Hamir, 2000; Dubey et al., 2001b; Thomas et al., 2007). The bird was concurrently infected with a second *Sarcocystis* species and the authors could not be sure of the identity of the single tissue cyst observed in the neuropil. The cat died due to post-surgical encephalomyelitis attributed to *S. neurona*; cysts observed in the brain were comparable in size and morphology to prior descriptions of *S. neurona* tissue cysts (Table 3), but confirmation was not possible because they were not present in subsequent paraffin sections. Putative immature *S. neurona* tissue cysts were described from the brains of sea otters by Thomas et al. (2007). However, confirmation of the identity of these tissue cysts as *S. neurona* was based solely on immunohistochemistry; no PCR or ultrastructural confirmation was reported.

For *T. gondii* and *N. caninum*, the dominant antigenic epitopes expressed by tachyzoites continue to be expressed by bradyzoites. As a result, immunohistochemistry using polyclonal antisera raised against tachyzoites is an effective means for detection of tachyzoites, bradyzoites and tissue cyst walls in infected tissues (Table 2 and Fig. 3B) (Conley et al., 1981;Uggla et al., 1987;Barr et al., 1991). In contrast, a more dramatic loss of expression of dominant merozoite epitopes is apparent for bradyzoites and tissue cyst walls of *S. neurona*, *S. falcatula* and possibly other *Sarcocystis* spp. As a result, monoclonal and polyclonal antisera raised against merozoite antigens may not reliably label bradyzoites and cyst walls of the same *Sarcocystis* spp., and may cross-react with those of closely related species. For example, monoclonal and polyclonal antisera derived against culture-derived merozoites of *S. neurona* and *S. falcatula* react variably and inconsistently with bradyzoites and tissue cyst walls of these same parasites (Fig. 2B, D and F) (Dubey et al., 2001a;Stanek et al., 2002). The *S. neurona* monoclonal antibody 2G5 did not react with bradyzoites or the cyst wall of *S. neurona* tissue cysts from experimentally infected animals (data not shown) and for brain tissue cysts from SO1, this same monoclonal antibody failed to react with the tissue cyst wall but reacted weakly with bradyzoites (Fig. 2D). This lack of reactivity is comparable with that observed for known *S. neurona* tissue cysts from experimentally infected cats (Marsh et al., 2002). Conversely, the *S neurona* polyclonal antiserum used in the current study reacts positively with tissue cyst walls but not bradyzoites of well characterized *S. neurona* tissue cysts (Butcher et al., 2002) and it reacts with the walls of *S. falcatula* tissue cysts (Dubey et al., 2001a). Evaluation of two polyclonal antisera directed against *S. falcatula* merozoites revealed negative or weak reactions to known *S. falcatula* bradyzoites (Dubey et al., 2001a). “Down-modulation” of dominant surface antigens with increasing zoite maturity might explain the variable bradyzoite reactivity using polyclonal antisera that has ranged from negative, to weak (B. Barr, personal communication), to positive, with the latter reported for bradyzoites

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only in immature *S. neurona* tissue cysts by Stanek et al. (2002). As a result, immunohistochemistry is of limited diagnostic value when evaluating tissue cyst stages of *S. neurona* and *S. falcatula*; parasite identity should be confirmed using TEM or PCR.

Of note, SO1 was treated with antiprotozoal medication for 5 weeks post-stranding. Merozoites were rare, but were still apparent in the brain after 5 weeks and numerous histologically unremarkable tissue cysts were observed in skeletal muscle and brain, demonstrating that tissue cysts are refractory to Ponazuril therapy. The potential for these cysts to reactivate under natural conditions or after cessation of anti-protozoal therapy is debated, but remains unproven (Dubey et al., 1989; Thomas et al., 2007; Miller, 2008). Recrudescence from tissue cysts is reported for the related apicomplexans *T. gondii* and *N. caninum* (Tenter et al., 2000; Guy et al., 2001). If recrudescence from tissue cysts occurs in *S. neurona*-infected marine species, it would significantly reduce the clinical value of antiprotozoal therapy.

Prolonged antiprotozoal therapy may also explain why parasites resembling *S. neurona* were initially isolated from the brain of SO1 but then disappeared. SO2 was co-infected with *T. gondii* and *S. neurona*; isolation of *T. gondii*, but not *S. neurona*, on cell culture from SO2 may reflect differences in parasite growth properties and/or cell line compatibility. When both parasites are present concurrently in cell monolayers, *T. gondii* can overwhelm *S. neurona* parasites in culture unless limiting dilutions are prepared to separate the two (Miller et al., 2001a).

Given the recent introduction of the definitive hosts (opossums) to coastal California (Grinnell, 1915), the development of *S. neurona* tissue cysts in the brains of sea otters could be a product of evolving host-parasite relationships or unique strain(s) of *S. neurona* infecting otters. Interestingly, all three otters stranded in 2004 during a localized, *S. neurona*-associated epizootic. Many of the tissue cyst profiles for SO1, and the only tissue cyst profiles observed in the brains of SO2 and SO3, were located within or adjacent to the hippocampus and pyriform lobe. Parasite-associated inflammation and vascular congestion also appeared particularly severe within this region. Given recent reports on the ability of *T. gondii* to moderate behavior of infected laboratory animals and humans (Berdoy et al., 2000; Flegr et al., 2002; Wang et al., 2007), the potential for apicomplexan parasites to preferentially infect the archipallium and paleopallium, resulting in disturbances in behavior and mentation, merits careful evaluation.

In conclusion, here we present, to our knowledge, the first ultrastructural and molecular confirmation of the development of *S. neurona* tissue cysts in the CNS of any animal. We confirmed the development of *S. neurona* tissue cysts in the brain of wild sea otters with protozoal meningoencephalitis using immunohistochemical, ultrastructural and molecular techniques. Tissue cysts were also examined using TEM and ultrastructural features were consistent with prior descriptions of putative *S. neurona* tissue cysts. Brain tissues from all three sea otters were confirmed positive for *S. neurona* via PCR and DNA sequencing of the *18S rDNA* and $ITS1_{500}$ loci. No additional *Sarcocystis* spp. were detected via histopathology, immunohistochemistry or PCR. Variation in *S. neurona* tissue cyst reactivity to antibodies raised to well-characterized *S. neurona* and *S. falcatula* strains was also demonstrated, underscoring the importance of completing ultrastructural or molecular characterization of suspect tissue cysts.

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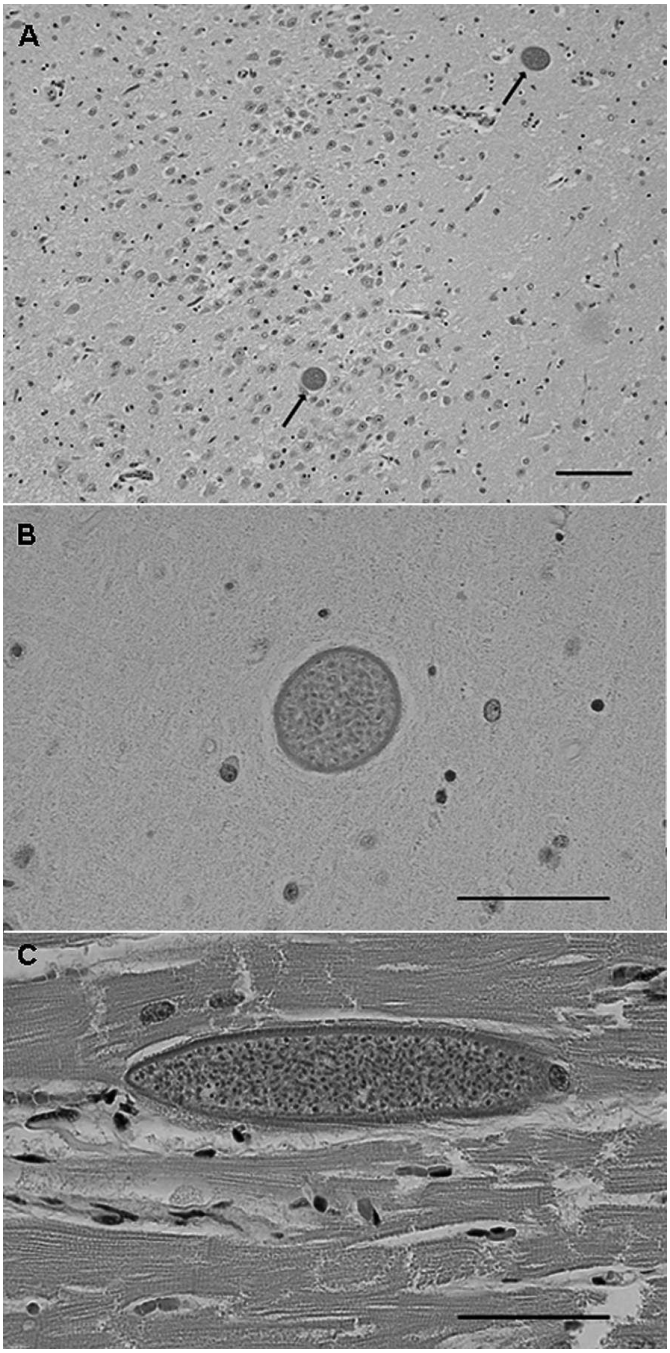

Fig. 1.

Sarcocystis neurona tissue cysts from formalin fixed, paraffin-embedded brain and heart from sea otter #1 (SO1), stained with H&E. A) Hippocampus; densely packed neurons extend from top to bottom in the center of the figure together with two, 30 to 40 μm diameter, thick-walled protozoal tissue cysts (arrows) (bar = 100 μm). B) Higher magnification view of a 45 × 40 μm tissue cyst from the brain of SO1, demonstrating the 2.5 μm thick, pink to amphophilic cyst wall with fine surface villi. Within the cyst are hundreds of 3 to 5 μm diameter bradyzoites, characterized by an outer rim of pale basophilic cytoplasm and a central round, deeply basophilic nucleus (bar = 50 μm). C) Cardiac myofiber from SO1 containing a 175 × 40 μm tissue cyst. Note the presence of fine surface villi and numerous bradyzoites (bar = 65 μm).

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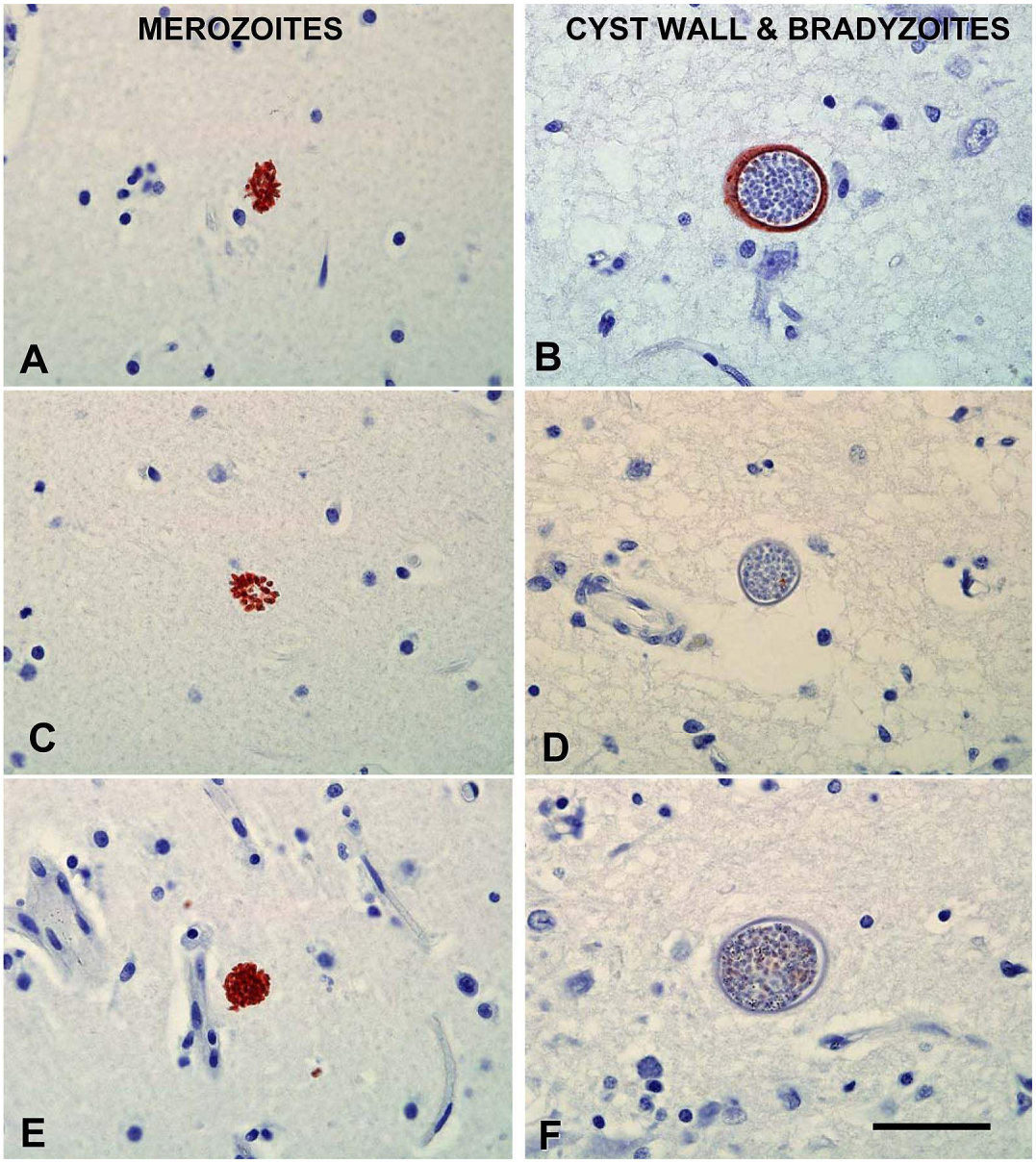

Fig. 2.

Variation in immunohistochemical staining properties of various protozoal structures (merozoites, bradyzoites and the outer tissue cyst wall) from the brain of sea otter #1, an animal with PCR-confirmed *Sarcocystis neurona* infection. A and B) Application of polyclonal antiserum to *S. neurona*: strong positive staining of merozoites (A) and the tissue cyst wall (B) is noted but with negative staining of bradyzoites (B). C and D) Application of monoclonal antibody (SN2G5) to *S. neurona*: strong positive staining of merozoites (C) contrasts with negative staining of the tissue cyst wall (D) and sparse, patchy labeling of the enclosed bradyzoites (D). E and F) Application of polyclonal antiserum to *Sarcocystis falcatula*: results are similar to those from application of the *S. neurona* monoclonal antibody, with strong positive staining of merozoites (E) contrasting with negative staining of the tissue cyst wall (F) and sparse, patchy labeling of the enclosed bradyzoites (F) (bar = 50 μm).

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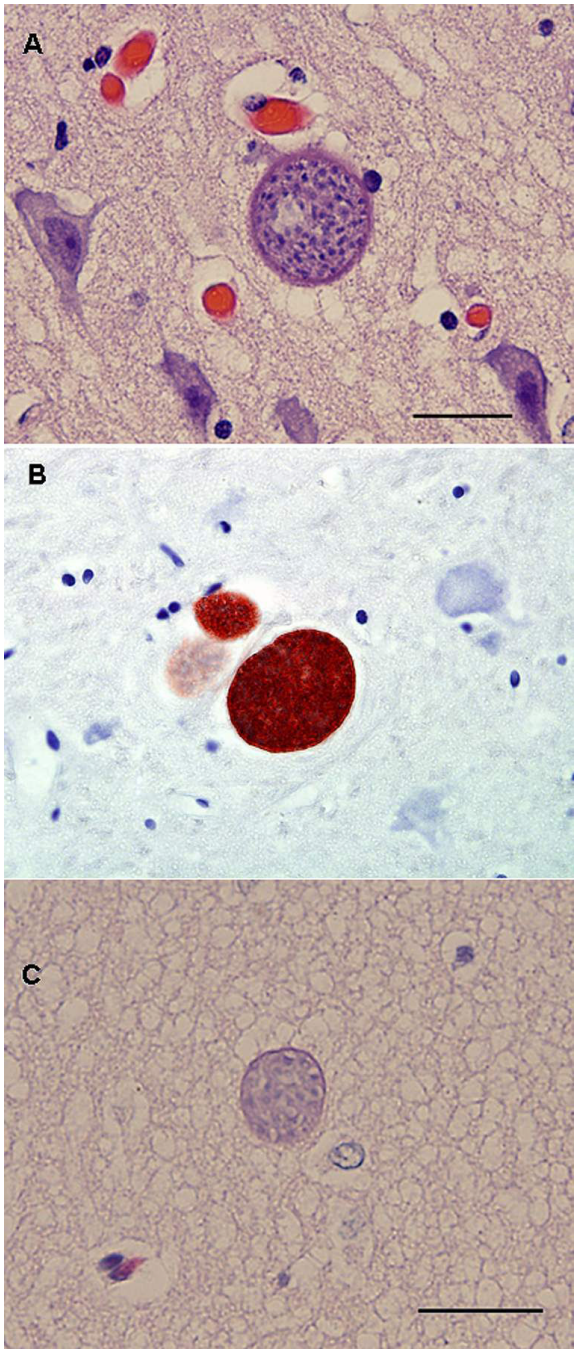

Fig. 3.

Tissue cysts from formalin-fixed, paraffin-embedded brains of sea otters #2 (SO2) and #3 (SO3). A) A single 38 μm diameter tissue cyst was observed in the pyriform lobe of SO2. Fine villous protrusions were barely discernable at the top center of the thick, pink to amphophilic, 2.5 μm thick outer cyst wall. (H&E stain, bar = 35 μm). B) Tissue cysts from another area of the cerebrum of SO2, showing strong labeling with polyclonal antiserum to *Toxoplasma gondii* (immunoperoxidase reaction, bar = 35 μm). C) Putative immature *Sarcocystis neurona* tissue cyst in cerebrum of SO3. Compared with those from SO1 and SO2, this cyst is smaller (21 μm diameter) with a thin cyst wall and no visible surface villi, similar to immature

S. neurona tissue cysts that have been described in other studies (Table 3). (H&E stain, bar = 30 μm).

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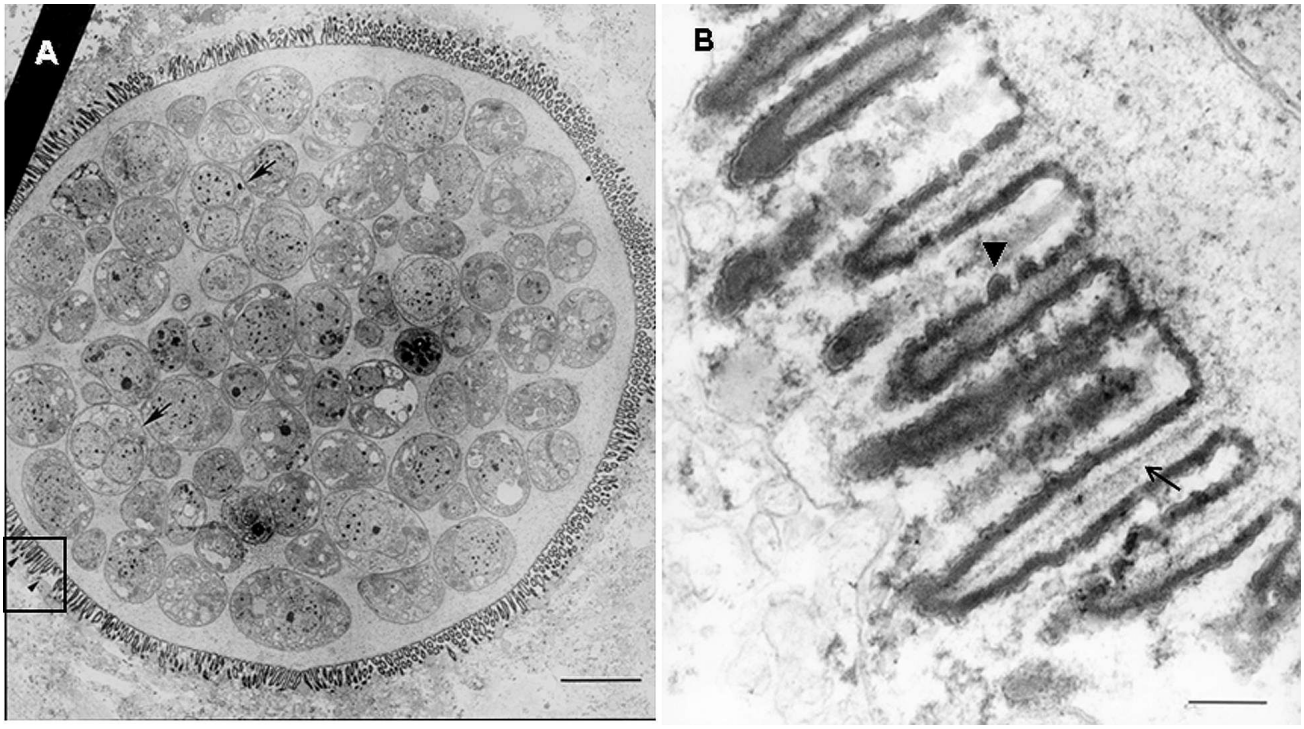

Fig. 4.

Transmission electron micrograph (TEM) of a 20 μm diameter tissue cyst from the brain of sea otter #1 (SO1). A) Within the tissue cyst are large numbers of metrocytes, including some that are dividing, indicated by the presence of two nuclear profiles within a single metrocyte (arrows). Prominent surface villi are apparent along the outer cyst wall (bar = 2.5 μm). B) Higher magnification view of the outer wall of the same tissue cyst (enlarged area indicated by box and small arrowheads at the lower left corner of A). Note the slight basal constriction of surface villi, with an increase in diameter at the midpoint, then narrowing to a tapered tip. The villous surface has a prominent, electron-dense outer layer with a “hobnailed” or beaded appearance (arrowhead). Within each microvillus, two or more fine, linear, electron dense, filamentous structures (microtubules - arrow) extend longitudinally from the villous tip to the outer portion of the granular layer that comprises the inner cyst wall. Villi are approximately 1 μm long by 0.2 μm wide (bar = 0.25 μm).

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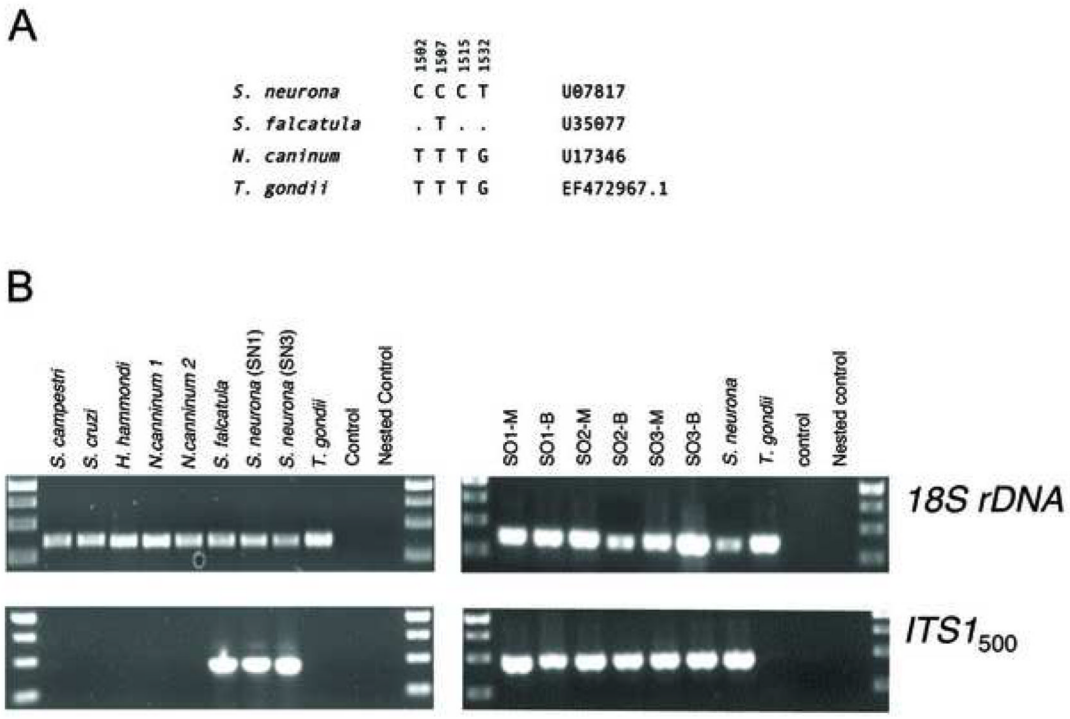

Fig. 5.

Differentiation among tissue cyst-forming parasites infecting sea otters by PCR and DNA sequencing. A) DNA sequence analysis using coccidia-specific *18S rDNA* locus primers amplifies a small polymorphic region that distinguishes among common coccidia infecting warm-blooded vertebrates. Polymorphic sites that exist between *Sarcocystis neurona* (Genbank accession number **U07817**), *Sarcocystis falcatula* (**U35077**), *Toxoplasma gondii* (**EF472967.1**) and *Neospora caninum* (**U17346**) are highlighted for the region amplified. Periods (.) indicate identity with *S. neurona*. The numerical position annotated refers to the numbered sites in the published sequence **U07817. 5**. B) *Sarcocystis* spp.-specific $ITS1_{500}$ primers were tested for specificity using DNA from S. neurona, Sarcocystis campestris, Sarcocystis cruzi, S. falcatula, T. gondii, N. caninum, and *Hammondia hammondi 18S rDNA* control primers established that parasite DNA was present and the $ITS1_{500}$ primers were highly specific for only *S. falcatula* and *S. neurona*. DNA extracts from skeletal muscle (M) and brain (B) of SO1, SO2 and SO3 were amplified using both the *18S rDNA* and $ITS1_{500}$ primers, PCR products were separated in a 1% ethidium bromide-stained agarose gel.

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Table 1

Stranding data and test results for three sea otters with putative *Sarcocystis neurona* tissue cysts in brain tissue.

Otter number	Sex/ age	Strand date	Stranded alive/dead	IFAT titer: TG[a]	IFAT titer: SN[a]	Parasite isolation	Histopathology		IHC[a] (Brain)	PCR[a] (Brain)	TEM[a] (Brain)
							Brain	Muscle			
1	Male/ adult	22 April 2004	Alive	81,920	10,240	SN, lost early[b]	SN	SN	SN	SN	SN
2	Male/ adult	19 April 2004	Dead	81,920	10,240	TG	SN	SN	SN, TG	SN, TG	ND[a]
3	Male/ subadult	10 April 2004	Dead	320	81,920	SN	SN	SN	SN	SN	ND

[a] IFAT = indirect fluorescent antibody test, TG = Findings consistent with *Toxoplasma gondii*, SN = Findings consistent with *S. neurona*, IHC = immunohistochemistry, PCR = PCR + restriction length polymorphisms and sequencing of ITS1 and SnSAG3 genomic DNA, TEM = transmission electron microscopy, ND = not done.

[b] Otter was treated for 5 weeks with antiprotozoal medication (Ponazuril 5 mg/kg orally once daily) priorto euthanasia.

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Table 2

Immunohistochemical staining of protozoa in tissue from laboratory-infected controls and sea otters with encephalitis using antisera directed against Sarcocystis neurona, Sarcocystis falcatula, Toxoplasma gondii and Neospora caninum.

Organism (and host tissue)		SN Polyab[a]	SN2G5 Mab[a]	SF Polyab[a]	TG Polyab[a]	NC Polyab[a]	Comments
Positive and negative controls							
Sarcocystis neurona (mouse brain)	merozoites	++	++	++	-	-	
Sarcocystis falcatula (budgerigar lung)	merozoites	-/+	-	++	-	-	
Toxoplasma gondii (mouse brain/lung)	tachyzoites	-	-	-	+	-	
	bradyzoites	-	-	-	+	-	
	tissue cyst wall	-	-	-	+	-	
Neospora caninum (mouse brain)	tachyzoites	-	-	-	-	+	
	bradyzoites	-	-	-	-	+	
	tissue cyst wall	-	-	-	-	+	
Sea otter #1 (SO1) (brain)	merozoites	++	++	+/++	-	-	Numerous SN tissue cysts on H&E and IHC[a]. Cysts also examined via transmission electron microscopy.
	bradyzoites	-	-(rare faint +)	-(some faint +)	-	-	
	tissue cyst wall	-/++ (most ++)	-	-	-	-	
Sea otter #2 (SO2) (brain)	merozoites	++	++	++	++[b]	-	1 SN tissue cyst on H&E sections[b]. Cyst not visible on IHC recuts
Sea otter #3 (SO3) (brain)	merozoites	++	++	++	-	-	1 SN tissue cyst on H&E sections. Cyst not visible on IHC recuts.

[a] SN = *S. neurona*, SF = *S. falcatula*, TG = *T. gondii*, NC = *N. caninum*, Polyab = polyclonal antiserum, Mab = monoclonal antibody, IHC = immunohistochemistry.

[b] Otter with H&E, PCR and culture-confirmed SN and TG infection: Rare tissue cysts that were TG-positive on IHC did not stain with antisera to SN or SF.

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Table 3

Prior reports of putative *Sarcocystis neurona* tissue cysts in brain or muscle: microscopic and ultrastructural features.

Host species	Experimental infection?	Tissue cyst location	Cyst size	Surface Villi?	Microtubules extend to granular layer?	Electron dense surface protrusions	Wall thickness	Reference
Raccoon	Yes	Muscle	≤ 270×50 μm	Y (≤ 5×0.7 μm)	Y	Y	≤ 1.5 μm	Stanek et al. 2002
Raccoon	Yes	Muscle	≤ 125×10μm	Y	NR[a]	NR	<0.5 μm	Dubey, et al., 2001g
Skunk	Yes	Muscle	27.7 μm	Y (3.0 μm)	Y	Y	~1.5 μm	Cheadle et al., 2001b
Skunk	No	Muscle	≤ 500×40 μm	Y	Y	Y	1-2 μm	Dubey et al., 2002
Fisher	No	Muscle	≤ 200×30 μm	Y (≤ 2.2×0.4μm)	Y	Y	1-3 μm	Gerhold et al., 2005
Domestic cat	Yes	Muscle	NR	Y (≤ 2.5μm)	Y	Y	NR	Dubey et al., 2000
Domestic cat	Yes	Muscle	≤ 700×50 μm	Y (≤ 2.8×0.4μm)	Y (sometimes)	Y	1-2 μm	Dubey et al., 2001c
Domestic cat	Yes	Muscle	NR, > 50 μm in photo	Y (≤2.4 μm)	NR	NR	~2 μm	Butcher et al., 2002
Domestic cat[b, c]	No	Brain	90×80 μm	Y	NR	NR	~2 μm	Dubey et al., 2003a
Dog	No	Muscle	~100×40 μm	Y (≤ 0.8×0.2μm)	No	Y	NR	Vashist et al., 2005
Armadillo	No	Muscle	NR	Y	NR	Y	NR	Cheadle et al., 2001a
Straw-necked ibis[b, c, d]	No	Brain	~60 × 20 μm	Y	NR	NR	~1.5-2 μm	Dubey et al., 2001b
Cowbird[b]	No	Muscle	~600 × 20 μm	Y	NR	NR	NR	Mansfield et al., 2008
Harbor seal	No[c]	Muscle	30×20 μm	NR	NR	NR	2.5 μm	LaPointe et al., 1998
Sea otter (*Enhydra lutris kenyoni*)	No	Muscle	20×90 μm range	Y(~2 μm)	NR	NR	NR	Rosonke et al., 1999
Sea otter (*E. l. kenyoni*)	No	Muscle	~350×200 μm	Y (1.3×0.25μm)	No	Y (50-75nm)	~1.5 μm	Dubey et al., 2001f
Sea otter[b, c] (unspecified)	No	Brain and muscle	17 to 40 μm diameter	NR	NR	NR	NR	Thomas et al., 2007

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Host species	Experimental infection?	Tissue cyst location	Cyst size	Surface Villi?	Microtubules extend to granular layer?	Electron dense surface protrusions	Wall thickness	Reference
Sea otter (*Enhydra lutris nereis*)	No	Brain and muscle	15 to 60 μm diameter	Y (2 × 0.2 μm)	Y	Y	1-4 μm	This report

[a] NR = not reported.

[b] Description based on light microscopy only.

[c] Tissue cysts were noted in the cerebrum on histopathology or immunohistochemistry, but parasite identity as *S. neurona* was not confirmed using PCR or transmission electron microscopy

[d] Species not determined-may not be *S. neurona*.