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DISCOVERY OF THREE NOVEL COCCIDIAN PARASITES INFECTING CALIFORNIA SEA LIONS (*ZALOPHUS CALIFORNIANUS*), WITH EVIDENCE OF SEXUAL REPLICATION AND INTERSPECIES PATHOGENICITY

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Abstract

Enteric protozoal infection was identified in 5 stranded California sea lions (*Zalophus californianus*). Microscopically, the apical cytoplasm of distal jejunal enterocytes contained multiple stages of coccidian parasites, including schizonts with merozoites and spherical gametocytes, which were morphologically similar to coccidians. By histopathology, organisms appeared to be confined to the intestine and accompanied by only mild enteritis. Using electron microscopy, both sexual (microgametocytes, macrogamonts) and asexual (schizonts, merozoites) coccidian stages were identified in enterocytes within parasitophorous vacuoles, consistent with apicomplexan development in a definitive host. Serology was negative for tissue cyst-forming coccidians, and immunohistochemistry for *Toxoplasma gondii* was inconclusive and negative for *Neospora caninum* and *Sarcocystis neurona*. Analysis of ITS-1 gene sequences amplified from frozen or formalin-fixed paraffin-embedded intestinal sections identified DNA sequences with closest homology to *Neospora* sp. (80%); these novel sequences were referred to as belonging to coccidian parasites ‘‘A,’’ ‘‘B,’’ and ‘‘C.’’ Subsequent molecular analyses completed on a neonatal harbor seal (*Phoca vitulina*) with protozoal lymphadenitis, hepatitis, myocarditis, and encephalitis showed that it was infected with a coccidian parasite bearing the ‘‘C’’ sequence type. Our results indicate that sea lions likely serve as definitive hosts for 3 newly described coccidian parasites, at least 1 of which is pathogenic in a marine mammal intermediate host species.

Marine mammals serve as definitive or intermediate hosts for a variety of apicomplexan protozoan parasites, most notably *Eimeria phocae*, *Toxoplasma gondii*, and *Sarcocystis*

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neurona (Miller, 2008). Very little is known about the extent and diversity of coccidian parasites infecting pinnipeds. Among pinniped species, *T. gondii* infection has been documented in a Hawaiian monk seal (*Monachus schauinslandi*), a northern elephant seal (*Mirounga angustirostris*), a northern fur seal (*Callorhinus ursinus*), harbor seals (*Phoca vitulina*), and California sea lions (*Zalophus californianus*), which all serve as intermediate hosts (Dubey et al., 2003; Conrad et al., 2005; Honnold et al., 2005). *Sarcocystis* spp. infection has most commonly been reported in Pacific harbor seals (Lapointe et al., 1998; Colegrove et al., 2005). Serologic analysis using a recombinant antigen derived from the major surface antigen NcSAG1 detected antibodies consistent with infection by *Neospora caninum* in harbor seals and spotted seals (*Phoca largha*) in Japan (Fujii et al., 2007). Exposure to *N. caninum* has been postulated in walrus (*Odobenus rosmarus*), sea otters *(Enhydra lutris nereis*), harbor seals, sea lions, ringed seals (*Phoca hispida*), bearded seals (*Erignathus barbatus*), and bottlenose dolphin (*Tursiops truncatus*) based on serology using an *N. caninum* agglutination test (Dubey et al., 2003). Enteric apicomplexan parasites reported in pinnipeds include *E. phocae* and *Cryptosporidium* spp. (Deng et al., 2000; Van Bolhuis et al., 2007; Dixon et al., 2008). Both fatal enterocolitis and self-limiting enterocolitis have been associated with *E. phocae* infections in harbor seals, and both sexual stages and oocysts have been noted in affected intestines, indicating that harbor seals are a definitive host (Van Bolhuis et al., 2007). The pathogenicity of *E. phocae* in other species has not been described. Coccidian oocysts have also been identified in fecal samples from several species from the Antarctic, including southern elephant seals (*Mirounga leonine*) and Weddell seals (*Leptonychotes wedelli*) (Drozdz, 1987).

Serologic evidence for infection with *T. gondii* has been found in a number of marine mammal species and has been associated with disease in mustelids, sirenians, cetaceans, and pinnipeds (Buergelt and Bonde, 1983; Dubey et al., 2003; Miller, 2008). Serosurveys have shown that there is a high level of exposure to *T. gondii* in the southern sea otter of California (Miller et al., 2002; Conrad et al., 2005), and *T. gondii* meningoencephalitis has been shown to be a significant cause of mortality, accounting for 16% of mortalities in 1 study (Kreuder et al., 2003). Evidence exists to indicate that *T. gondii* infections in southern sea otters are related to exposure to environmentally resistant oocysts shed in felid feces and transported to the marine environment by freshwater runoff (Miller et al., 2002, 2008), where prey species such as mussels (Arkush et al., 2003; Miller et al., 2008) and turban snails (Johnson et al., 2009) serve as a source of *T. gondii* oocysts in the marine environment. However, *T. gondii* infections in pelagic marine mammal species have also been noted, and the source of these infections remains enigmatic (Conrad et al., 2005). The diversity of *T. gondii* infections noted in marine mammal species suggests that transmission of *T. gondii* may not be completely explained by land-to-sea transport of infective oocysts. The present study investigated whether sea lions could serve as definitive hosts for tissue cyst-forming coccidian parasites.

We recently observed coccidian parasites within the small intestine of 5 free-ranging California sea lions during routine postmortem examinations. In all cases, both sexual and asexual stages resembling a coccidian parasite were found within enter-ocytes. These unexpected findings compelled further investigation to determine whether the sea lion could serve as a definitive host for coccidian species and their possible relationship to known cyst-

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forming coccidians, e.g., *T. gondii, N. caninum,* and *S. neurona*. Our results suggest that sea lions serve as definitive hosts for 3 previously undescribed apicomplexan protozoan parasites, at least 1 of which appears to be pathogenic to harbor seals.

MATERIALS AND METHODS

Animal information and serology

Details of the 5 cases of enteric protozoal infection (cases 1–5) in stranded California sea lions are included in Table I. Case 6 was a neonatal harbor seal that was found lethargic and that died shortly after stranding. Following stranding along different areas of the central California coast, animals were housed at The Marine Mammal Center (TMMC), Sausalito, California, for rehabilitation and medical care for a period of up to 36 days prior to death or being killed due to poor prognosis. Age class determination was based on standard length, weight, and tooth size (Greig et al., 2005). Clinical signs in sea lions prior to death or being killed included seizures, abnormal behavior, vomiting, and diarrhea. Sera samples collected during rehabilitation, or at the time of being killed, or both, were tested for the presence of IgG to *T. gondii*, *S. neurona*, and *N. caninum* via indirect immunofluorescent antibody testing (IFAT), as previously described (Miller et al., 2002).

Necropsy and histology

Necropsy of all sea lions and the neonatal harbor seal was performed within 12 hr of death at TMMC. Representative tissue samples from all organs, including between 3 and 6 separate sections of small intestine, were fixed in 10% neutral buffered formalin and sent either to the Zoological Pathology Service, College of Veterinary Medicine, University of Illinois at Urbana-Champaign, the Pathology Service, Veterinary Medical Teaching Hospital, School of Veterinary Medicine, University of California at Davis, or the Armed Forces Institute for Pathology (AFIP), Washington, D.C., for processing and analysis. Tissues were embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin.

Immunohistochemistry

Immunohistochemistry for *T. gondii* (3 differently sourced polyclonal anti–*T. gondii* antibodies were used; [1] rabbit polyclonal, AR125-5R, Biogenex Laboratories, Inc., San Ramon, California; [2] rabbit polyclonal produced from Me49 *T. gondii* isolate, California Animal Health and Food Safety Laboratory, Davis California; and [3] rabbit polyclonal, Statens Serumistitut, Copenhagen, Denmark), *S. neurona* (monoclonal clone 2G5-2T75) (Marsh et al., 2002), and *N. caninum* (rabbit polyclonal, produced from bovine fetal isolate #66, California Animal Health and Food Safety Laboratory, Davis, California) (Conrad et al., 1993) was performed on sections of intestine for each of the 5 sea lion cases of enteric infection using established methods (Miller et al., 2001; March et al., 2002; Ferguson, 2004). Positive controls for *T. gondii* immunohistochemistry included *T. gondii*–infected cat lymph node and intestine (source 1), *T. gondii*–infected cat brain and lung (source 2), and *T. gondii*–infected cat intestine with sexual and asexual stages present (source 3). Positive controls for *S. neurona* and *N. caninum* immunohistochemistry included brain stem from a *S. neurona*–infected horse and brain from a *N. caninum*–infected nude mouse, respectively. In addition, sections of hippocampus and mesenteric lymph node from cases 1 and 2 were

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stained using *T. gondii* antibodies from source 2. Immunohistochemistry for *T. gondii*, *S. neurona*, and *N. caninum* was also performed on sections of affected lymph node from case 6.

For 1 of the 5 affected sea lions (case 2), immunohistochemistry was conducted as previously described on intestine sections using a suite of antibodies previously characterized for their staining characteristics against the stages found in both the definitive and intermediate hosts, including anti–SAG 1, anti–BAG 1, anti-enolase (ENO) isoforms 1 and 2, lactic dehydrogenase (LDH) isoforms 1 and 2, anti–Rop 2.3.4, anti–GRA 7, anti–Tg ENR, and anti–Tg MORN 1. Antibodies are known to recognize molecules and iso-enzymes expressed during different stages of development (Ferguson, Cesbron-Delauw et al., 1999; Ferguson, Jacobs et al., 1999; Ferguson et al., 2002; Ferguson, 2004; Ferguson et al., 2008).

Transmission electron microscopy

For electron microscopy, portions of formalinfixed intestine from 2 of the 5 sea lions (cases 1 and 2) with enteric infection were placed in modified half-strength Karnovosky's fixative. The tissue was washed in 0.2 M sodium cacodylate after osmium fixation and dehydrated through a graded ethanol series, transitioned through propylene oxide, and infiltrated and embedded in Spurr epoxy formulation. Thick sections were mounted onto glass slides, stained by toluidine blue O, and examined by light microscopy to determine appropriate areas for thin section examination. Thin sections were cut, mounted onto 150-mesh copper grids, stained briefly by 6% methanolic uranyl acetate, and counterstained with Reynold lead citrate before examination by transmission electron microscopy at 60 kV accelerating voltage (Jsoimg, 1982).

DNA extraction and PCR amplification

DNA was extracted from paraffin-embedded, formalin-fixed samples of intestine (cases 1–5) and fresh frozen samples of duodenum, jejunum, mesenteric lymph node, heart, and brain (cases 3–5) from affected sea lions using either the formalin-fixed paraffin-embedded (FFPE) or DNA tissue extraction protocol, respectively (Qiagen DNeasy blood and tissue kit; Qiagen, Valencia, California). DNA was extracted from the neonatal harbor seal (case 6) from fresh frozen brain, liver, lung, kidney, tonsil, uterus, spleen, and inguinal and tracheobronchial lymph nodes. Primers flanking the internal transcribed spacer 1 (ITS1) region were used in order to detect multiple coccidian parasite infections (Wendte et al., 2010). Two microliters of eluted DNA from all extracted tissues were used in the first round of nested 50-μl PCR reactions. PCR reactions were carried out in an Eppendorf master cycler under the following reaction conditions: 5.0 μL 10X PCR BUFFER WITH $MGCL_2$ (15 MM), 5.0 μl of 100 μM dNTPs, 0.5 μM of each primer, and 1.5 U Taq polymerase (Sigma-Aldrich, St. Louis, Missouri). After initial denaturation of templates and primers (94 C, 5 min), 35 cycles of the following conditions were used: 95 C for 40 sec, 58 C for 40 sec, and 72 C for 90 sec, followed by a 10 min extension at 72 C. In the second round of the nested reaction, 1 μl of product DNA from the first round was used as the DNA template. Reaction times and conditions were identical to the first round. Additionally, *T. gondii*–specific primers targeting the B1 gene were used to amplify *T. gondii* DNA from frozen and paraffin-embedded samples (Grigg and Boothroyd, 2001).

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Five-microliter samples of PCR product were electrophoretically separated in a 1% agarose gel stained with GelRed (Biotium, Inc., Hayward, California) and visualized under UV light. PCR products were incubated for 15 min with ExoSAP-IT (USB Corporation, Cleveland, Ohio) prior to DNA sequencing. DNA sequencing was carried out by the RML Genomics Unit, Hamilton, Montana. A BLAST search was used to compare these sequences to similar sequences available in GenBank, and sequences were aligned using the SeqMan software (DNASTAR, Inc., Madison, Wisconsin).

RESULTS

Serology

IFAT IgG serum titers to *T. gondii*, *S. neurona*, and *N. caninum* in cases 1, 2, 3, 5, and 6 were all <1:40 (Table II) and considered negative. Serum was not available for analysis for case 4.

Pathology

In all 5 sea lions with enteric infection, multiple stages of coccidian parasites were noted within the apical cytoplasm of enterocytes in the small intestine (Fig. 1). Coccidian parasites were most commonly found in the distal small intestine and ileum. Within affected sections of intestine, infection was highly segmental. Organisms ranged from oval, 3- to 8-μm-diameter schizonts within clusters to parallel-arranged, pyriform, and approximately 2–3-μm by 6–8-μm merozoites within a 7–10-μm-diameter parasitophorous vacuole. Some schizonts were up to approximately 15 μm in diameter and had peripherally arranged merozoites with central pale basophilic regions or parallel-arranged merozoites that appeared to be budding (Fig. 1B). Up to approximately 7-μm-diameter round gametes were occasionally observed within parasitophorous vacuoles in the apical enterocyte cytoplasm. Some gametes had a single large distinct eosinophilic nucleus most consistent with macrogamonts (Fig. 1C). Others contained multiple small dark basophilic structures and were most consistent with microgamonts (Fig. 1D). Intestinal infections varied greatly in intensity among affected sea lions, and merozoites and schizonts were the most numerous protozoal forms noted. Sexual coccidian stages were more commonly noted in animals with heavier parasite loads, and macrogamonts were more easily identified histologically compared to microgamonts. Coccidian infection was accompanied by only mild inflammation, similar to what is commonly found in many free-ranging sea lions. The adjacent lamina propria contained small numbers of lymphocytes, plasma cells, and rare neutrophils or eosinophils. Mesenteric lymph nodes exhibited moderate to marked cortical lymphoid hyperplasia.

In the 5 affected sea lions (cases 1–5), protozoan infections were determined to be of no clinical significance, and death was attributed to non-parasite-related health problems. Protozoan organisms were not observed in extra-intestinal tissues in any of the 5 affected sea lions. In 2 of the 5 sea lions (cases 1 and 2), death was attributed to domoic acid toxicosis with characteristic hippocampal lesions (Silvangi et al., 2005). Severe pneumonia and concurrent emaciation were determined to be the causes of death for the remaining 3 animals (cases 4–6).

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In the harbor seal (case 6), neutrophilic and granulomatous inflammation and necrosis were noted in multiple lymph nodes associated with rare, 2–3-μm, round to oval, intrahistiocytic protozoan zoites (Fig. 2). Additionally there was multifocal necrotizing hepatitis, encephalitis, and myocarditis; however, no protozoal organisms were noted within the heart, liver, intestine, or brain. The gastrointestinal tract was devoid of ingesta, indicating that the seal pup had not nursed prior to death and infection occurred in utero.

Immunohistochemistry

In all 5 of the affected sea lions, both asexual and sexual stages of protozoal parasites, including schizonts, merozoites, and gametes, in intestine mucosal cells were strongly immunoreactive to all 3 of the *T. gondii* polyclonal antibodies tested (Fig. 3). It should be noted, however, that the specificity of the anti–*T. gondii* antibodies utilized has not been evaluated against the enteric stages of other coccidian parasites. Immunohistochemical results were evaluated in more detail by staining sea lion and *T. gondii*–infected cat intestinal sections in parallel with monospecific antibodies that recognize various stages of *T. gondii*. Of the 6 antibodies known to stain the coccidian stages of *T. gondii* (ENO2, LDH1, ROP2, 3, 4, GRA7, and NTPase), only ENO2 showed any evidence of positive staining, with all the others being negative compared to the *T. gondii* stages in control sections (Figs. 3B–E). However, the ENO2 staining in the sea lion intestine differed from that seen with *T. gondii* staining in the felid definitive host, with the absence of strong nuclear staining (Figs. 3B, C).

Protozoal stages did not react with polyclonal antibodies to *N. caninum* or monoclonal antibodies to *S. neurona*. Sections of hippocampus and mesenteric lymph node in cases 1 and 2 were negative using the *T. gondii* antibody. In the harbor seal, case 6, immunohistochemistry for *T. gondii*, *S. neurona*, and *N. caninum* in sections of affected lymph node was negative; however, protozoal organisms were not definitively visualized on immuno-stained slides and, therefore, may not have been present in the replicate sections made for immunohistochemical analysis.

Transmission electron microscopy

Both merozoites and sexual coccidian stages were noted within apical cytoplasm of enterocytes above the host cell nucleus (Fig. 4). No zoites were noted within the lamina propria or within inflammatory cells.

Many schizonts and merozoites were situated within a para-sitophorous vacuole limited by a thin, well-developed membrane. Merozoites contained a conoid, micronemes, amylopectin granules, dense granules, and a nucleus and nucleolus (Fig. 4B). Rhoptries were difficult to definitively identify. Some schizonts showed developing merozoites. Microgamonts (N = 2) were 5.5–6 μm in diameter and surrounded by a parasitophorous vacuole membrane. Profiles of microtubules with a 9 + 2 arrangement could be visualized adjacent to the microgamonts within the parasitophorous vacuole and were considered to be microgamete flagellum. Microgametes in the early stages of development were adjacent to the surface of the microgamont (Fig. 4C). Macro-gamonts (N = 5) ranged from 5.5 to 5.8 μm in diameter and were surrounded by a parasitophorous vacuolar membrane. Macro-gamonts contained a

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nucleus, nucleolus, wall-forming bodies, polysaccharide granules, and occasionally visible canaliculi (Fig. 4D).

PCR amplification of ITS-1 and B1 sequences

Amplification of extracted DNA from frozen tissues (3 sea lions and 1 harbor seal) and formalin-fixed, paraffin-embedded intestine using pan-coccidian primers anchored in the 110-copy small subunit (SSU) 18S nuclear ribosomal gene complex, which amplifies across the size polymorphic ITS-1 locus, produced bands of < 400 bp. DNA sequence analysis of these amplicons identified three sequence types, which were designated ''A,'' ''B,'' and ''C'' (Table III; Figs. 5, 6). Sequences ''A,'' ''B,'' and ''C'' were single, homogeneous sequence profiles, and no di-nucleotide sites were identified at this multicopy gene locus (Fig. 6). Each sequence type was distinct from each other but closely (< 80%) orthologous to *N. caninum* and deposited in GenBank with the following accession numbers GU936629, GU936630, and GU936631, respectively. DNA consistent with sequences ''A'' and ''B'' was concurrently amplified in all frozen tissues examined from sea lion 5. Although coccidian parasites were observed by histology and immunohistochemistry only within the intestine of affected sea lions, coccidian DNA was amplified from the brain, heart, and mesenteric lymph node of sea lions 4 and 5. The DNA sequence amplified from tissues from the neonatal harbor seal with disseminated infection (case 6) was identical to a sequence amplified from the intestine of sea lion case 2 (''C''). PCR using *T. gondii*–specific primers targeting the B1 gene failed to amplify *T. gondii* DNA on multiple repeat attempts.

DISCUSSION

This study describes the first report of an enteric coccidian parasite infection in California sea lions with morphological and ultrastructural evidence of asexual (schizonts and merozoites) and sexual (macrogamonts, microgamonts) coccidian stages. The presence of all of these stages concurrently in the epithelium confirms the presence of a sexual cycle of replication occurring in the intestinal tract of affected sea lions, indicating that sea lions are definitive hosts of these coccidian parasites. Morphologically and ultrastructurally, these organisms were of similar size and morphologic appearance to sexual and asexual stages of both tissue cyst-forming and non-cyst-forming coccidian parasites (Ferguson et al., 1974, 1975; Dubey and Sreekumar, 2003; Speer and Dubey, 2005; Ferguson and Dubremetz, 2007). PCR analyses (cases 1–5) identified 3 previously unrecognized coccidian parasites, designated ''A,'' ''B,'' and ''C,'' suggesting that multiple coccidian organisms can infect the sea lion intestine.

Although immunohistochemistry revealed strong positive immunostaining for *T. gondii* using polyclonal antibodies from all 3 of the sources utilized, the polyclonal antibodies could potentially be cross reacting with closely related coccidians. For instance, cross reaction has been noted using the polyclonal antiserum from source 2 with *Hammondia hammondii* (B. Barr, unpubl. obs.) and with a *Lankesterella* species reported in white tree frogs (Gericota et al., 2010). Due to the possibility of cross reaction with the *T. gondii* polyclonal antibodies, a panel of well-characterized *T. gondii* antibodies was used to evaluate the specificity of the initial immunohistochemical results. The antibodies known to

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stain the coccidian stages of *T. gondii* failed to stain the parasites in the sea lion intestine, although formalin fixation may have potentially affected tissue reactions. Although ENO2 antibodies did stain the parasites in the sea lion intestine, staining of parasite nuclei was not strong, as would be expected with *T. gondii*. Enolase is a glycolytic enzyme and will be present in all coccidian parasites; therefore, this staining likely represents a cross reaction. The molecular data support the conclusion that novel coccidian parasites were found infecting these marine mammals and highlight the caution that should be taken when using immunohistochemistry alone to diagnose protozoal infections, especially when diagnostic immunohistochemical stains are based on polyclonal antibodies.

In this study, DNA amplification and sequence analysis indicated that coccidian infections in the sea lion intestine are complex, with multiple genotypically distinct parasites present in the intestine of the 5 affected sea lions involving 3 previously unrecognized distinct organisms. Additionally, in cases 4 and 5, DNA from 2 different organisms was amplified from the intestine of the same individual. Coccidia are important and common enteric parasites in many mammalian species; however, intestinal coccidia have not been previously reported in California sea lions despite their overwhelming popularity as display animals in zoo and aquaria. In pinnipeds, enteric coccidian parasites have only been previously documented in harbor seals, with infection attributed to *Eimeria phocae* (Van Bolhuis et al., 2007). With the exception of *E. phocae*, the possibility that pinnipeds serve as definitive or transport hosts for enteric or systemic coccidians is unknown.

Histologically identified parasites in the different cases could represent closely related, but morphologically similar, coccidian species that co-infect the sea lion intestine. Although histology and electron microscopy verified the presence of both sexual and asexual stages within the affected sea lions, further identification of the coccidian organisms through histological and ultrastructural features was not possible. Sea lion intestinal coccidian forms were much smaller than those described for *E. phocae* infections, where microgamonts range from approximately 89 to 123 × 57 to 135 μm and macrogamonts range from 17 to 24 × 17 to 22 μm (Van Bolhuis et al., 2007). Unfortunately, neither mature oocysts or oocyst wall formation were noted histologically or ultrastructurally, which precluded more exact species identification (Ferguson et al., 1974, 1975; Ferguson, 2004; Ferguson and Dubremetz, 2007). The parasitophorous vacuole noted surrounding organisms was limited by a thin membrane, which is more consistent with the parasites belonging to *Eimeria*, since *Toxoplasma* and *Isospora* spp. coccidian stages are typically enclosed by a thickened membrane. Due to the proven ability of *T. gondii* oocysts to survive in seawater (Lindsay and Dubey, 2009) and prey species such as northern anchovies (*Engraulis mordax*) and Pacific sardines (*Sardinops sagax*) (Massie et al., 2010), we considered the possibility that the amplified DNA originated from histologically undetected coccidian stages located only within intestinal contents resulting in a false diagnosis of co-infection. False positive results were considered unlikely, however, given that DNA from several organisms (coccidia ‘‘A’’ and ‘‘B’’ in cases 4 and 5) was amplified from multiple extra-intestinal tissues, indicating disseminated infection in those animals. Initial attempts at laser capture microdissection to extract DNA specifically from the intestinal epithelial cells containing sexual macrogamont or microgamont stages were unsuccessful.

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Systemic infection in cases 4 and 5 suggests that sea lions may act as both definitive and intermediate hosts for coccidians ''A'' and ''B,'' similar to felids, which serve as both the definitive and intermediate hosts for *T. gondii* (Dubey et al., 1970; Dubey, 1976). Although there was no histologic evidence of extra-intestinal infection in these sea lions, DNA can be amplified in many tissues from animals with early infections of tissue cyst-forming coccidia, such as *N. caninum* and *T. gondii*, prior to histologic evidence of infection (Esteban-Redondo and Innes, 1998; Kang et al., 2009). Free-ranging sea lions that die from various causes occasionally have evidence of mild meningoencephalitis that is morphologically consistent with protozoal infection (K. Colegrove, unpubl. obs.). The cause of this inflammation is often undetermined because no protozoal organisms can be found on routine histologic examination, and immunohistochemical stains for protozoal organisms are negative (K. Colegrove, unpubl. obs.). It is possible that the newly identified protozoa in this study are the cause of some of the mild systemic infections that have been previously noted in sea lions. Further research on the prevalence of tissue cyst-forming coccidian infections in free-ranging sea lions is needed, and this report highlights the utility of incorporating PCR using ITS-1 primers to specifically identify previously uncharacterized protozoal organisms.

The possibility that other marine organisms could serve as definitive hosts for *T. gondii* or organisms closely related to *N. caninum* has been previously postulated to explain the presence of protozoal organisms in the marine environment (Conrad et al., 2005). Amplification of coccidian ''C'' DNA from the harbor seal (case 6) further suggests that sea lions are definitive hosts for at least 1 coccidian species that may be pathogenic to other marine mammal species that share the same ecosystem. Lapointe et al. (2003) reported protozoal infection due to an unidentified organism in a harbor seal, which indicated that protozoans other than *T. gondii* and *S. neurona* may cause disease in free-ranging pinnipeds. Although several marine mammal species were reported to be serologically positive to *N. caninum* in previous studies (Dubey et al., 2003; Fujii et al., 2007), the amplification of *N. caninum* DNA from case 4 represents, to our knowledge, the first direct evidence of infection in a sea lion.

Based on the time between the admittance of the affected sea lions to rehabilitation and their death, infections were acquired both in the wild and during rehabilitation. One sea lion (case 4) died during transport to the rehabilitation center prior to being exposed to other animals. Therefore, the *N. caninum* and newly identified coccidia ''A'' and ''B'' identified in this animal must have been obtained in the wild prior to stranding. The other 4 sea lions were in rehabilitation for 9–36 days, during which time they were exposed to other sea lions and were fed fresh frozen and thawed herring (*Clupea pallasii*) prior to death. None of the affected sea lions was housed at the rehabilitation center during the same time period. The transmission dynamics of these newly discovered coccidian parasites requires further investigation so

In conclusion, this study identified previously undescribed enteric protozoan species infecting 5 free-ranging California sea lions with evidence of asexual and sexual coccidian forms, suggesting that sea lions are the definitive host of some of these organisms. Polymerase chain reaction using primers targeting the ITS-1 gene identified 3 previously uncharacterized sequences likely representing 3 new coccidian species that are most closely

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related to *N. caninum*. Further analysis of intestinal infections in California sea lions, including the application of laser capture microdissection methods to specifically isolate enterocytes containing protozoa for PCR analysis, will be critically important to better understand the pathogenicity of coccidian species that affect marine mammals. Although no coccidian oocysts were identified in the affected sea lions, it is likely that sexual replication will produce oocysts that are shed from the intestine of these definitive hosts (Dubey et al., 1970). Examination of sea lion feces for coccidian oocysts should allow for a better understanding of the prevalence of infection in sea lions. The identification of a pathogenic strain ''C'' causing fatal disease in a neonatal harbor seal with disseminated in utero infection highlights the urgency for better understanding of transmission dynamics of these coccidian pathogens infecting California sea lions in the marine environment.

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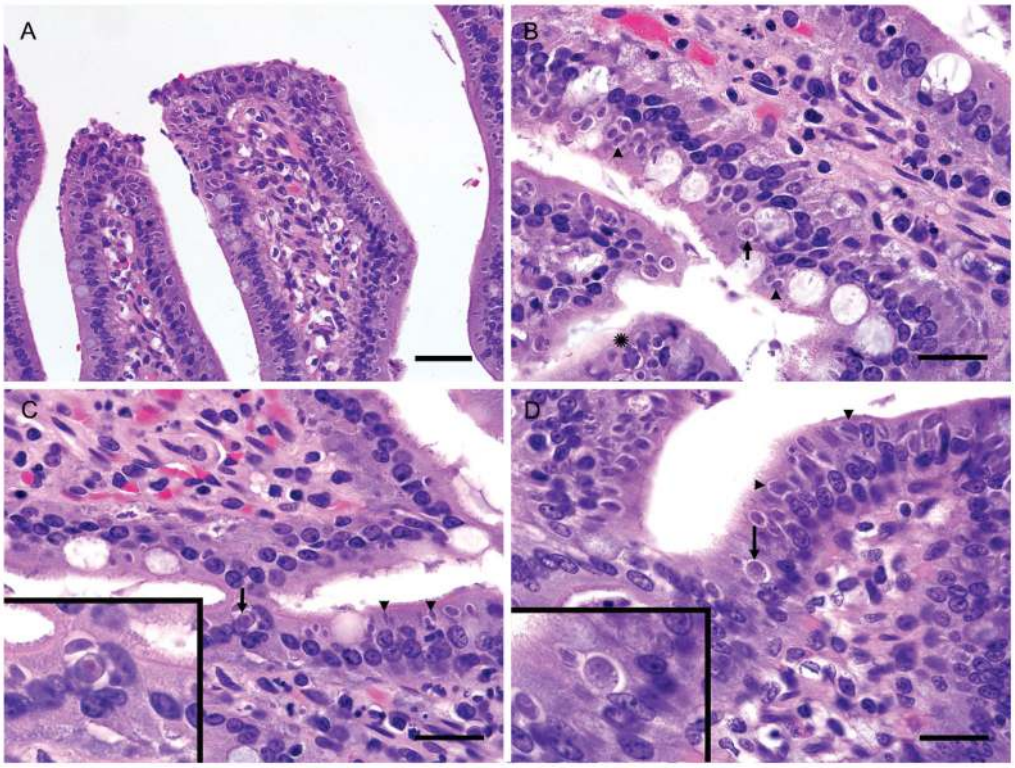

Figure 1.

Sections of California sea lion (*Zalophus californianus*) (case 2) small intestinal villi containing sexual and asexual coccidian stages. All sections were stained with hematoxylin and eosin. (**A**) Numerous coccidian stages within the apical cytoplasm of enterocytes (arrows). Bar = 50 μm. (**B**) Oval to cigar-shaped schizonts (arrowheads), a mature schizont with peripherally arranged developing merozoites (star), and a macrogamont (arrow). Bar = 20 μm. (**C**) Multiple coccidian stages within enterocytes including schizonts (arrowheads) and a macrogamont (arrow). Inset: Higher magnification of macrogamont. Bar = 20 μm. (**D**) Schizonts (arrowheads) and a microgamont (arrow) within enterocytes. Inset: Higher magnification of microgamont. Bar = 20 μm.

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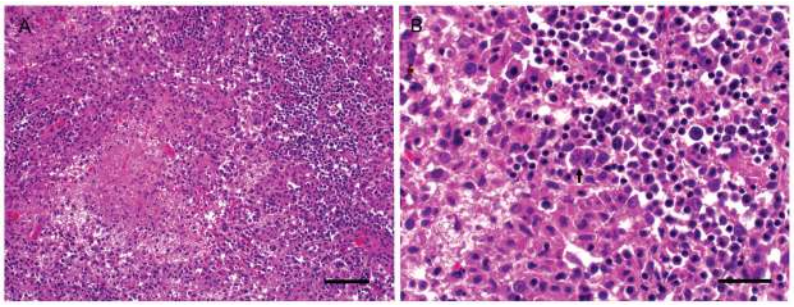

Figure 2.

Section of lymph node from a neonatal harbor seal (*Phoca vitulina*) with in utero protozoal infection (case 6). All slides were stained with hematoxylin and eosin. (**A**) Area of necrosis and granulomatous inflammation. Bar = 100 μm. (**B**) Multiple protozoal zoites within a macrophage (arrow). Bar = 50 μm.

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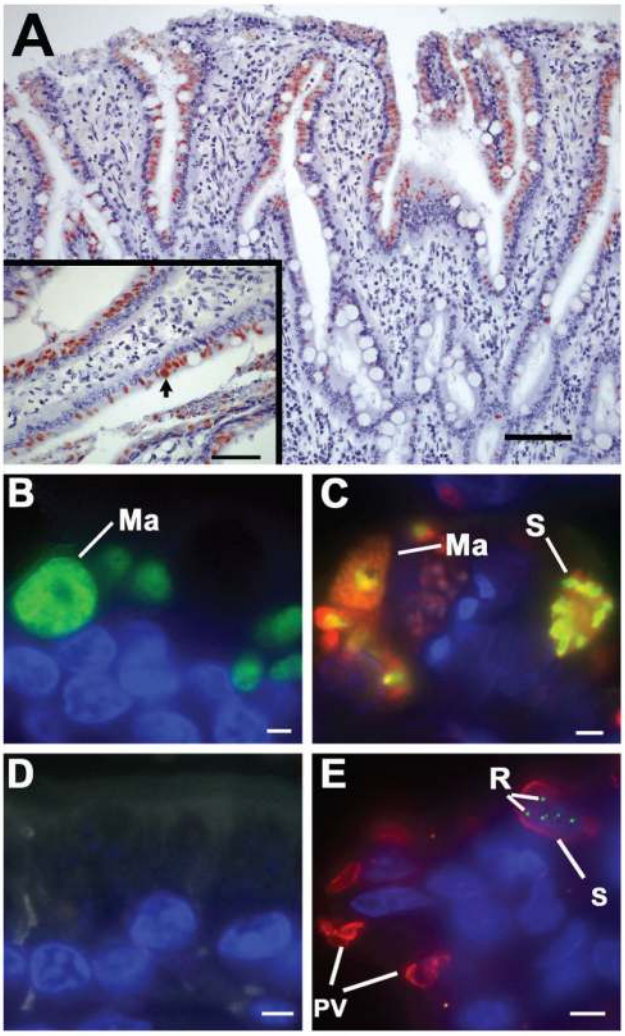

Figure 3.

Sections of California sea lion small intestine (case 2) (**A, B, D**) and cat intestine infected with the coccidian stages of *Toxoplasma gondii* (**C, E**) stained with various anti–*Toxoplasma* antibodies. In (**A**), section was labeled with anti–*Toxoplasma* polyclonal antibody from source 2 and visualized with 3-amino-9-ethyl carbazole (AEC) chromogen. In (**B–E**), sections were double labeled with two antibodies (see below), visualized using FITC (green) and Texas red (red), and observed with a fluorescent microscope. (**A**) Section of sea lion intestine stained with the polyclonal anti–*Toxoplasma* antibody showing apparently positive staining of the enteric parasites. Bar = 50 μm. Inset: Higher magnification illustrating an apparent immunopositive round macrogamont (arrow). Bar = 20 μm. (**B, C**). Sections of sea lion (**B**) and cat (**C**) intestine double labeled with ENO2 (green) and LDH1 (red). There is some labeling of the parasites in both sections with ENO2, while LDH1 staining is limited to the *Toxoplasma* parasites in the cat intestine. Note that staining with ENO2 in the sea lion intestine differs from the staining of *T. gondii* in the cat intestine in that parasite nuclei in the sea lion intestine are not strongly reactive to the antibody, suggesting nonspecific cross reaction. Ma, macrogamont; S, schizont. Bar = 1 μm. (**D, E**)

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Sections of sea lion (**D**) and cat (**E**) intestine double labeled with Rop2,3,4 (green) and NTPase (red). Note that the parasites in the sea lion intestine are unstained, while the parasitophorous vacuoles and rhoptries of the *Toxoplasma* parasites in the cat intestine are strongly labeled. PV, parasitophorous vacuole; R, rhoptry; S, schizont. Bar = 1 μm.

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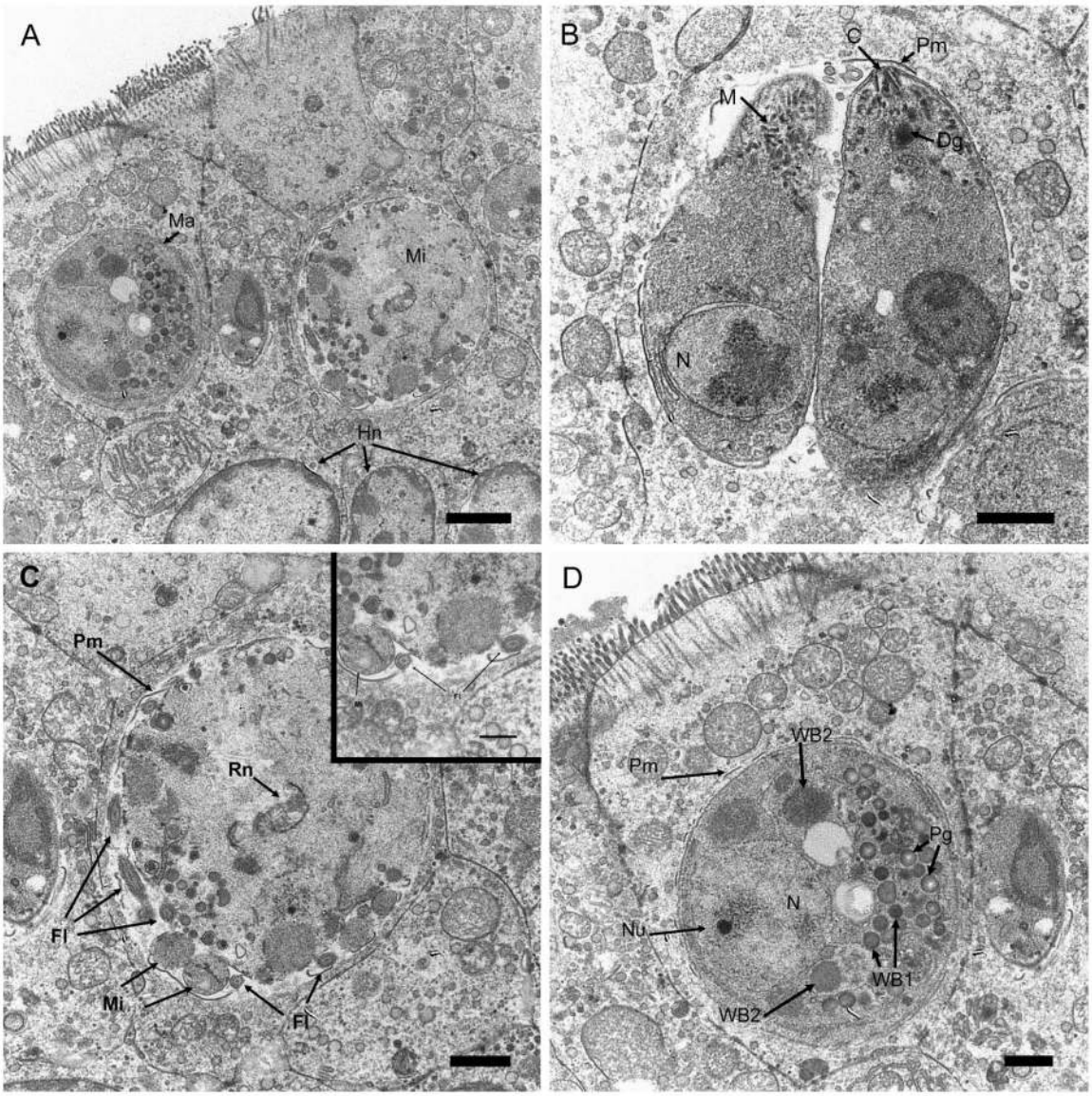

Figure 4.

Transmission electron micrographs of sea lion enterocytes containing sexual and asexual coccidian stages. (**A**) Enterocytes containing a microgamont (Mi) and a macrogamont (Ma); Hn, nucleus of host enterocytes. Bar = 2 μm. (**B**) Two merozoites with various organelles. N, nucleus; C, conoid; Dg, dense granules; M, microneme; Pm, parasitophorous vacuolar membrane. Bar= 1 μm. (**C**) Microgamont in the early stage of microgamete (Mi) formation. Fl, microgamete flagellum; Pm, parasitophorous vacuolar membrane; Rn, residual nucleus of microgamont. Bar= 1 μm. Inset: Higher magnification illustrating microgamete flagellum (Fl). Bar = 500 nm. (**D**) Macrogamont with a large nucleus (N); Nu, nucleolus; Pm, parasitophorous vacuolar membrane; WB1, wall-forming body type 1; WB2, wall-forming body type 2; Pg, polysaccharide granules. Bar = 1 μm.

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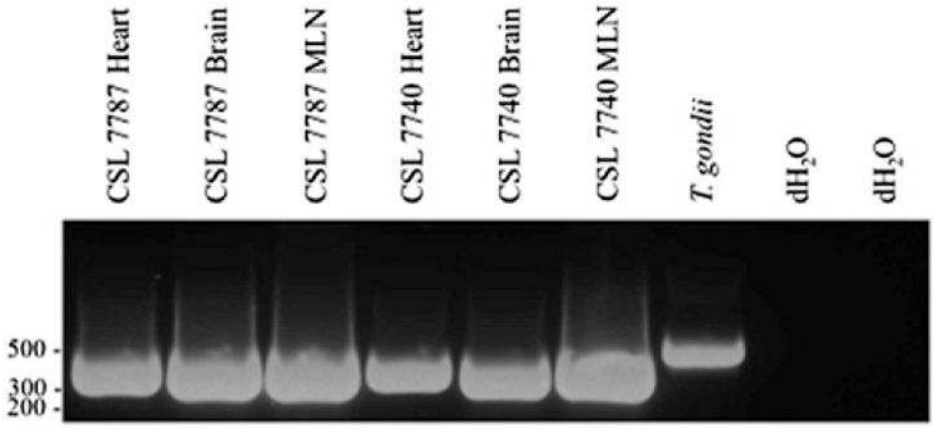

Figure 5.

Representative agarose gel of ITS-1 PCR amplicons produced from California sea lion (CSL) tissues (400 bp) and control *T. gondii* tachyzoite DNA (500 bp) and water. DNA sequencing analysis of the 400-bp amplicons identified that CSL 7787 heart, frontal lobe, mesenteric lymph node, and CSL 7740 occipital lobe and mesenteric lymph node contained DNA with 2 distinct sequence types, designated coccidia ''A'' (GenBank accession no. GU936629) and coccidia ''B'' (GenBank accession no. GU936630).

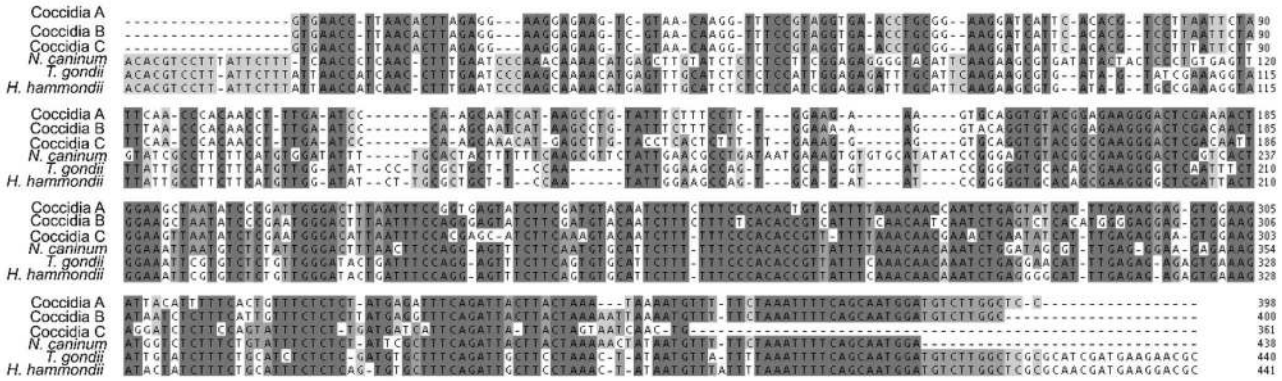

Figure 6.

Clustal ITS-1 sequence alignment viewed using JALView alignment software for the coccidia ‘‘A,’’ ‘‘B,’’ and ‘‘C’’ (GenBank accession no. GU936631) sequence types against *T. gondii* (AF2552408), *Neospora caninum* (AF038861), and *Hammondia hammondii* (AF096499). The closest orthologous sequence was that of *N. caninum*, and ITS-1 DNA sequences from the genus *Sarcocystis* sp. were too divergent to align. that potential sources of infection for sea lions and other marine mammals can be ascertained.

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Table I

Details of the 5 California sea lion (CSL) and 1 harbor seal (HS) cases with protozoal infection.

Case no.	Species	Animal ID	Gender	Age class	Stranding county	Time in rehabilitation before death (days)	Cause of death
1	Sea lion	CSL 6489	Female	Juvenile	San Luis Obisbo	26	Domoic acid toxicity
2	Sea lion	CSL 6457	Female	Juvenile	Sonoma	36	Domoic acid toxicity
3	Sea lion	CSL 7753	Male	Yearling	San Luis Obisbo	9	Pneumonia
4	Sea lion	CSL 7740	Male	Yearling	Santa Cruz	0	Pneumonia
5	Sea lion	CSL 7787	Male	Yearling	Marin	27	Pneumonia
6	Harbor seal	HS 1634	Female	Neonate	San Mateo	0	Disseminated protozoal infection

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Table II

Results of Indirect Fluorescent Antibody Test (IFAT) IgG serology for the 5 sea lions and 1 harbor seal with protozoal infection.

Case no.	Species	Dates blood sample taken	Date of death	*Toxoplasma gondii* titer	*Sarcocystis neurona* titer	*Neospora caninum* titer
1	Sea lion	5 March	19 March 2005	1<1:40	1<1:40	1<1:40
2	Sea lion	12 December, 15 January	15 January 2005	1<1:40	1<1:40	1<1:40
3	Sea lion	19 July	19 July 2008	1<1:40	1<1:40	1<1:40
4	Sea lion	None available				
5	Sea lion	22 August	22 August 2008	1<1:40	1<1:40	1<1:40
6	Harbor seal	14 March	14 March 2006	1<1:40	1<1:40	1<1:40

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Table III

Detail of data from ITS-1 locus PCR and DNA sequence analysis for the 5 sea lions with enteric protozoal infection.

	Tissues tested*							
Case no.	**Duodenum**	**Jejunum**	**Mesenteric lymph node**	**Heart**	**Brain—frontal lobe**	**Brain—occipital lobe**	**Brain—cerebellum**	**Formalin-fixed paraffin-embedded intestine**
1 CSL 6489	NS	NS	NS	NS	NS	NS	NS	B
2 CSL 6457	NS	NS	NS	NS	NS	NS	NS	C
3 CSL 7753	A	A	NS	Neg	Neg	Neg	Neg	Neg
4 CSL 7740	A *Neospora caninum*	A *Neospora caninum*	A, B	A	Neg	A, B	Neg	Neg
5 CSL 7787	A, B	A, B	A, B	A, B	A, B	A, B	A, B	Neg

* NS, not sampled; Neg, no protozoal DNA amplified; A, newly described coccidian ‘‘A’’; B, newly described coccidian ‘‘B’’; C, newly described coccidian ‘‘C.’’

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