

Transplacental toxoplasmosis in a wild southern sea otter (*Enhydra lutris nereis*)

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Abstract

In September 2004, a neonatal sea otter pup was found alive on the beach in northern Monterey Bay, CA. Efforts to locate the mother were unsuccessful. Due to a poor prognosis for successful rehabilitation, the pup was euthanized. Postmortem examination revealed emaciation, systemic lymphadenopathy and a malformation of the left cerebral temporal lobe. On histopathology, free tachyzoites and tissue cysts compatible with *Toxoplasma gondii* were observed in the brain, heart, thymus, liver, lymph nodes and peri-umbilical adipose. The presence of *T. gondii* within host tissues was associated with lymphoplasmacytic inflammation and tissue necrosis. Immunofluorescent antibody tests using postmortem serum were positive for anti-*T. gondii* IgM and IgG (at 1:320 and 1:1280 serum dilution, respectively), but were negative for IgG directed against *Sarcocystis neurona* and *Neospora caninum* (<1:40 each). Brain immunohistochemistry revealed positive staining for tachyzoites and tissue cysts using antiserum raised to *T. gondii*, but not *S. neurona* or *N. caninum*. *T. gondii* parasite DNA was obtained from extracts of brain and muscle by PCR amplification using the diagnostic B1 locus. Restriction enzyme digestion followed by gel electrophoresis and DNA sequencing confirmed the presence of Type X *T. gondii*, the strain identified in the majority of southern sea otter infections.

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1. Introduction

Toxoplasmosis, caused by the protozoan parasite *Toxoplasma gondii* is an important cause of morbidity and mortality in humans and terrestrial animals (Tenter

et al., 2000). In these species, transplacental transmission of *T. gondii*, especially if occurring during early gestation, may result in placentitis, embryonic death, stillbirth, abortion, congenital malformations or post-natal blindness and retardation (Dubey and Beattie, 1988; Dubey, 1993; Tenter et al., 2000). Increasing recognition of *T. gondii* as a waterborne pathogen traced to drinking water raises concerns for risks to pregnant women and their unborn fetuses (Benenson et al., 1982; Bowie et al., 1997; de Moura et al., 2006; Heukelbach

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et al., 2007). The high prevalence of *T. gondii* infection in some marine mammal species also emphasizes the importance of aqueous transport of oocysts as an important source of exposure (Miller et al., 2002; Conrad et al., 2005). Although *T. gondii* infection is common, reports of transplacental transmission of *T. gondii* in marine mammals are sparse, when compared to humans and terrestrial animals (Jardine and Dubey, 2002; Resendes et al., 2002). This may be due in part to decreased surveillance, as aborted marine mammal fetuses are unlikely to be examined by pathologists, or may be missed due to fetal reabsorption *in utero*. Fetal death and congenital *T. gondii* infections have been reported from wild and captive cetaceans (Jardine and Dubey, 2002; Resendes et al., 2002). Chief findings among these cases included abortion, stillbirth and neonatal death. Gross placentitis has not been reported from marine mammals infected with *T. gondii*. Reproductive impairment due to *T. gondii* infection could prove to be important at the population level for some cetaceans, but currently documentation is sparse. Vertical (transplacental or transmammary) transmission could provide one additional means for these parasites to gain access to new hosts and spread to diverse habitats.

Despite 15 years of detailed histopathology, parasite isolation in cell culture, molecular analysis and/or serological testing of >750 animals, no confirmed transplacental infections of *T. gondii* have been reported previously from wild or captive sea otters (*Enhydra lutris*). Although *T. gondii* is an important cause of southern sea otter (*E. lutris nereis*) infection and mortality (Cole et al., 2000; Miller et al., 2002; Kreuder et al., 2003), the vast majority of culture and PCR-confirmed infections and fatalities have been noted in otters >6 months of age (Conrad et al., 2005). Although transplacental transmission seems likely to occur in wild otters, given the ability of *T. gondii* to pass transplacentally in other mammals, evidence to confirm this mode of infection was lacking. Here we report on a stranded, wild southern sea otter pup with disseminated toxoplasmosis resulting from transplacental infection. Further, we reveal the genotype of *T. gondii* infecting this otter and compare it with those previously reported from Pacific coastal marine mammals.

2. Material studied, area descriptions, methods, techniques

In September 2004, a neonatal female sea otter was found alive on the beach at Capitola, CA in northern Monterey Bay. Efforts to locate the mother were

unsuccessful; due to a poor prognosis for successful rehabilitation and release and a lack of available surrogates to facilitate pup care, the animal was euthanized. The pup was sedated with intramuscular fentanyl citrate (Fentanyl Citrate for Injection, Central Avenue Pharmacy, Pacific Grove, CA) and diazepam (Diazepam Injection USP, Abbott Laboratories, Abbott Park, IL) (Monson et al., 2001) for blood collection prior to euthanasia with intravenous pentobarbital sodium (Beuthanasia D Solution, Schering-Plough Animal Health Corp., Summit, NJ). The carcass was placed on ice and transported to the Marine Wildlife Veterinary Care and Research Center (MWVCRC) in Santa Cruz for necropsy. The animal was radiographed and a detailed necropsy was performed as previously described (Miller et al., 2001b). Samples of all major tissues were placed in 10% neutral-buffered formalin, fixed, trimmed and paraffin-embedded. Five-micron sections were cut on a rotary microtome and stained using hematoxylin and eosin (H&E). Immunohistochemistry for *T. gondii*, *Sarcocystis neurona* and *Neospora caninum* was performed for selected tissues using established methods (Miller et al., 2001b). Whole blood was collected from the heart and great vessels and centrifuged. The resulting serum was tested for the presence and titer of IgG to *T. gondii*, *S. neurona* and *N. caninum*, and IgM to *T. gondii* using indirect fluorescent antibody tests (IFAT), as previously described (Miller et al., 2002). Cerebrum and cerebellum were collected aseptically and processed for parasite isolation in cell culture as described (Miller et al., 2001a). Cryopreserved brain and skeletal muscle were tested for the presence of *T. gondii* using established primers to detect and amplify protozoal genomic DNA at the *B1* locus (Burg et al., 1989; Grigg and Boothroyd, 2001). The infecting genotype was then determined via restriction fragment length polymorphism (RFLP) and DNA sequencing at the *B1* locus (Grigg and Boothroyd, 2001; Miller et al., 2004). Positive controls consisted of genomic DNA from archetypal *T. gondii* Type I (RH), Type II (76K), and Type III (CEP) strains. Negative controls consisted of deionised water and/or genomic DNA from non-infected sea otter tissue extracts.

3. Results

Physical examination at the time of stranding revealed the pup to be thin, underweight and semi-comatose. The pup weighed 1.45 kg with a total body length (nose to tail) of 45 cm. Based on published length and weight criteria (Morejohn et al., 1975), the pup's small size and the presence of a moist, soft and pink

umbilicus that was firmly attached to the ventral body wall, the animal was determined to be between 1 and 3 days old. Antemortem blood chemistry and cytology revealed severe hypoglycemia (blood glucose = 7 mg/dl; normal range = 67–161 mg/dl), a mild increase in alkaline phosphatase, likely as a result of osteogenesis (373 IU/l; normal range 57–257 IU/l) and increased circulating immature (band stage) neutrophils. Prior to euthanasia with intravenous pentobarbital sodium (Beuthanasia D Solution, Schering Plough Animal Health Corporation, Summit, NJ) the pup was sedated with intramuscular fentanyl citrate (fentanyl citrate for injection, Central Avenue Pharmacy, Pacific Grove, CA,) and diazepam (Diazepam Injection USP, Abbot Laboratories, Abbot Park, IL) (Monson et al., 2001).

At necropsy, the presence of a fresh, firmly attached umbilicus was confirmed and moderate, diffuse lymphadenopathy was also identified. Affected lymph nodes were 1.5–2× normal size, solid, meaty and tan-white. Also noted was diffuse vascular congestion of the cerebral meninges. The left temporal lobe of the cerebrum was abnormally shaped; a large 3 cm × 1.5 cm × 1.2 cm, broad-based, pedunculated mass of grossly normal neuropil projected off the left caudolateral brain surface (Fig. 1). This lobe was attached to the adjacent brain tissue via a broad stalk and projected

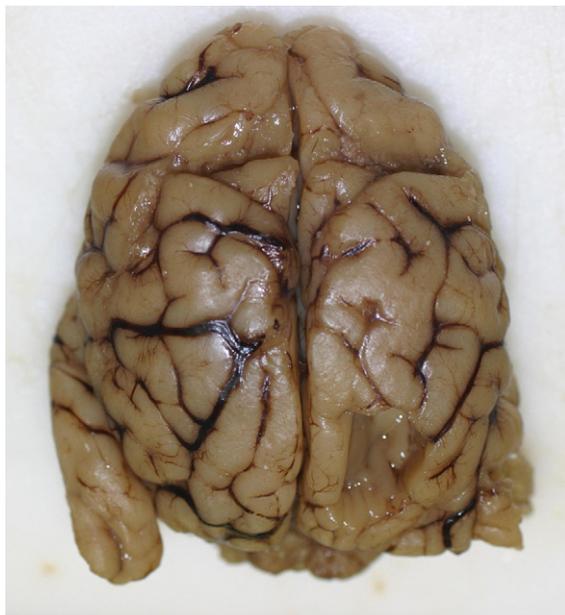


Fig. 1. Formalin-fixed brain from a neonatal southern sea otter with a malformation of the left temporal lobe. A large, broad-based, pedunculated mass of otherwise normal-appearing neuropil projects caudally from the left caudolateral aspect of the cerebrum. The two small holes in the right caudal aspect of the brain are artifacts resulting from pre-fixation tissue sampling.



Fig. 2. A left lateral view of the malformed brain tissue described in Fig. 1.

caudally from the point of attachment (Fig. 2). The brain was otherwise unremarkable grossly. Attempts to collect cerebrospinal fluid postmortem via cisternal tap were unsuccessful. Mild patchy bronzing and pallor of the hepatic capsule was noted grossly, along with mild diffuse pulmonary hyperinflation. Postmortem urine was below minimum detection limits (<5 ppb) for the marine biotoxin domoic acid via liquid chromatography–mass spectrophotometry and heart blood was negative on bacterial culture using nonselective media.

Microscopic examination of H&E-stained, paraffin-embedded sections of formalin-fixed tissues revealed the presence of intracytoplasmic 1.5–2.5 μm long stout, banana-shaped tachyzoites in the brain, heart, thymus, liver, multiple lymph nodes and subcutaneous brown fat. Parasite proliferation in the brain, heart and lymph nodes was associated with moderate to marked lymphoplasmacytic inflammation and tissue necrosis. A similar inflammatory infiltrate was observed in the tongue and adrenal cortex, but protozoal parasites were not found. All lymph nodes exhibited marked cortical lymphoid hyperplasia on histopathology, along with diffuse, moderate subcapsular and medullary histiocytic lymphadenitis. Proliferating tachyzoites were common in the subcapsular sinuses and superficial cortices of the right prescapular and mesenteric lymph nodes (Fig. 3). Parasite proliferation in the thymus was associated with mild stromal hemorrhage. Mild fibrinosuppurative inflammation was noted within the lumenae of the umbilical arteries and veins, but no bacteria or other pathogens were observed. Extramedullary hematopoiesis was prominent within the hepatic sinusoids and splenic red pulp. Scattered, thin-walled 80 μm × 40 μm tissue cysts were observed in the cytoplasm of infected cardiomyocytes (Fig. 4).

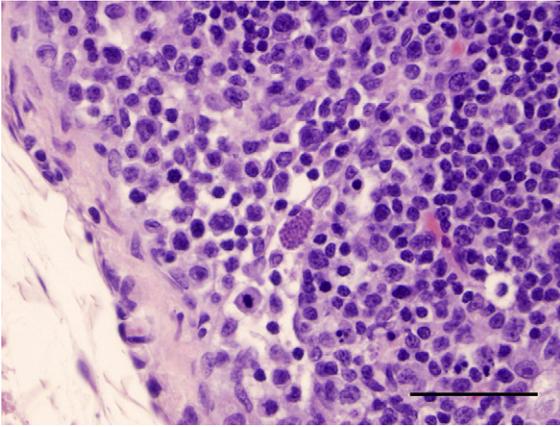


Fig. 3. H&E-stained, paraffin-embedded section of the right prescapular lymph node. Proliferating *Toxoplasma gondii* tachyzoites are forming a grapelike cluster within a subcapsular sinus (center of photo). The adjacent subcapsular sinus contains elevated numbers of lymphocytes and plasma cells and the cortex is moderately hyperplastic (bar = 100 μ m).

In the brain, multifocal areas of lymphoplasmacytic inflammation, tissue cavitation and necrosis were apparent in the cerebrum, medulla and brainstem (Fig. 5). This infiltrate was organized as multifocal, discrete 400–800 μ m diameter inflammatory nodules, composed predominantly of lymphocytes and plasma cells surrounding moderate numbers of 10–40 μ m diameter protozoal cyst or pre-cyst stages (Fig. 6). Protozoa were sometimes more concentrated at the lesion periphery, suggesting a centrifugal pattern of lesion expansion, as previously described (Miller, 2008). Findings on microscopic examination of the malformed left temporal lobe were consistent with the

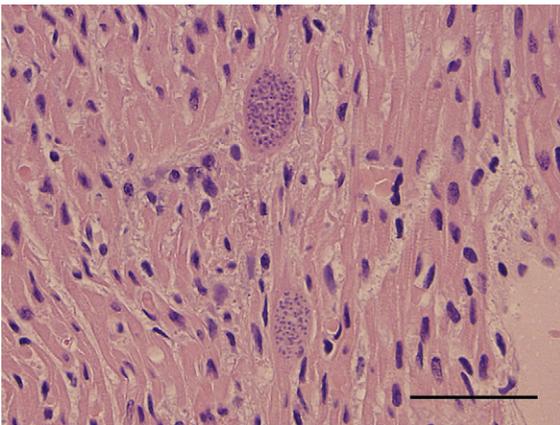


Fig. 4. H&E-stained, paraffin-embedded section of the left ventricular myocardium. A mild inflammatory infiltrate composed of lymphocytes and plasma cells is located adjacent to two elliptical *T. gondii* tissue cysts (top and bottom center) (bar = 100 μ m).

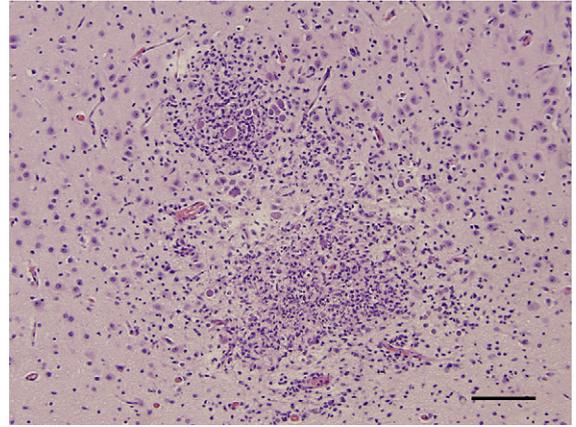


Fig. 5. H&E-stained, paraffin-embedded section of cerebrum. Two inflammatory nodules are composed primarily of small lymphocytes interspersed plasma cells and macrophages. Mild perilesional edema, characterized by pale, finely vacuolated foci, is apparent at the periphery of each nodule. Mild perivascular cuffing of adjacent small blood vessels by inflammatory cells is noted (bar = 200 μ m).

other examined areas: parasites, inflammation and necrosis were extensive, but no more than within the adjacent, normal neuropil, and the tissue architecture was within normal limits.

Immunohistochemistry of cerebrum revealed tachyzoites and tissue cysts that stained positive for *T. gondii*, but negative for *S. neurona* and *N. caninum*. Serology was negative for IgG directed against *S. neurona* and *N. caninum* (both at <1:40 serum dilution), but positive for anti-*T. gondii* IgM (1:320) and IgG (1:1280) using previously established cutoffs (Miller et al., 2002). An attempt to isolate parasites from trypsinized cerebrum and cerebellum collected at necropsy was unsuccessful.

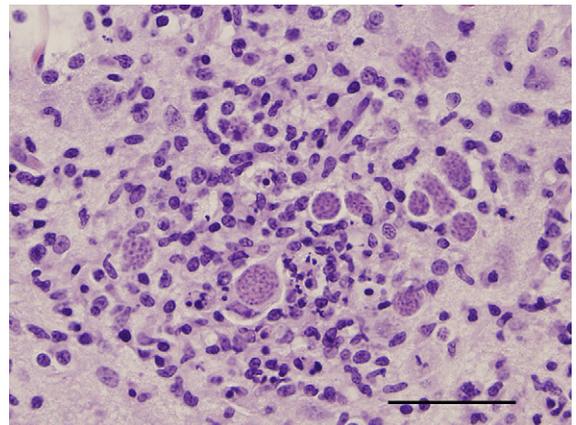


Fig. 6. A higher magnification view of the upper inflammatory nodule in Fig. 5 above. Large numbers of developing *T. gondii* tissue cysts are apparent (bar = 100 μ m).

However, DNA extracted from cryopreserved brain and skeletal muscle was positive for *T. gondii* by PCR amplification and enzyme digestion of the species-specific *B1* gene. Based on PCR-RFLP digestion patterns and DNA sequence analysis at the *B1* locus, a Type X allele was identified that possessed an identical nucleotide sequence to the previously characterized sea otter isolate 3160 (GenBank accession number AF179871). Hence, the *T. gondii* strain infecting the otter pup was consistent with infection by a Type X strain of *T. gondii*.

4. Discussion

Here we report the first case of disseminated toxoplasmosis resulting from transplacental infection in a neonatal sea otter from coastal California. Evidence to support transplacental transmission as the source of infection includes the animal's young age (≤ 3 days old), the presence of both IgM and IgG in the pup's serum and detection of numerous *T. gondii* tissue cysts in the brain on histopathology. Postpartum infection with *T. gondii* is furthermore unlikely because of the life history of otters; other than milk, solid food is not typically consumed by sea otter pups prior to 4 weeks of age (Riedman and Estes, 1990) and no food was present within the gastrointestinal tract at necropsy. In laboratory studies in rodents, *T. gondii* tissue cysts required ≥ 8 days to develop in the brain after parasite inoculation (Dubey et al., 1997) and tissue cysts are often not observed in significant numbers until ≥ 30 days postinfection. Other than obtundation, no specific neurological abnormalities were reported for this pup prior to euthanasia. However, detection of neurological impairment in neonates is often challenging and it is possible that clinical abnormalities were missed.

The most significant findings on histopathology were moderate to severe lymphoplasmacytic inflammation associated with proliferating tachyzoites in multiple tissues. For the majority of reports of *T. gondii* infection in sea otters, the primary focus of tissue parasitism is the central nervous system and tissue cysts are the most numerous parasite stage observed on histopathology (Kreuder et al., 2003; Dubey, 1993; Miller, 2008; Thomas et al., 2007), suggestive of chronic or recrudescent infection. However, for this animal, tachyzoites were numerous and disseminated throughout the body, suggestive of acute infection and/or inability of this neonatal otter to mount a protective immune response. Detection of both anti-*T. gondii* IgM and IgG in the serum is also supportive of recent infection. Although putative passive transfer of *T.*

gondii antibodies has been observed in PCR and culture-negative sea otter pups, the resulting titers are typically lower than was observed in this PCR confirmed, *T. gondii*-infected animal (data not shown).

PCR amplification and gel electrophoresis of genomic DNA at the *B1* locus from brain and skeletal muscle confirmed the identity of the parasite as *T. gondii*, and RFLP analysis and DNA sequencing revealed it to be a Type X strain, the predominant form infecting wild southern sea otters (Miller et al., 2004; Conrad et al., 2005). Sequencing of DNA isolated from the brain of this pup revealed the infecting parasite to be genetically identical with a Type X *T. gondii* detected in a wild mussel (*Mytilus californianus*) collected from the same region (Miller et al., in review). Mussels are a favored sea otter prey item; the identification of Type X strains in both sea otters and mussels may provide important insight into the transmission dynamics and flow of Type X strains within the marine environment.

A large malformation was found in the left temporal lobe of the cerebrum of this pup at gross necropsy (Fig. 1). Numerous reports link transplacental toxoplasmosis in humans and terrestrial animals with development of congenital brain defects (Dubey and Beattie, 1988; Tenter et al., 2000). The malformed tissue was positive for *T. gondii* on histopathology and immunohistochemistry, although the degree of inflammation and necrosis was not more severe than in adjacent, grossly normal neuropil. No other specific causes of brain malformation were identified. The causal relationship between fetal infection by *T. gondii* and development of this temporal lobe defect is unconfirmed; however, no prior reports exist for either fetal toxoplasmosis or congenital brain disease in southern sea otters. These observational data are supportive of, but not diagnostic for a link between the two conditions.

5. Conclusion

Transplacental toxoplasmosis and congenital cerebral malformation are reported for the first time in a wild neonatal sea otter that stranded in Monterey Bay, California. Elevated IgM and IgG titers against *T. gondii* were detected in serum and tachyzoites were observed and tissue cysts and tachyzoites consistent with *T. gondii* were observed in multiple tissues on histopathology. PCR amplification, gel electrophoresis and sequencing of the *B1* gene confirmed the *T. gondii* genotype as a Type X strain that was indistinguishable from that detected in a wild mussel collected in the same

area (Miller et al., in review). This otter also had a large congenital malformation of the left temporal cerebral lobe. Concurrent presentation of the brain defect and severe, *T. gondii*-associated meningoencephalitis in a neonatal otter suggests that these lesions may be causally linked.

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