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Detection and characterization of diverse coccidian protozoa shed by California sea lions

Yvette A. Girard [a, *], Christine K. Johnson [a], Heather M. Fritz [b, c], Karen Shapiro [b], Andrea E. Packham [b], Ann C. Melli [b], Daphne Carlson-Bremer [d], Frances M. Gulland [e], Daniel Rejmanek [f], Patricia A. Conrad [a, b]

[a] Karen C. Drayer Wildlife Health Center, School of Veterinary Medicine, University of California, Davis, CA, USA
[b] Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, CA, USA
[c] Department of Veterinary Pathology and Microbiology, College of Veterinary Medicine, Washington State University, Pullman, WA, USA
[d] Vanderbilt Institute for Global Health, Vanderbilt University, Nashville, TN, USA
[e] The Marine Mammal Center, Sausalito, CA, USA
[f] California Animal Health and Food Safety, Davis, CA, USA

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ABSTRACT

Tissue-cyst forming coccidia in the family Sarcocystidae are etiologic agents of protozoal encephalitis in marine mammals including the federally listed Southern sea otter (*Enhydra lutris*). California sea lions (*Zalophus californianus*), whose coastal habitat overlaps with sea otters, are definitive hosts for coccidian protozoa provisionally named Coccidia A, B and C. While Coccidia A and B have unknown clinical effects on aquatic wildlife hosts, Coccidia C is associated with severe protozoal disease in harbor seals (*Phoca vitulina*). In this study, we conducted surveillance for protozoal infection and fecal shedding in hospitalized and free-ranging California sea lions on the Pacific Coast and examined oocyst morphology and phenotypic characteristics of isolates via mouse bioassay and cell culture. Coccidia A and B were shed in similar frequency, particularly by yearlings. Oocysts shed by one free-ranging sea lion sampled at Año Nuevo State Park in California were previously unidentified in sea lions and were most similar to coccidia infecting Guadalupe fur seals (*Arctocephalus townsendi*) diagnosed with protozoal disease in Oregon (USA). Sporulated Coccidia A and B oocysts did not replicate in three strains of mice or in African green monkey kidney cells. However, cultivation experiments revealed that the inoculum of fecally-derived Coccidia A and B oocysts additionally contained organisms with genetic and antigenic similarity to *Sarcocystis neurona*; despite the absence of detectable free sporocysts in fecal samples by microscopic examination. In addition to the further characterization of Coccidia A and B in free-ranging and hospitalized sea lions, these results provide evidence of a new role for sea lions as putative mechanical vectors of *S. neurona*, or *S. neurona*-like species. Future work is needed to clarify the distribution, taxonomical status, and pathogenesis of these parasites in sea lions and other marine mammals that share their the near-shore marine environment.

1. Introduction

Coccidia are a diverse group of protozoan parasites within the phylum Apicomplexa and include pathogenic species of significance to animal and human health. Monoxenous coccidia, such as those in the genus *Eimeria* and *Isospora (syn. Atoxoplasma)*, primarily parasitize a single host throughout their life cycle and include important avian pathogens (McDougald, 1998; Barta et al., 2005; Berto et al., 2011). Heteroxenous coccidian protozoa including *Toxoplasma gondii*, *Neospora caninum* and *Sarcocystis neurona* infect multiple hosts throughout their life cycle and their

* Corresponding author. Karen C. Drayer Wildlife Health Center, 1089 Veterinary Medicine Drive, School of Veterinary Medicine, University of California, Davis, CA 95616, USA.
E-mail addresses: yagirard@ucdavis.edu (Y.A. Girard), ckjohnson@ucdavis.edu (C.K. Johnson), hmfritz@vetmed.wsu.edu (H.M. Fritz), kshapiro@ucdavis.edu (K. Shapiro), aepackham@ucdavis.edu (A.E. Packham), acmelli@ucdavis.edu (A.C. Melli), dpbremer@gmail.com (D. Carlson-Bremer), gullandf@tmmc.org (F.M. Gulland), drejmanek@ucdavis.edu (D. Rejmanek), paconrad@ucdavis.edu (P.A. Conrad).

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transmission is facilitated by predator–prey relationships (Sibley, 2003). In the intermediate host (often herbivorous or omnivorous), infectious stages are formed within tissue cysts that can be ingested by the definitive host (often a carnivorous predator). The definitive host is so called because the parasite life cycle is perpetuated through sexual multiplication in these animals. Definitive hosts shed environmentally-resistant oocysts, (or sporocysts from sporulated oocysts in the case of *S. neurona*), which are infective after sporulation for intermediate hosts that ingest fecally-contaminated food or water (Sibley, 2003).

Although the only known definitive hosts of *T. gondii*, *N. caninum* and *S. neurona* are terrestrial animals, specifically felids, canids and opossums (*Didelphis* spp.), respectively, there is evidence that marine mammals can also become infected with these and other related coccidia (Dubey et al., 2000, 2001a, 2001b; Tenter et al., 2000; Dubey et al., 2003; Colegrove et al., 2011; Gibson et al., 2011; Carlson-Bremer et al., 2012a; Goodswen et al., 2013). The most likely modes of transmission of these pathogens to aquatic animals are via ingestion of water-borne oocysts or sporocysts originating in land-based surface runoff, or infected prey (Miller et al., 2002; Conrad et al., 2005; Massie et al., 2010; Gibson et al., 2011; Shapiro et al., 2012). One of the most serious consequences of *S. neurona* and *T. gondii* infection in marine mammals is fatal protozoal encephalitis which has been described most commonly in cetaceans, including Southern sea otters (*Enhydra lutris nereis*), California sea lions (*Zalophus californianus*) and Pacific harbor seals (*Phoca vitulana richardsi*) (Thomas and Cole, 1996; Lapointe et al., 1998; Cole et al., 2000; Miller et al., 2001a; Dubey et al., 2003; Kreuder et al., 2003; Miller et al., 2004, 2009, 2010; Gibson et al., 2011). More recently, severe myositis in a hospitalized California sea lion was recognized as a new clinical syndrome associated with *S. neurona* infection (Carlson-Bremer et al., 2012b). Pathogenic infections with other, less well characterized, tissue-encysting coccidia have also been documented in marine mammals in association with protozoal lymphadenitis, hepatitis, myocarditis, encephalitis and non-suppurative necrotizing meningoencephalitis (Dubey et al., 2003; Lapointe et al., 2003; Colegrove et al., 2011; Gibson et al., 2011).

California sea lions inhabit waters of the Pacific coast of North America between southwestern Canada and Baja California (Lowry et al., 1992). As long-lived coastal residents with large fat stores and piscivorous prey preferences that are shared with humans, sea lions have the potential to act as sentinel species, or indicators of aquatic ecosystem and human health (Bossart, 2011). During postmortem examination of rescued California sea lions that died at The Marine Mammal Center (TMMC, Sausalito, California), sexual and asexual stages of three novel coccidia with genetic similarity to *Neospora* spp. were identified in sea lion enterocytes, and these organisms were putatively named Coccidia A, B and C (Colegrove et al., 2011; Carlson-Bremer et al., 2012a). Subsequent fecal analysis of stranded and rehabilitated sea lions sampled between 2007 and 2009 confirmed that sea lions shed Coccidia A and B, particularly as yearlings, and that shedding of Coccidia A could be detected in feces for up to 22 days (Carlson-Bremer et al., 2012a). Evidence to date suggests that California sea lions act as both definitive and intermediate hosts of novel protozoa in the absence of clinical signs or pathologic evidence of disseminated infection. Yet, the identification of severe protozoal hepatitis, myocarditis and encephalitis in a neonatal harbor seal infected with Coccidia C is of particular concern because it indicates that sea lions may be capable of shedding coccidian protozoa that are pathogenic to harbor seals and perhaps other aquatic wildlife (Colegrove et al., 2011).

A better understanding of the biology, epidemiology, and pathogenesis of tissue-encysting coccidian organisms that parasitize marine mammals is needed to properly assess the risks and burden of protozoal disease in aquatic ecosystems such as the Pacific coast of North America. As part of ongoing health surveillance in aquatic species of the Pacific Ocean, and accompanying studies of pathogen pollution in near-coastal California waters, we examined the diversity of coccidian parasites shed by hospitalized and free-ranging sea lions at coastal haul-out locations in central California. In addition, we characterized the phenotypes of sea lion-derived coccidian protozoa in both a mouse model of pathogenesis and in mammalian cell culture.

2. Materials and methods

2.1. Sample collection

Staff at TMMC collected fecal samples from individual California sea lions (CSL) stranded along the central and northern California coast between September 2010 and May 2012 as previously described (Carlson-Bremer et al., 2012a). Samples were typically collected within the first week of hospital admission, although some animals were sampled up to 3 weeks later. Animals and corresponding fecal samples were assigned unique identity numbers (CSL-). Multiple samples from each animal were collected when available for up to 9 weeks. When coccidial oocysts were identified at TMMC, one or more fecal samples from an individual animal were shipped on cold packs to the University of California Davis for oocyst harvesting and genetic characterization. Age classifications for sea lions (adult, subadult, juvenile, yearling, and pup) were estimated as previously described (Carlson-Bremer et al., 2012a). Between May 2011 and October 2012, sea lion fecal samples from free-ranging animals were also collected from coastal haul out sites at Año Nuevo State Park (37.108, −122.337), White Rock (35.532, −121.088), and Seal Rock at Point Lobos (36.516, −122.336), placed into individual 50 mL conical vials and kept at 4 °C during transport to UC Davis for oocyst detection, harvesting and genetic characterization. Samples were identified according to location (Año-, WR- and PL-).

2.2. Oocyst harvesting, sporulation and excystation

Coccidial oocysts were harvested from fecal samples by a double centrifugation flotation method as described by Dabritz and co-authors (Dabritz et al., 2007) with some modifications. A detailed protocol for oocyst harvesting from sea lions is available in the supplemental information associated with this article (See Supplementary data). When quantities were sufficient, a portion of freshly-harvested oocysts were not frozen, but were reserved and sporulated for bioassay in mice and inoculation into cell cultures. Oocysts for cell culture were additionally excysted.

For sporulation, newly-harvested oocysts were washed two times in sterile PBS, and the final pellet resuspended in 5 mL PBS, to which was added Clorox bleach at a final concentration of 10%. Following 20 min incubation with occasional gentle mixing, the sample was washed 3 times in 50 mL molecular biology grade water, with each centrifugation at 1000 *g* for 10 min. After final centrifugation, supernatant was carefully removed and the pellet mixed with 3–5 mL PBS (higher volume for samples with higher oocyst concentration). Approximately 1 mL of oocyst suspension in PBS was added to 5 mL filtered seawater (0.22 μm, Acrodisc® Syringe Filters, Pall Corporation) with 250 μl amphotericin B (250 μg/mL) in a T-25 tissue culture flask with ventilated lid. Each flask was set on a gentle rocker at RT and visually inspected every 2 days by light microscopy for evidence of sporulation. Incubation continued until 45%–72% sporulation was observed and stabilized (typically 3–8 days). Sporulated oocysts were washed two times in ~40 mL sterile PBS, counted, and prepared for oral inoculation into mice.