

FIGURE 2. Immunoelectron micrograph of *E. tenella* sporozoites. (A) Negative control, wherein no specific antibody was applied, showing a sporozoite section devoid of positive reaction for flotillin-1. (B) Sporozoite showing flotillin-1-positive patches on the parasite membrane. Note that the expression of flotillin-1 on the cellular membrane of sporozoites concentrates at the apical tip. Bar = 2 μ m.

via the apical complex present at the apex of invasive forms. As soon as sporozoites come into contact with a host cell surface, a signal is transduced from the surface to the apex. The signal induces reorientation, microneme exocytosis, apical binding to the host cell, and formation of the parasitophorous vacuole (Dubremetz et al., 1998). Therefore, many biological processes are involved in the host cell invasion, including numerous signal transduction pathways, cell migration, organization of the cytoskeleton, protein sorting, and membrane trafficking. It has been proposed that flotillin-1 plays a structural role in all of these cellular mechanisms (Edidin, 2003). Therefore, the presence of flotillin-1 on the cellular membrane of sporozoites predominantly at the

apical tip suggests that flotillin-1 belongs to the invasion machinery of *E. tenella*.

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Cryptosporidium and *Giardia* in Marine-Foraging River Otters (*Lontra canadensis*) From the Puget Sound Georgia Basin Ecosystem

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ABSTRACT: Species of *Cryptosporidium* and *Giardia* can infect humans and wildlife and have the potential to be transmitted between these 2 groups; yet, very little is known about these protozoans in marine wildlife. Feces of river otters (*Lontra canadensis*), a common marine wildlife species in the Puget Sound Georgia Basin, were examined for species of *Cryptosporidium* and *Giardia* to determine their role in the ep-

idemiology of these pathogens. Using ZnSO₄ flotation and immunomagnetic separation, followed by direct immunofluorescent antibody detection (IMS/DFA), we identified *Cryptosporidium* sp. oocysts in 9 fecal samples from 6 locations and *Giardia* sp. cysts in 11 fecal samples from 7 locations. The putative risk factors of proximate human population and degree of anthropogenic shoreline modification were not as-

sociated with the detection of *Cryptosporidium* or *Giardia* spp. in river otter feces. Amplification of DNA from the IMS/DFA slide scrapings was successful for 1 sample containing >500 *Cryptosporidium* sp. oocysts. Sequences from the *Cryptosporidium* 18S rRNA and the COWP loci were most similar to the ferret *Cryptosporidium* sp. genotype. River otters could serve as reservoirs for *Cryptosporidium* and *Giardia* species in marine ecosystems. More work is needed to better understand the zoonotic potential of the genotypes they carry as well as their implications for river otter health.

Cryptosporidium parvum and *Giardia duodenalis* are protozoans that can infect humans as well as numerous species of wildlife and domestic animals (Fayer et al., 2000; Thompson, 2000). Little is known about the epidemiology of these pathogens at the human-wildlife interface; yet, often wildlife are assumed to be transmitting pathogens to humans, especially *Giardia* species (Dykes et al., 1980; Erlandsen et al., 1988). Human recreational water quality standards are being developed and enforced for beaches in Washington and other states; yet, virtually nothing is known about the epidemiology and the potential for zoonotic transmission of *Cryptosporidium* and *Giardia* species in marine ecosystems. Oocysts of *C. parvum* can survive for at least 1 yr in salt water (Tamburrini and Pozio, 1999) and can be concentrated by filter-feeding bivalves, including mussels (Tamburrini and Pozio, 1999) and oysters (Fayer et al., 1999). Similarly, *G. duodenalis* cysts can persist in aquatic environments and are concentrated by bivalves such as mussels and clams (Graczyk et al., 2003).

Cryptosporidium and *Giardia* spp. have been identified in California sea lions (*Zalophus californianus*) (Deng et al., 2000) and ringed seals (*Phoca hispida*) (Olson et al., 1997; Fayer et al., 2004; Hughes-Hanks et al., 2005; Santin et al., 2005) as well as in North Atlantic right whales (*Eubalaena glacialis*) and bowhead whales (*Balaena mysticetus*) (Hughes-Hanks et al., 2005). *Cryptosporidium* sp. has been identified in dugong (*Dugong dugon*) (Hill et al., 1997) and *Giardia* spp. have been identified in harp seals (*P. groenlandica*), grey seals (*Halichoerus grypus*), and a harbor seal (*P. vitulina*) from eastern coastal Canada (Measures and Olson, 1999), but not from other mammals that use marine waters.

The Puget Sound Georgia Basin (PSGB) marine ecosystem is a highly productive inland sea in the transboundary region between Washington state, U.S.A., and British Columbia, Canada (48°30'N, 123°40'W). Nearly 7 million people reside along the shores of this ecosystem where humans, wildlife, and domestic animals share habitat and marine resources (Fraser et al., 2006). To determine whether *Cryptosporidium* and *Giardia* species are present in marine wildlife from this ecosystem, we examined the feces of free-ranging marine-foraging river otters (*Lontra canadensis*) for *Cryptosporidium* and *Giardia* spp. River otters are widely distributed throughout the marine waters of the PSGB where they primarily feed on numerous species of marine fish and invertebrates (Stenson et al., 1984; Jones, 2000). They have a high metabolic rate and defecate at latrine sites on docks and on shorelines (Ben-David et al., 1998), presenting the opportunity to evaluate fecal samples collected at the land-sea interface around the region. Specifically, our objectives were to (1) determine whether river otters in the region were infected with these protozoans, (2) characterize the isolates by using molecular tools, and (3) evaluate whether *Cryptosporidium* and *Giardia* spp. infections in river otters are associated with otter proximity to larger human populations or degree of adjacent shoreline modification.

In Washington state, river otter fecal samples were collected from docks and along the marine shoreline between February and June 2003. Fecal samples were identified as river otter based on size, conformation, content, and location, and were collected individually for refrigeration until testing. Only samples less than 24 hr old were collected as determined by degree of desiccation relative to weather conditions over the past few days. To take replicate samples at a spatial location, but avoid repeat sampling of the same animal, samples were collected from communal latrine sites when 3 to 5 fresh fecal samples were deposited during the last 24 hr and could be individually identified as being from different animals by gross examination of fish bones and shellfish remains in the samples. Latrine study sites were spaced at least 20 km apart by water (shoreline or open water) or 15 km apart over land. Adjacent sites that were less than 40 km apart were sampled on the same day or the following day to prevent resampling of the same animal at 2 adjacent sites. Sites were classified by their percentage of shoreline

modification as determined by the Washington state ShoreZone Inventory (<http://www.sharesalmonstrategy.org/images/maps/shoreline.jpg>) and size of the proximal resident human population by using the 2000 U.S. Census (<http://quickfacts.census.gov/>).

In British Columbia, latrines were identified from land and water. Using the same criteria for identification and freshness, fecal samples were collected along the marine shoreline of southern Vancouver Island from the city of Victoria and outlying islets to the community of Port Renfrew between June and November 2004. Individual samples were collected opportunistically from small as well as communal latrine sites and stored chilled until testing. No efforts were made to prevent resampling of the same animal.

Fresh fecal samples collected in Washington state were tested for *Cryptosporidium* sp. oocysts and *Giardia* sp. cysts by using 2 techniques, i.e., double centrifugation flotation with ZnSO₄ (specific gravity 1.2) and immunomagnetic separation followed by direct immunofluorescent antibody (IMS/DFA) detection (Pereira et al., 1999; Zajac et al., 2002). When positively labeled *Cryptosporidium* sp. oocysts or *Giardia* sp. cysts were identified by IMS/DFA, the slide contents were scraped and washed for DNA extraction and amplification. *Cryptosporidium* sp. DNA in positive samples was amplified using established primers designed specifically to amplify genomic DNA sequences for a segment of the 18S rRNA gene (Xiao et al., 1999) and a segment of the *Cryptosporidium* sp. oocyst wall protein (COWP) gene (Spano et al., 1997). The primers used on *Giardia* sp.-positive samples amplified a segment of the 18S rRNA gene (McGlade et al., 2003). Amplified products were sequenced and aligned using GeneDoc software (Nicholas et al., 1997) for comparison with GenBank reference genotypes.

Fresh fecal samples collected in British Columbia were tested only for *Cryptosporidium* sp. oocysts and *Giardia* sp. cysts by using ZnSO₄ flotation (specific gravity 1.2) and sucrose flotation (Gajadhar, 1994). Molecular characterization was not performed.

Putative risk factors that could be associated with detection of *Cryptosporidium* sp. or *Giardia* sp. in river otter feces were identified, and the data were analyzed for all Washington study sites and time points. The sample collection month was recorded, ranging from February through June 2003. The coastal human population living adjacent to each river otter fecal collection site was categorized as low (<5,000 people), medium (5–15,000 people), or high (>15,000 people), based on data from the 2000 Census (<http://quickfacts.census.gov/>). The extent of shoreline modified for human habitation in the region of each river otter fecal collection site was categorized as low (<40% modification), medium (41–60% modification), or high (>60% modification). Logistic regression methods were used to assess the strength of association between each putative risk factor and the separate outcomes of detecting species of *Cryptosporidium* or *Giardia* in river otter feces, while adjusting for repeated sampling within sites by using a cluster variable. Statistical analyses were conducted using Stata software (Stata Corporation, College Station, Texas), and significant *P* values were defined as <0.1.

In Washington state, 57 fresh river otter fecal samples were collected from 13 locations throughout the Puget Sound region (Fig. 1). *Cryptosporidium* sp. oocysts were detected in 4 samples from 4 locations (Fig. 1). *Giardia* sp. cysts were detected in 11 samples from 7 locations (Fig. 1). Fecal samples coinfecting with species of *Cryptosporidium* and *Giardia* were not detected, although animals infected with *Cryptosporidium* sp. and animals infected with *Giardia* sp. were detected concurrently at 3 locations (Seattle, Union, and Shelton; Fig. 1). Three *Cryptosporidium* sp.-positive samples were detected by IMS/DFA but not fecal flotation, and 1 positive was detected by flotation but not by IMS/DFA. Of the IMS/DFA-positive samples, 2 positive samples had fewer than 5 oocysts, and 1 sample contained >500 oocysts. *Giardia* sp. cysts were detected by IMS/DFA but not by fecal flotation in 6 samples and by flotation but not IMS/DFA in 5 samples. Cysts per IMS/DFA sample ranged from as few as 2 to as many as 203. Frank blood was noted in the 1 sample containing >200 *Giardia* sp. cysts.

Amplification of DNA from the IMS/DFA slide scrapings was successful for the sample containing >500 *Cryptosporidium* sp. oocysts but not for the other *Cryptosporidium* sp. or *Giardia* sp.-positive samples that contained fewer parasites. For both the *Cryptosporidium* sp. 18S rRNA and the COWP loci, polymerase chain reaction (PCR) products were sequenced in 2 directions and found to be most similar to the ferret, *Cryptosporidium* sp., genotype in a nBLAST search and by using

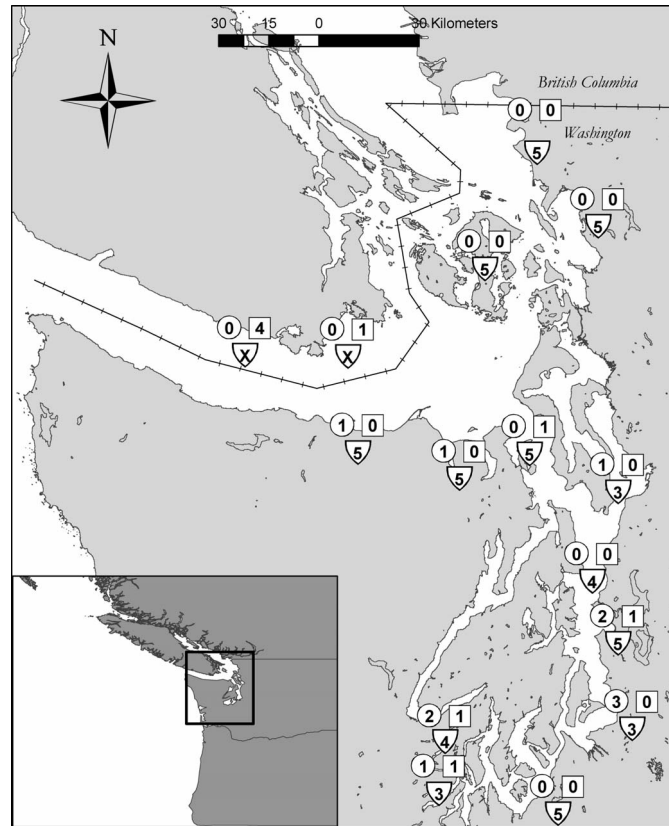


FIGURE 1. Location and number of *Cryptosporidium* sp.- (in square) and *Giardia* sp. (in circle)-positive fecal samples and the total number of river otter fecal samples collected per site (in triangle). Due to differences in sampling, the number of fecal samples collected is not given for Canadian sites. Actual locations of sample are represented by circles; squares, and triangles are off set slightly for easier reading. Inset identifies Puget Sound Georgia Basin location relative to Oregon, Washington, and British Columbia.

GeneDoc alignment software. Differences along the 816-bp 18S rRNA locus were noted when comparing the river otter sequence (GenBank DQ288166) and the ferret *Cryptosporidium* sp. genotype (AF112572). Specifically, in the region from bp 198 to bp 1017, the river otter 18S sequence differed from the ferret genotype at bp 229 and in the bp 690-700 variable region. Similarly, differences were noted between the river otter sequence (GenBank DQ288167) and ferret genotype (AF266267) along the 538-bp COWP locus. The river otter COWP locus differed from the ferret genotype with T-C conversions at bp 354 and bp 747 bp as well as a C-T conversion at bp 819.

Risk factor analysis of the variables sample collection months, adjacent human population, and extent of shoreline modification for association with detecting species of *Cryptosporidium* or *Giardia* in river otter feces were assessed using the results of all 57 fecal samples and by using a cluster variable to adjust for repeated sampling within sites. None of the putative risk factors was found to be significantly associated ($P < 0.1$) with detection of *Cryptosporidium* or *Giardia* spp. in river otter fecal samples.

In British Columbia, 36 river otter fecal samples were collected from 30 sites along the southern tip of Vancouver Island. *Cryptosporidium* sp. oocysts were detected in 5 samples from 2 locations (Fig. 1). *Giardia* sp. cysts were not detected.

Human, companion animal, and agricultural-related fecal material are discharged, dumped, or carried in runoff into marine waters all over the world (Fayer et al., 2004). In the PSGB, for example, untreated sewage effluent from an estimated 210,000 people living on the southern end of Vancouver Island is discharged from 2 marine outfalls averaging 80,000 m³/day and 50,000 m³/day in wet winter months (Hodgins et al., 1998), highlighting the need to better understand the epidemiology of *Cryptosporidium* and *Giardia* spp. in marine wildlife and their ecosystems. We identified species of *Cryptosporidium* and *Giardia* in marine-foraging river otters throughout the PSGB, highlighting for the first

time that this widely distributed species can be infected with and shed these protozoans throughout this marine ecosystem. River otters drink fresh water from streams and culverts that drain urbanized shorelines and empty into the marine waters of the PSGB. They also defecate on docks and boats and on shorelines adjacent to where people grow and harvest shellfish, which have been shown to concentrate these potential pathogens (Fayer et al., 1999; Tamburrini and Pozio, 1999; Graczyk et al., 2003). Because some *Cryptosporidium* and *Giardia* spp. have health implications and can be transmitted amongst humans and animals, it is important to consider whether human discharge could be infecting river otters or whether river otters could be contaminating shellfish beds and thereby infecting humans.

Four of 57 or 7% of river otters in the marine waters of the Puget Sound region were shedding *Cryptosporidium* sp. at the time of sampling, and 11 of 57 or 19% were shedding *Giardia* sp. In Washington, attempts were made to minimize resampling of the same individual by gross evaluation of fecal contents at each site, by selecting adjacent sites that were at least 20 km apart by water or 15 km apart over land, and by sampling adjacent sites on the same or subsequent days. Data on marine-foraging river otter home range in Washington are not available, but home range for marine-foraging river otters in Alaska varies between 10 and 40 km of shoreline, depending on the sex of the animal and site (Bowyer et al., 2003). Assuming that home range is similar in Washington state, it is highly unlikely that the same animal was sampled at adjacent sites due to the distances between selected sites and that adjacent sites that were less than 40 km apart were sampled on the same or following days. The British Columbia river otter survey showed that a *Cryptosporidium* sp. also is present in otters using similar habitats nearby in Canada. Using different laboratory methods and varying sample sizes, other work has identified species of *Giardia* and *Cryptosporidium* infection of varying prevalence in different species of marine mammals. *Giardia* sp. prevalence was estimated at 20% (Olson et al.,

1997) and 64.5% (Hughes-Hanks et al., 2005) in ringed seals, 50% in harp seals (Measures and Olson, 1999), 71.4% in right whales (Hughes-Hanks et al., 2005), and 33.3% in bowhead whales (Hughes-Hanks et al., 2005). *Cryptosporidium* sp. prevalence ranges from 5.1% in bowhead whales to 22.6% in ringed seals and 24.5% in right whales (Hughes-Hanks et al., 2005).

River otters can be found in urban and rural areas throughout the PSGB. We used human population and the degree of anthropogenic shoreline modification as putative risk factors in an effort to determine whether human proximity or development could be influencing river otter infection with species of *Cryptosporidium* or *Giardia*. We found no significant association, but this does not negate the possibility that anthropogenic factors not evaluated in this study could be involved in marine mammal infections. Using mussels (*Mytilus* sp.) and oysters (*Crassostrea* spp.) as bioindicators of marine and estuarine contamination, Miller et al. (2005) and Fayer et al. (2002) found that freshwater outflow and precipitation events were associated with increased odds for detecting *Cryptosporidium* sp. Due to the relatively large home range of river otters compared with sessile organisms and lack of information about *Giardia* and *Cryptosporidium* spp. infection and shedding duration, these environmental risk factors were not evaluated for river otters. More work is needed to characterize the *Cryptosporidium* and *Giardia* species genotypes found in river otters and other wildlife species to better evaluate anthropogenic and environmental factors before this issue can be resolved in the Pacific Northwest.

Analysis of the river otter *Cryptosporidium* sp. isolate at 2 loci revealed a novel genotype that most closely resembles the *C. parvum* ferret genotype (AF266267 and AF112572). Ferrets and river otters are closely related mustelids, and it is possible that the *Cryptosporidium* sp. identified represents a genotype unique to river otters. If this genotype is host adapted to river otters and is the genotype most often associated with marine-foraging river otters in the PSGB, then it is less likely that river otter infection is zoonotic and associated with human habitation, shoreline modification, or fecal waste discharged into marine waters. Additional biological and molecular characterization is needed to determine whether the river otter genotype can be transmitted to humans or other animals. Other *Cryptosporidium* sp. genotypes have been shown to be host adapted and only present a major health risk to immunocompromised humans outside the host range (Gajadhar, 1994; Fayer et al., 2000).

Mere detection of infectious agents does not imply their ability to cause morbidity or mortality in the host, and the significance of *Cryptosporidium* and *Giardia* spp. infection to the health of marine-foraging river otters is unknown. Although the potential exists for these organisms to cause disease in river otters, neither parasite has been previously identified as a pathogen in this species (Kimber and Kollias, 2000). Frank blood was present in the 1 sample containing >200 *Giardia* sp. cysts, but this could have been coincidental and does not prove that *Giardia* sp. causes disease in river otters. To better understand the role of *Cryptosporidium* and *Giardia* spp. in causing disease, otters from zoological collections with gastrointestinal disease should be tested for species of *Cryptosporidium* and *Giardia* by using IMS/DFA as well as ZnSO₄ flotation. Isolates should be characterized molecularly and the remission of clinical signs should follow treatment with appropriate antiprotozoal medication to confirm protozoal involvement in causing disease.

This study used the most sensitive methods currently available for detection of *Cryptosporidium* sp. oocysts and *Giardia* sp. cysts in fecal samples. Traditional methods such as fecal flotation and DFA alone have been shown to detect 1,000 or more oocysts or cysts/g feces (Xiao and Herd, 1993). Immunomagnetic separation is a concentration method that allows for analysis of a 0.5-ml fecal pellet before DFA quantitation, in contrast to the 10 µl that is analyzed when DFA is used alone. The IMS method has been shown to improve sensitivity by 1–2 log₁₀ units, from 1,000 oocysts to 10 oocysts/g feces (Pereira et al., 1999). With the increased sensitivity of IMS/DFA detection, we can expect to detect both newly infected high shedding animals as well as chronic low shedding animals. In addition, this study used a serial fecal processing protocol to directly combine IMS concentration with quantitative fluorescent microscopy, followed by DNA amplification, so that molecular characterization could be carried out without requiring a separate IMS concentration step. Amplification of samples positive by fecal flotation was not attempted because the IMS/DFA preparation was expected to

be more sensitive, specific, and efficient for providing clean DNA template for PCR analysis. It is interesting that 5 samples were positive for *Giardia* sp. cysts by fecal flotation but negative by IMS/DFA testing. The *Giardia* sp. cysts seen in the flotation slide could have been an antigenically different *Giardia* species than the *G. duodenalis* against which the IMS antibodies were designed.

Adding marine-foraging river otters to the list of marine species that can be infected with and shed *Cryptosporidium* and *Giardia* spp. provides support that these protozoan parasites are probably more common in marine waters and in marine wildlife than was previously thought. Additional work is warranted to better understand the taxonomy, epidemiology, and zoonotic potential of *Cryptosporidium* and *Giardia* spp. in river otters and other marine wildlife.

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Evidence of Experimental Postcyclic Transmission of *Bothriocephalus acheilognathi* in Bonytail Chub (*Gila elegans*)

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ABSTRACT: We examined the role that predation of infected conspecific fish and postcyclic transmission might play in the life cycle of the Asian fish tapeworm, *Bothriocephalus acheilognathi* (Cestoda: Pseudophyllidae) Yamaguti, 1934. Young-of-the-year (YOY) bonytail chub (*Gila elegans*) were exposed to copepods infected with *B. acheilognathi* and subsequently fed to subadult bonytail chub. Within 1 wk after consumption of the YOY chub, subadults were necropsied and found infected with gravid and nongravid tapeworms. This study provides evidence that postcyclic transfer of *B. acheilognathi* can occur. Postcyclic transmission may be an important life history trait of *B. acheilognathi* that merits consideration when studying the impact and distribution of this invasive and potentially pathogenic tapeworm.

Postcyclic parasite transmission, as proposed by Bozhkov (1969), occurs when an adult parasite is ingested indirectly by its definitive host's predator and subsequently survives to parasitize the predator (Odening, 1976; Nickol, 1985). The phenomenon has been demonstrated experimentally in fish for a number of acanthocephalan species (Lassiere and Crompton, 1988; Kennedy, 1999; Rauque et al., 2002; McCormick and Nickol, 2004). This mode of parasite transmission was also demonstrat-

ed through mechanical transplantation for different stages of several species of *Proteocephalus* (Willemse, 1969) and has been suggested to occur in several other species of this genus (Scholz and Hanzelova, 1998). It is also a likely route of transmission for the fish tapeworm *Bothriocephalus pearsei* (Scholz et al., 1996). Reports of postcyclic transmission are often suggestive and derive from observations of parasites infecting fish that are not likely to consume the proper intermediate host to acquire the infection.

Pseudophyllidean tapeworms utilize a copepod first intermediate host for the larval proceroid stage and generally require a fish as the second intermediate host for the plerocercoid stage. Plerocercoids typically embed and develop in muscle or visceral mass of the fish. Larger fish or, in some cases, a mammal is host to the adult stage of the worm. The life cycle of the Asian fish tapeworm (*Bothriocephalus acheilognathi* Yamaguti, 1934) (*Bothriocephalus gowkongensis* Yeh, 1955), however, does not require a second intermediate or paratenic host, and transmission to the final fish host takes place directly via ingestion of copepods infected with the proceroid stage (Körting, 1975). Postcyclic transmission has not been reported as a mode of transmission for *B. acheilognathi*, although small fish have been reported as "carriers" (Hoffman, 1999).