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Prevalence, Environmental Loading, and Molecular Characterization of *Cryptosporidium* and *Giardia* Isolates from Domestic and Wild Animals along the Central California Coast

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The risk of disease transmission from waterborne protozoa is often dependent on the origin (e.g., domestic animals versus wildlife), overall parasite load in contaminated waterways, and parasite genotype, with infections being linked to runoff or direct deposition of domestic animal and wildlife feces. Fecal samples collected from domestic animals and wildlife along the central California coast were screened to (i) compare the prevalence and associated risk factors for fecal shedding of *Cryptosporidium* and *Giardia* species parasites, (ii) evaluate the relative importance of animal host groups that contribute to pathogen loading in coastal ecosystems, and (iii) characterize zoonotic and host-specific genotypes. Overall, 6% of fecal samples tested during 2007 to 2010 were positive for *Cryptosporidium* oocysts and 15% were positive for *Giardia* cysts. Animal host group and age class were significantly associated with detection of *Cryptosporidium* and *Giardia* parasites in animal feces. Fecal loading analysis revealed that infected beef cattle potentially contribute the greatest parasite load relative to other host groups, followed by wild canids. Beef cattle, however, shed host-specific, minimally zoonotic *Cryptosporidium* and *Giardia duodenalis* genotypes, whereas wild canids shed potentially zoonotic genotypes, including *G. duodenalis* assemblages A and B. Given that the parasite genotypes detected in cattle were not zoonotic, the public health risk posed by protozoan parasite shedding in cattle feces may be lower than that posed by other animals, such as wild canids, that routinely shed zoonotic genotypes.

Waterborne transmission of *Cryptosporidium parvum* and *Giardia duodenalis* (synonymous with *G. intestinalis* and *G. lamblia*) has emerged as an important public health concern in developed and developing countries (16). In the United States alone, 10,500 cases of *Cryptosporidium* and 19,140 cases of *Giardia* infection from drinking water or recreational contact with untreated water were reported by the Centers for Disease Control and Prevention during 2008 (80). Because these environmentally resistant parasites can infect a wide range of mammalian hosts (23), there are numerous contributing host sources of oocysts and cysts in water supplies impacted by surface runoff. Previous epidemiological studies have linked human infections with exposure to domestic animal feces in agricultural runoff (35), runoff from naturally vegetated areas (74), and direct exposure to domestic animal and wildlife feces (19).

The risk of waterborne disease transmission is affected not only by origin (e.g., domestic animals versus wildlife) but also by parasite load in contaminated water sources and by parasite genotype. Numerous studies have focused on detection and enumeration of *Cryptosporidium* and *Giardia* spp. in feces of domestic animals, particularly livestock (25). There are limited reports, however, on the concentration and environmental loading of waterborne protozoan pathogens as a result of fecal contamination by wildlife (31), although these animal groups commonly utilize man-made and natural surface water sources (17).

Furthermore, many *Cryptosporidium* and *Giardia* parasites are morphologically similar, and thus light microscopy does not provide sufficient information to assess the zoonotic risk. Instead, molecular methods are required to identify parasite genotypes and the resulting public health implications due to fecal contamination from various host animals. Many *Cryptosporidium* and *Giardia* parasite lineages are highly host specific; however, zoonotic genotypes have been detected in domestic animals and wildlife (81, 12). For *Cryptosporidium*, the primary zoonotic species is *C. parvum*, whereas *C. canis*, *C. felis*, *C. meleagridis*, *C. muris*, and *C. suis* species and the *Cryptosporidium* cervine genotype appear to be nonzoonotic or weakly zoonotic (78). For *Giardia duodenalis*, assemblages A and B have the widest host ranges, infecting humans, domestic animals and livestock, terrestrial wildlife (62), and marine mammals and birds (38). Other *G. duodenalis* assemblages have more restricted host ranges, with assemblages C and D typically isolated from dogs, assemblage E associated with hoofed livestock, assemblage F infecting cats, and assemblage G infecting rats (45).

In response to these data gaps and as part of a larger fecal pathogen pollution research program, this study was designed to (i) determine the prevalence of protozoan pathogens and assess risk factors for *Cryptosporidium* and *Giardia* detection in sympatric terrestrial and marine animals; (ii) compare the various animal hosts with respect to their ability to load coastal ecosystems with pathogenic and/or zoonotic *Cryptosporidium* and *Giardia* strains; and (iii) characterize zoonotic and host-specific *Crypto-*

Received 2 August 2012 **Accepted** 2 October 2012
Published ahead of print 5 October 2012
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doi:10.1128/AEM.02422-12

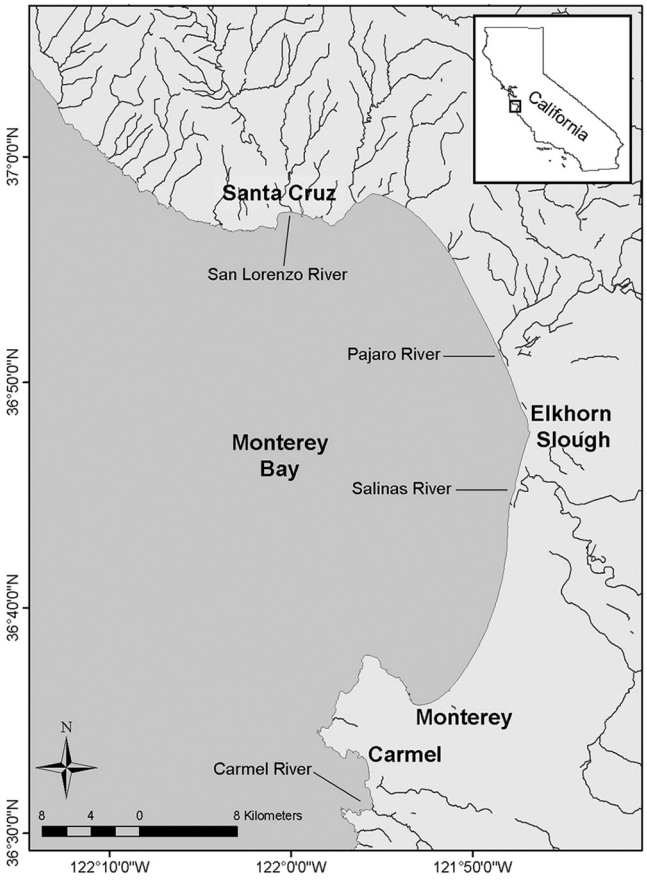

FIG 1 Map of the Monterey Bay study area showing four regional rivers impacted by mixed land use, including significant agricultural activity and interspersed urban and rural residential areas, 2007 to 2010. Map created with ArcMap 9.3 (ESRI).

sporidium and *Giardia* genotypes in sympatric terrestrial and marine animals.

MATERIALS AND METHODS

Sample collection. Fecal samples were collected from terrestrial and marine animals residing in the Monterey Bay region of California between March 2007 and March 2010 (Fig. 1). Sampled watersheds were characterized by mixed land uses, including significant agricultural activity and interspersed urban and rural residential areas. Terrestrial animals selected for fecal screening represented both urban and rural land uses, including domestic dogs (*Canis lupus familiaris*), domestic cats (*Felis silvestris catus*), beef cattle (*Bos taurus*), wild canids (coyotes [*Canis latrans*] and gray foxes [*Urocyon cinereoargenteus*]), wild felids (mountain lions [*Puma concolor*] and bobcats [*Lynx rufus*]), and Virginia opossums (*Didelphis virginiana*). Sampled marine wildlife included gulls (*Larus* spp.) and southern sea otters (*Enhydra lutris nereis*).

Feces from live domestic dogs, cats, beef cattle, gulls, and sea otters were collected from the ground within minutes to ≤6 h after defecation and in some cases off plastic sheets containing interspersed bait stations to attract the animals. Samples from live opossums were collected from the interior of live traps (Tomahawk, Hazelhurst, WI) that had been previously disinfected using 10% bleach. To minimize the risk of environmental contamination, all samples were collected from surfaces that had not contacted the substratum whenever possible. Sample freshness was ascertained by observation of defecation and/or the presence of high moisture sheen on the sample surface (17). Feces were also collected during necropsy of domestic cats, wild canids, wild felids, opossums, and sea otters (≤72-h postmortem interval at refrigerated temperatures) at the California Department of Fish and Game (CDFG) Marine Wildlife Veterinary Care and Research Center (MWVCRC). All domestic cats that were necropsied were humanely euthanized, nonadoptable animals that were obtained through cooperation with local animal shelters. All fecal samples were collected aseptically in sterile 50-ml conical vials, refrigerated, and processed at MWVCRC for protozoan identification and enumeration.

Protozoan detection. Direct immunofluorescent antibody microscopy (DFA) was used for detecting and enumerating *Cryptosporidium* oocysts and *Giardia* cysts, as previously described (54). Briefly, 5 g of each fecal sample was mixed with approximately 30 ml of 0.1% Tween 80 (ICI Americas, Inc., Wilmington, DE) and washed through a fine-mesh sieve into a 50-ml conical tube. Fecal suspensions were centrifuged at 1,000 × *g* for 10 min, the supernatant was aspirated and discarded, and the top 1 to 3 mm of the residual fecal sediment was resuspended 1:1 (vol/vol) in deionized water to a final volume of between 1 and 3 ml. A 10-μl transfer loop was used to deposit a drop of fecal material onto a treated glass slide well (Waterborne Inc., New Orleans, LA). The slide was air dried overnight, and oocysts/cysts were labeled by DFA using fluorescein isothiocyanate-labeled anti-*Cryptosporidium* or anti-*Giardia* monoclonal antibodies (A100FLK, Aqua-Glo, G/C Direct Comprehensive kit; Waterborne Inc., New Orleans, LA). Protozoa were visualized at a magnification of ×20, with identification confirmed at a magnification of ×40. Samples containing one or more 4- to 6-μm-diameter oocysts (*C. parvum*-like) or one or more 10- to 15-μm cysts (*G. duodenalis*-like) were recorded as positive, and the number of oocysts and/or cysts per slide well was determined. Samples containing *Cryptosporidium andersoni*-like oocysts (6- to 8-μm diameter) were also recorded as positive and were grouped with *C. parvum*-like samples for statistical analyses at the genus level. If no fluorescent oocysts or cysts were observed, the sample was recorded as negative. Fecal samples positive by DFA were then subjected to immunomagnetic separation (IMS) (Dynabeads; Dynal, Inc., Lake Success, NY) to concentrate *Cryptosporidium* oocysts and *Giardia* cysts, followed by DFA to obtain additional more accurate concentration data and by DNA extraction to facilitate genotyping efforts. In cases where IMS was not performed or was inconclusive (<1% of samples), the original DFA results were used for analyses.

A parasite spiking trial was conducted with adult beef cattle fecal samples to characterize the general method performance in the laboratory. For fecal sample preparation, 24 adult beef cattle samples were obtained fresh from California livestock operations. Ten of the 24 samples had 5,000 parasites of both *Cryptosporidium* and *Giardia* mixed into 1 ml of fecal sample. From the well-mixed 1-ml sample, a measured 20-μl volume was transferred to a DFA slide. All slides were read in a blinded fashion by the same microscopist. Recovery rates (%) were calculated by dividing the number of recovered oocysts or cysts per 1 ml by the expected number of oocysts or cysts per 1 ml and multiplying by 100 (75).

Prevalence and risk factor analyses. Statistical relationships were assessed between fecal shedding of *Cryptosporidium* and *Giardia* spp. and defined risk factors, such as animal group (domestic dogs, domestic cats, beef cattle, wild canids, wild felids, opossums, gulls, or sea otters), sex (male, female, or unknown), age (immature, adult, or unknown), season (dry or wet), location (Elkhorn Slough watershed, Carmel River watershed, or Monterey Bay area), collection year (2007, 2008 and 2009, or 2010), and animal status at the time of fecal sampling (live or dead). Temporal delineations for season were based on average climatic patterns for the central California coast with respect to rainfall, air and water temperature, and salinity (13). Wet-season samples were collected from November through April, and dry-season samples were obtained from May through October. Because meteorological conditions were similar during 2008 and 2009 and only one sample was collected during 2009, samples from the two years were pooled for analyses. The overall parasite prevalence was determined by dividing the number of parasite-positive fecal samples by the total number of samples collected within each risk factor

category (15). The 95% exact binomial confidence intervals (CI) around each point estimate were calculated using Stata/LC 11.1 (Stata-Corp., College Station, TX).

Simple and multiple logistic regression approaches were used to investigate associations between host-specific, demographic, and environmental risk factors with respect to test outcome (e.g., parasite positive or negative). Simple (bivariate) logistic regression evaluated associations between each risk factor (e.g., animal species, age, sex, etc.) and *Cryptosporidium* and *Giardia* detection in individual fecal samples. Risk factors that were significant at a P level of <0.1 were then incorporated in a forward-stepping manner into multiple logistic regression models. These multivariable models yielded adjusted odds ratios that simultaneously measured the strength of associations between multiple risk factors and the parasite outcome of interest. All regression analyses were adjusted for data dependence within each watershed by including a cluster variable for sampling area. Analyses were performed using Stata/LC 11.1 (Stata-Corp.).

Environmental loading. Loading estimates were calculated conservatively as the arithmetic mean of amount of feces (g) produced per animal per day multiplied by the arithmetic mean intensity of oocyst shedding for each test-positive animal multiplied by the prevalence of test-positive samples within an animal group (6). The mean feces amount (g) produced per animal per day was calculated as the mean weight of an individual scat multiplied by the mean number of defecations per day for each animal species. Mean feces estimates were based on the published literature (see Table 4). Published estimates were unavailable for gray foxes; thus, fecal output measurements for coyotes were used (6) for all wild canid calculations. As no bobcats tested positive for the parasites, fecal output measurements for mountain lions were used (1) for all wild felid calculations. The mean intensity of oocyst shedding was defined as the average number of observed oocysts and/or cysts per 19.4 mg of feces (mean weight of feces/slide well) per test-positive animal (32). The parasite prevalence of *Cryptosporidium* spp. and/or *Giardia* spp. for each tested animal species was obtained by dividing the number of parasite-positive fecal samples by the total number of samples tested in each animal group. Environmental loading estimates per individual were then compared among animal groups. Because a high number of cases were without parasites and parasite-negative samples resulted in a skewed data distribution for some animal species, comparisons were made using the zero-inflated negative binomial regression model with a 95% confidence interval using Stata/LC 11.1 (Stata-Corp.). To further examine the overall impact that specific animal groups may have had on watersheds, population-based environmental loading estimates were also generated for species with known abundance estimates in Monterey County and California by multiplying the environmental loading per animal [(oo)cysts individual^{-1} day^{-1}] by the estimated number of individuals in the population.

Molecular characterization. In preparation for PCR, DFA-positive fecal samples were subjected to IMS per the manufacturer's instructions (Dynal, Inc.). The resulting product of concentrated *Cryptosporidium* oocysts and *Giardia* cysts in 100 μl of neutralized 0.1 N HCl was collected in a 2-ml microcentrifuge tube and spun at ~8,000 × *g* for 2 min. Supernatant was carefully removed and extraction begun with the addition of 50 μl of lysis buffer ATL (Qiagen Inc., Valencia, CA) to the remaining 20- to 50-μl pellet. The sample was subjected to a single freeze-thaw cycle (4 min in liquid N_2, followed immediately by 4 min in boiling water). The freeze-thaw was followed by the addition of 130 μl of ATL and 40 μl of proteinase K. The remainder of the extraction was carried out per the manufacturer's instructions (QIAmp Mini Kit; Qiagen Inc.). Final elution was with 80 μl 10% AE buffer in PCR water heated to 95°C. DNA was stored at −80°C until PCR was performed.

Cryptosporidium genotyping was performed by targeting a broadly conserved 18S ribosomal DNA (rDNA) region using PCR in conjunction with DNA sequence analysis. *Cryptosporidium* PCR was initially performed with primers from Morgan et al. (47), producing a 298-bp product from the 18S rRNA gene that was amplified using forward primer 18SiF, 5′ AGTGACAAGAAATAACAATACAGG 3′, and reverse primer 18SiR, 5′-CCTGCTTTAAGCACTCTAATTTTC-3′. Amplification was performed in volumes of 50 μl containing 5 μl 10× PCR buffer, 6 mM $MgCl_2$, 0.2 mM dinucleoside triphosphates (dNTP), 0.2 μl 10% bovine serum albumin (BSA) (0.4 μg/μl), 200 nM each primer, 1.25 units HotStarTaq Plus DNA polymerase (Qiagen Inc.), and from 2 to 5 μl template DNA. Thermal cycling conditions were as follows: samples were heated to 95°C for 5 min followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and 1 cycle of 72°C for 7 min. All samples with PCR-positive results using primers from the Morgan et al. publication (47) were also tested with a different 18S primer set from Xiao et al. (76). These external primers were eF, 5′-TTCTAGAGCTAATACATGCG-3′, and eR, 5′-CCCATTTCCTTCGAAACAGGA-3′ (~1,325 bp) (76). All PCRs were performed in a 50-μl final volume consisting of 5 μl 10× PCR buffer, 6 mM $MgCl_2$ · 2 mM dNTP mixture, 0.2 μl 10% BSA (0.4 μg/μl), 200 nM each primer, 1.25 μl HotStarTaq Plus DNA polymerase (Qiagen Inc.), and 2 to 3 μl template DNA. Amplification was as follows: 1 cycle of 95°C for 5 min followed by 35 cycles of 9°C for 45 s, 55°C for 45 s, and 72°C for 1 min, followed by 1 cycle of 72°C for 7 min. A secondary PCR product of 826 to 864 bp was amplified using primers 5′-GGAAGGGTTGTATTTATTAGATAAAG-3′ and 5′-AAGGAGTAAGGAACAACCTCCA-3′. PCR mix and thermal cycler conditions were the same for the two PCRs, with the exception that 3 mM $MgCl_2$ was used in the second-round mixture (76). All PCR assays in this study were initially optimized for the matrix type (e.g., feces) and assay type (e.g., IMS-DFA), including inhibition testing, and then each set of PCR samples run during the study included positive controls consisting of PCR-positive *Cryptosporidium* or *Giardia* DNA and negative controls consisting of PCR water instead of DNA.

Similarly, *Giardia* isolates were characterized using a seminested PCR and DNA sequence analysis of the glutamate dehydrogenase (GDH) gene (57). A second *Giardia* locus, the beta-giardin (β-giardin) gene (11), was utilized to confirm the identity of isolates testing PCR positive with GDH primers. DNA extraction and storage were as described above. The GDH external primers were GDHeF, 5′ RCAACGTYAAYCGYGGYTTCCGT 3′, and GDHiR, 5′ GTTRTCCTTGCACATCTCC 3′. Internal primers were GDHiF, 5′ CAGTACAACTCYGCTCTCGG 3′, and GDHiR (the same primer as the one used in the first reaction), used to amplify a 432-bp region of the target gene. The first-round β-giardin primers were G7, 5′-AAGCCCGACGACCTCACCCGCAGTGC-3′, and the reverse primer G759, 5′-GAGGCCGCCCTGGATCTTCGAGACGAC-3′. Second-round primers amplified a 171-bp region of the β-giardin gene and consisted of forward primer G376, 5′-CATAACGACGCCATCGCGGCTCTCAGGAA-3′, and reverse primer G759. Gel electrophoresis was used to separate the PCR products on a 2% agarose gel that contained 0.005% ethidium bromide.

All PCR products were purified using EXOzapIT (USB Corporation, Cleveland, OH) or extracted from the gel by use of the QIAquick gel extraction kit (Qiagen Inc.) according to the manufacturer's instructions. Nucleotide sequences were analyzed using Chromas Lite 2.01 (Technelysium Pty Ltd.) and nBLAST searches of the GenBank database and aligned against reference sequences using the Clustal X2 sequence alignment program (61).

Nucleotide sequence accession numbers. Sequences determined in this study have been deposited in GenBank under accession numbers JX437075 through JX437085 and JX448621 through JX448645.

RESULTS

Pathogen detection and risk factor analyses. Fecal samples were collected and tested from 802 terrestrial and marine animals living in the Monterey Bay region of California from March 2007 through March 2010. Six percent of all tested samples were positive for *Cryptosporidium* oocysts, whereas 15% were positive for *Giardia* cysts, and 3% of the samples were positive for both parasites. The mean percent recovery and range of *Cryptosporidium* and *Giardia* parasites from the spiking trial were 4% (range, 0 to 9%) and 23% (range, 0 to 225%), respectively. Table 1 summa-

TABLE 1 Prevalence with 95% binomial confidence intervals of *Cryptosporidium* and *Giardia* pathogens isolated from terrestrial and marine animal species in the Monterey Bay region of California, 2007 to 2010[a]

Variable	*n*	*Cryptosporidium*		*Giardia*	
		% Positive	95% CI	% Positive	95% CI
Animal group					
Dogs	182	1.1	0.1–3.9	8.8	5.1–13.9
Cats	74	10.8	4.8–20.2	14.9	7.7–25.0
Beef cattle	201	6.5	3.5–10.8	34.3	27.8–41.3
Wild canids	18	22.2	6.4–47.6	38.9	17.3–64.2
Wild felids	11	0.0	0–28.5*	18.2	2.3–51.8
Opossums	68	25.0	15.3–37.0	14.7	7.3–25.4
Gulls	145	0.0	0–2.8*	2.1	0.4–5.9
Sea otters	103	1.0	0.02–5.3	1.0	0.02–5.3
Age					
Immature	182	13.7	9.1–19.6	39.0	31.9–46.5
Adult	174	7.5	4.0–12.4	12.1	7.6–17.8
Unknown	446	1.6	0.6–3.2	6.1	4.0–8.7
Sex					
Male	107	13.1	7.3–21.0	18.8	11.8–27.4
Female	148	10.1	5.8–16.2	12.8	7.9–19.3
Unknown	547	2.9	1.7–4.7	14.6	11.8–17.9
Watershed					
Elkhorn Slough	498	6.4	4.4–8.9	17.7	14.4–21.3
Carmel River	280	4.6	2.5–7.8	11.1	7.6–15.3
Monterey Bay	24	0.0	0–14.2*	0.0	0–14.2*
Season					
Dry (May–October)	453	5.7	3.8–8.3	11.7	8.9–15.0
Wet (November–April)	349	5.4	3.3–8.4	18.9	14.9–23.4
Yr					
2007	342	3.2	1.6–5.7	10.5	7.5–14.3
2008-2009	449	7.6	5.3–10.4	18.5	15.0–22.4
2010	11	0.0	0–28.5*	0.0	0–28.5*
Live/dead status					
Live	693	4.9	3.4–6.8	14.8	12.3–17.7
Dead	109	10.1	5.1–17.3	14.7	8.6–22.7

[a] Asterisks indicate one-sided, 97.5% confidence interval.

rizes the prevalence and frequency of parasite-positive fecal samples by risk factor category. All animal groups, with the exception of wild felids and gulls, tested positive for *Cryptosporidium* spp. The greatest prevalence of *Cryptosporidium* species oocyst shedding was observed in feces from opossums (25% positive) and wild canids (22% positive). For *Giardia*, all animal groups tested positive. The greatest *Giardia* species cyst prevalence was observed in wild canids (39% positive) and beef cattle (34% positive). Overall, feces from younger (immature) animals were more likely to test positive for *Cryptosporidium* spp. (14%) and *Giardia* spp. (39%) than feces from adult animals (*Cryptosporidium* spp., 7%; *Giardia* spp., 12%). Additionally, parasite prevalence was greater in feces from males (*Cryptosporidium* spp., 13%; *Giardia* spp., 19%) than females (*Cryptosporidium* spp., 10%; *Giardia* spp., 13%).

When tested individually in univariable analyses, all defined risk factors (animal group, age, gender, sample season, sample location, collection year, and live/dead status at the time of sampling) were significantly associated with *Cryptosporidium* and/or *Giardia* spp. detection in feces (Table 2). For example, domestic cats, beef cattle, wild canids, and opossums were all significantly more likely to shed both *Cryptosporidium* and *Giardia* than were domestic dogs. Wild felids also were more likely to shed *Giardia* (but not *Cryptosporidium*) than were domestic dogs. However, these trends differed for sympatric marine species; gulls were less likely to test positive for *Giardia* spp. than dogs, and no significant differences between dogs and sea otters were observed for either parasite.

When significant risk factors were simultaneously incorporated into multiple logistic regression models, detection of both *Cryptosporidium* oocysts and *Giardia* cysts was significantly associated with animal group and age class (Table 3). Both domestic and wild animals, including domestic cats, beef cattle, wild canids, and opossums, were significantly more likely to test positive for *Cryptosporidium* spp. than domestic dogs ($P < 0.001$). Wild canids were approximately four times more likely to shed *Giardia* than domestic dogs ($P = 0.027$). In contrast, no significant differences were observed for *Giardia* detection in domestic cats, beef

TABLE 2 Demographic and environmental risk factors associated with detection of *Cryptosporidium* and *Giardia* in feces from terrestrial and marine animals living near Monterey Bay, CA (univariable analysis)[a]

Variable	*n*	*Cryptosporidium* spp.		*Giardia* spp.		Both pathogens	
		Odds ratio	*P* value	Odds ratio	*P* value	Odds ratio	*P* value
Animal group							
Dogs	182	1.0		1.0		1.0	
Cats	74	10.9	<0.001*	1.8	<0.001*	15.9	<0.001*
Beef cattle	201	6.2	<0.001*	5.4	0.062*	7.5	<0.001*
Wild canids	18	25.7	<0.001*	6.6	<0.001*	51.7	0.002*
Wild felids	11	—[b]	—	2.3	0.03*	—	—
Opossums	68	30.0	<0.001*	1.7	0.088*	14.3	0.008*
Gulls	145	—	—	0.2	0.004*	—	—
Sea otters	103	0.9	0.932	0.1	0.120	1.8	0.805
Age							
Adult	174	1.0		1.0		1.0	
Immature	182	2.0	0.162	4.7	<0.001*	2.7	0.073*
Unknown	446	0.2	<0.001*	0.5	0.088*	0.2	0.004*
Sex							
Male	107	1.0		1.0		1.0	
Female	148	0.7	0.733	0.6	0.052*	0.6	0.470
Unknown	547	0.2	<0.001*	0.7	0.272	0.2	0.021*
Season							
Dry (May–October)	456	1.0		1.0		1.0	
Wet (November–April)	352	0.9	0.760	1.8	0.016*	1.4	0.385
Year							
2007	342	1.0		1.0		1.0	
2008-2009	449	2.5	<0.001*	0.5	<0.001*	3.1	0.027*
2010	11	—	—	—	—	—	—
Live/dead status							
Live	697	1.0		1.0		1.0	
Dead	111	2.2	0.018*	1.0	0.969	3.1	0.055*

[a] Asterisks indicate odds ratios significantly different from the reference category based on *P* values of ≤0.1.
[b] —, not detected.

cattle, or opossums compared with domestic dogs. Young animals were approximately twice as likely to shed *Cryptosporidium* ($P < 0.001$) and *Giardia* ($P = 0.003$) in their feces as were adults. Other important risk factors for *Cryptosporidium* and/or *Giardia* detection in feces included live/dead status and gender; the odds of *Cryptosporidium* detection were 40% lower for fecal samples collected from dead animals ($P < 0.001$), and males were 1.2 times more likely to test positive for *Giardia* spp. than were females ($P < 0.001$).

Environmental loading. Environmental loading estimates for sampled species are summarized in Table 4. The loading estimates suggest that beef cattle contribute the most to environmental loading of *Cryptosporidium* (3.0×10^7 oocysts individual^{-1} day^{-1}; regression model coefficient $b = 12.26$; $P < 0.001$) in the Monterey Bay region, followed by domestic cats (75,768 oocysts individual^{-1} day^{-1}; $b = 6.08$; $P < 0.001$), wild canids (34,666 oocysts individual^{-1} day^{-1}; $b = 5.50$; $P < 0.001$), and opossums (13,146 oocysts individual^{-1} day^{-1}; $b = 4.54$; $P < 0.001$), compared to the reference category of domestic dogs (141 oocysts individual^{-1} day^{-1}). The daily environmental contribution of oocysts from sea otters also differed significantly from that of oocysts from domestic dogs (403 oocysts individual^{-1} day^{-1}; $b = -3.58$; $P = 0.014$), although both species exhibited a low prevalence of oocyst shedding compared to the other terrestrial species.

Beef cattle also were a significant environmental contributor of *Giardia* cysts (7.6×10^6 cysts individual^{-1} day^{-1}; $b = 3.27$; $P < 0.001$), followed by wild canids (1.0×10^6 cysts individual^{-1} day^{-1}; $b = 1.23$; $P = 0.027$), compared to domestic dogs (291,075 cysts individual^{-1} day^{-1}). Domestic cats (130,447 cysts individual^{-1} day^{-1}; $b = -0.080$; $P < 0.001$), wild felids (56,119 cysts individual^{-1} day^{-1}; $b = -1.65$; $P = 0.039$), sea otters (18,157 cysts individual^{-1} day^{-1}; $b = -7.41$; $P < 0.001$), opossums (637 cysts individual^{-1} day^{-1}; $b = -6.12$; $P < 0.001$), and gulls (24 cysts individual^{-1} day^{-1}; $b = -9.42$; $P < 0.001$) shed fewer cysts than domestic dogs and did not appear to contribute significantly to environmental loading by *Giardia* in the Monterey Bay region.

To scale up from the individual animal level, population estimates were compiled (where available) at the county and state levels as seen in Table 4. Cows exhibited the greatest fecal loading contribution for both parasites, followed by domestic cats for *Cryptosporidium* and domestic dogs for *Giardia* (Table 4). Population-based contributions for California overall could be made for all animal groups except for opossums and gulls, for which

TABLE 3 Significant demographic and environmental risk factors associated with detection of *Cryptosporidium* and *Giardia* in feces from terrestrial and marine animals living near Monterey Bay, CA, 2007 to 2010 (multivariable logistic regression)[a]

Variable	*n*	*Cryptosporidium* spp.		*Giardia* spp.		Both pathogens	
		Odds ratio	*P* value	Odds ratio	*P* value	Odds ratio	*P* value
Animal group							
Dogs	182	1.0		1.0		1.0	
Cats	74	10.4	<0.001*	1.0	0.672	6.6	0.002*
Beef cattle	201	4.2	0.013*	1.7	0.384	2.6	0.076*
Wild canids	18	31.9	<0.001*	4.2	0.027*	26.3	<0.001*
Wild felids	11	—[b]	—	1.2	0.638	—	—
Opossums	68	28.7	<0.001*	1.0	0.943	5.6	<0.001*
Gulls	145	—	—	0.2	0.003*	—	—
Sea otters	103	0.9	0.959	0.1	0.089*	1.3	0.874
Age							
Adult	174	1.0		1.0		1.0	
Immature	182	2.4	<0.001*	2.1	0.001*	3.2	0.028*
Unknown	446	1.2	0.744	0.3	<0.001*	0.7	0.750
Sex							
Male	107			1.0			
Female	148			0.768	<0.001*		
Unknown	547			2.2	0.149		
Live/dead status							
Live	697	1.0					
Dead	111	0.6	<0.001*				

[a] Asterisks indicate odds ratios significantly different from the reference category based on *P* values of ≤0.1.
[b] —, not detected.

data were not available. Again, beef cattle appeared to contribute the most for both parasites, followed by domestic cats for *Cryptosporidium* and domestic dogs for *Giardia* (Table 4). Wild canids, although fewer than domestic dogs and cats, also contributed significantly to the environmental loading of both parasites.

Molecular characterization. Table 4 summarizes the genotyping results for 11 *Cryptosporidium* and 26 *Giardia* isolates from domestic animals and terrestrial wildlife. All *Cryptosporidium* genotypes isolated from domestic animals were host specific. *Cryptosporidium* isolates from domestic cats were identical to the feline-origin *C. felis* reference sequence (GenBank AF108862) from bp 457 to 782, whereas isolates from beef cattle were identical to the livestock origin *C. andersoni* reference sequence (GenBank AF093496) from bp 511 to 977. All positive and negative PCR controls produced acceptable quality control results during this study.

Most *Giardia* genotypes obtained from domestic animals also were host specific. Analysis of *Giardia* isolates from domestic dogs indicated that 43% were assemblage C and 57% were assemblage D. Two assemblage C isolates (Dog252 and Dog566) were identical to each other and to the canine reference genotype C sequence (GenBank U60985) from bp 73 to 485, whereas one assemblage C isolate (Dog179) differed from these strains by a single base pair (nucleotide substitution of C to G at bp 159). Assemblage D isolates from domestic dogs shared 99% homology with each other and the canine reference genotype D sequence (GenBank U60986) from bp 76 to 489. Isolates Dog431 and Dog575 were identical and differed from the reference sequence by two base pairs (C to T at bp 192 and T to C at bp 241). Isolates Dog569 and Dog572 were identical and differed from the reference sequence by one base pair (T to C at bp 241).

Sequence analysis of domestic cat *Giardia* isolates indicated that 80% were assemblage B and 20% were assemblage E. Assemblage B isolates from domestic cats (Cat676, Cat880, and Cat901) were identical to each other and to the human reference genotype B sequence (GenBank L40508) from bp 76 to 485. However, isolate Cat820 differed by one base pair (T to C at bp 183).

Sequence analysis of *Giardia* isolated from beef cattle revealed that all were comprised of assemblage E. All beef cattle isolates shared 99% homology with each other and the reference genotype E sequence (GenBank AY178740) from bp 59 to 482. Isolates Cattle236 and Cattle240 differed by one base pair (C to T at 447 and A to G at bp 382, respectively). Isolate Cattle032 differed by two base pairs (A to G at bp 382 and C to T at bp 447).

Some terrestrial wildlife species like opossums exhibited only host-specific *Cryptosporidium* genotypes, whereas wild canids and wild felids exhibited both host-specific and potentially zoonotic *Giardia* genotypes. All five *Cryptosporidium* isolates from opossums were identical to each other and the opossum genotype II reference sequence (GenBank AY120906) from bp 235 to 587. At bp 588, however, the five isolates from this study differed by one nucleotide substitution from the reference sequence (C to A). Analyses of *Giardia* isolates for wild canids revealed genotypes comprised of assemblages A (33%), B (33%), and D (33%). One assemblage A isolate from a gray fox (Fox922) was identical to the human reference sequence (GenBank L40509) from bp 76 to 484, and another canid isolate (Fox888) was identical to the bobcat reference sequence (GenBank GQ426099) from bp 12 to 420. An assemblage B isolate from a gray fox (Fox887) was identical to the human reference genotype B sequence (GenBank L40508) from bp 76 to 485, whereas a second gray fox isolate (Fox890) differed by only one base pair (T to C at bp 183). Wild canid genotype D

TABLE 4 Mean intensity of *Cryptosporidium* and *Giardia* (oo)cyst shedding, estimated environmental loading, and parasite genotypes detected for terrestrial and marine animals living near Monterey Bay, CA[a]

Pathogen and animal group	Mean intensity of shedding per test-positive animal [no. of (oo)cysts g of feces^{-1}]	Mean amt of feces produced individual^{-1} day^{-1} (g)[b]	Environmental loading per animal [no. of (oo)cysts individual^{-1} day^{-1}]	Animal abundance estimates for Monterey County[c]	Environmental loading per Monterey County population [no. of (oo)cysts population^{-1} day^{-1}]	Animal abundance estimates for California[d]	Environmental loading per California population [no. of (oo)cysts population^{-1} day^{-1}]	Genotype(s) detected (*n*)
Cryptosporidium spp.								
Dogs	0.6	249.0	141.2	79,597.9	1.1E + 07	7,948,978.7	1.1E + 09	NA
Cats	1,884.8	40.2	75,768.3	89,799.5	6.8E + 09	8,967,756.1	6.8E + 11	*C. felis* (2)
Beef cattle	1,870.7	16,000.0	3.0E + 07	27,784.0	8.3E + 11	620,000.0	1.9E + 13	*C. andersoni* (4)
Wild canids (coyotes)	337.6	200.0	67,515.5	NA	NA	500,000.0	3.4E + 10	NA
Wild felids (mountain lions)	0.0	203.0	0.0	NA	NA	5,000.00	0.0	NA
Opossums	798.2	17.5	13,967.9	NA	NA	NA	NA	Opossum genotype II (5)
Gulls	0.0	22.2	0.0	NA	NA	NA	NA	NA
Sea otters	1.0	403.5	403.5	NA	NA	2,711.0	1.1E + 06	NA
Giardia spp.								
Dogs	1,169.0	249.0	291,075.9	79,597.9	2.3E + 10	7,948,978.7	2.3E + 12	C (3), D (4)
Cats	3,245.0	40.2	130,447.7	89,799.5	1.2E + 10	8,967,756.1	1.2E + 12	B (4), E (1)
Beef cattle	483.9	16,000.0	7.7E + 06	27,784.0	2.2E + 11	620,000.0	4.8E + 12	E (7)
Wild canids (coyote)	9,759.7	200.0	2.0E + 06	NA	NA	500,000.0	9.8E + 11	A (2), B (2), D (2)
Wild felids (mountain lions)	276.4	203.0	56,119.0	NA	NA	5,000.00	2.8E + 08	E (1)
Opossums	36.4	17.5	636.9	NA	NA	NA	NA	NA
Gulls	1.1	22.2	23.7	NA	NA	NA	NA	NA
Sea otters	45.0	403.5	18,157.5	NA	NA	2,711.0	4.9E + 07	NA

[a] NA, no estimate or too few oocysts or cysts for PCR analysis.
[b] Mean amount of feces day^{-1} (g) is based on wet weight measurements reported in the literature for domestic dogs (22), domestic cats (18), beef cattle (2), wild canids (6), wild felids (1), opossums (55), gulls (28), and sea otters (21; S. C. Oates, unpublished data).
[c] Based on Monterey County abundance estimates for domestic dogs and cats (3, 67) and beef cattle (69).
[d] Based on California abundance estimates for domestic dogs and cats (3, 67), beef cattle (70), coyotes (68), mountain lions (14), and sea otters (71).

Giardia sequences (Coyote639 and Fox897) shared 99% homology with each other and the canine reference genotype D sequence (GenBank U60986) from bp 76 to 489. Isolate Coyote639 from a coyote differed from the reference sequence by two base pairs (C to T at bp 87 and T to C at bp 241). Isolate Fox897 from a gray fox was identical to domestic dog isolates Dog431 and Dog575. One domestic cat isolate and one wild felid (mountain lion) isolate were positive for *Giardia* assemblage E. Both assemblage E felid isolates were identical to each other and the reference sequence (GenBank AY178740) and were confirmed using β-giardin gene sequence analysis. Parasite DNA sequence information was not obtained from other host groups due to low parasite numbers.

DISCUSSION

This study has provided important insights into the epidemiology and environmental loading of *Cryptosporidium* and *Giardia* for a variety of coast-dwelling species, especially domestic animals and terrestrial wildlife. Animal group-specific environmental loading estimates for host-adapted and zoonotic protozoan strains of *Cryptosporidium* and *Giardia* illustrate the variability of public health risk associated with environmental contamination by differing fecal sources. Collectively, our findings will help guide stakeholder groups charged with managing animal health, public health, and protection of natural resources by integrating diverse pieces of information (e.g., species, age, population abundance and distribution, intensity of cyst and oocyst shedding, and zoonotic potential of protozoan genotype) into a valid watershed management risk assessment. In addition, these techniques may help identify routes of pathogen transfer between humans, domestic animals, and wildlife populations and help to unravel nonpoint sources of protozoan contamination.

The prevalences of *Cryptosporidium* in this study fell within previously reported ranges for most animals. *Cryptosporidium* prevalences of 1.1 to 7.1% have been reported in range beef cattle (4, 5, 33), and prevalences of 27% were noted for coyotes (66). Similarly, we observed a prevalence of 6.5% in beef cattle, and a comparable prevalence (22%) was observed in wild canids, which included both coyotes and gray foxes. *Cryptosporidium* was not detected in the wild felids and gulls tested during our study. These low prevalences were consistent with previous studies in which low *Cryptosporidium* prevalences were reported for bobcats (7%) (82) and gulls (0 to 5%) (46, 60). In contrast, *Cryptosporidium* prevalences of 11% were reported in opossums (24), whereas during our study, detection was greatest in opossums (25%).

Prevalences of *Giardia* in this study also fell within previously reported ranges for most animals. For example, wild canids, including both coyotes and gray foxes, during our study exhibited a similar prevalence (39%) to that of coyotes (32%) as previously reported by Trout et al. (66). Moreover, a prevalence of 4% was noted for gulls (27), and a comparable prevalence (2%) was observed in gulls during the present study. However, prior studies of *Giardia* spp. shedding by adult beef cattle on pasture reported prevalences of 0 to 11% (10, 23, 52), whereas during the present study, prevalences in beef cattle were 34%. The greater prevalence observed in this study than in previous studies could reflect differences in livestock susceptibility due to variations in management practices, environmental exposure, or herd age structure within the cattle population. All cattle that were sampled during this study were maintained under free-range conditions, with the majority of samples collected from 1- to 2-year-olds and adults. However, some calves (≤6 months) were sampled, which may have increased prevalence. Young cattle generally have greater rates of infection; Ralston et al. (56) demonstrated that the cumulative *Giardia* infection rate may reach 100% in range-fed beef calves.

The feces from one sea otter tested positive for both *Cryptosporidium* and *Giardia*. Although *Cryptosporidium* and *Giardia* have been identified in a variety of marine species (34), this is the first report of a sea otter testing positive for either of these parasites. The *Cryptosporidium*- and *Giardia*-infected individual was an adult female sea otter (1021-06) whose home range spanned the south and west side of the Monterey peninsula, including an area of intensive use near the outflow of the Carmel River. Individual variation in prey selection is a notable feature of sea otters in central California, with individual otters specializing in a limited subset of the diverse suite of prey species consumed by the population as a whole (64), and this particular female specialized primarily in large crabs of the genus *Cancer* and abalone, with a few other species including urchins, mussels, and sea stars comprising <5% of the diet. Although only one individual was found to be shedding *Cryptosporidium* or *Giardia* parasites during our study, this may be due to the sporadic nature of fecal parasite shedding; sea otters may be at a significant risk of exposure because of several unique aspects of their biology, including a preference to feed in nearshore habitat, often close to freshwater inputs, and consumption of 25 to 30% of their body weight per day in benthic invertebrates (41). Previous studies have demonstrated the ability of shellfish to filter and/or bioconcentrate *Cryptosporidium* and *Giardia* (43, 44) and have linked protozoan parasites including *Toxoplasma gondii* (40) and *Sarcocystis neurona* (42) to sea otter deaths along the central California coast.

Overall, animal group was an important risk factor for enhanced shedding of *Cryptosporidium* and *Giardia*. For example, wild canids were 27 times more likely to test positive for *Cryptosporidium*, 6 times more likely to test positive for *Giardia*, and 24 times more likely to test positive for both pathogens than were domestic dogs. Only 1% and 9% of domestic dogs tested during this study tested positive for *Cryptosporidium* and *Giardia*, respectively. These findings are similar to those of previous studies of domestic dogs that reported ranges from 0 to 20% for *Cryptosporidium* (48) and from 3 to 36% for *Giardia* (9).

Across all species tested, feces from immature animals were 3 times more likely to test positive for *Cryptosporidium* and *Giardia* and 4 times more likely to test positive for both parasites than samples originating from adults. These results are similar to those from other studies that have observed greater prevalence of protozoan parasites in younger animals (51, 73). Similar to Oliveira-Sequeira et al. (50), sex also was a significant risk factor for *Giardia* detection, with significantly more males testing positive than females. Differences in fecal shedding of *Cryptosporidium* and *Giardia* among species, age classes, and sexes may reflect variation in food sources, habitat use, and immune system function. For example, the greater prevalence of protozoan parasites in younger animals has been linked to the lack of specific immune functions that are required to combat many gastrointestinal and systemic infections (8, 65).

Environmental loading rates for *Cryptosporidium* and *Giardia* spp. were compared for a range of terrestrial and marine animals residing in the Monterey Bay area in order to evaluate their relative contribution to pathogen loading of coastal ecosystems. In our study, fecal loading estimates for beef cattle were slightly

greater than previous estimates and appeared to substantially contribute to environmental burdens of *Cryptosporidium* and *Giardia* in the Monterey Bay area. Previous loading estimates for *Cryptosporidium* in beef cattle varied from 3,900 oocysts cow^{-1} day^{-1} (5) to 2.3×10^5 oocysts cow^{-1} day^{-1} (32). The high prevalence and intensity of *Cryptosporidium* oocyst shedding in the present study compared to those in prior reports suggest that variations exist in the medical ecology (e.g., survival, transmission, and infectivity) of *Cryptosporidium* genotypes that infect cattle in different geographical regions (5).

Although the percent recovery from our DFA parasite spiking trial was lower than that reported in some previous studies (54, 75), variation across assays is not expected to fully explain the differences in host patterns when the same assay type is used in multiple studies. Cattle feces were selected for the DFA spiking trial as a conservative estimate for parasite recovery because they typically contain more debris that can interfere with parasite detection than feces from other animal groups. The percent recovery of *Giardia* cysts was greater than the percent recovery for *Cryptosporidium* oocysts and may have been due to background interference, uneven distribution of parasites, or test cross-reactivity. It should be noted that the limit of detection for DFA is 1 to 2 log higher than when IMS is used to concentrate parasites from the fecal samples prior to DFA screening (54), and so studies that use DFA alone may report false-negative results for parasite infection from animals that were shedding numbers below the limit of detection. As with the spiking experiment, we initially screened all fecal samples with DFA. To obtain more accurate concentration data and facilitate genotyping efforts, fecal samples positive by DFA were then subjected to IMS to concentrate *Cryptosporidium* oocysts and *Giardia* cysts, and thus our recovery rates may actually have been greater than indicated by the DFA spiking trial.

Few environmental loading estimates exist for *Cryptosporidium* or *Giardia* shedding by species other than livestock. Two studies have examined environmental loading of *Cryptosporidium* by California ground squirrels (*Spermophilus beecheyi*) (6, 7), striped skunks (*Mephitis mephitis*), yellow-bellied marmots (*Marmota flaviventris*), and coyotes (6). Similar to the study by Atwill et al. (6), where environmental loading of *Cryptosporidium* for coyotes ranged between 35,000 oocysts $individual^{-1}$ day^{-1} and 41,000 oocysts $individual^{-1}$ day^{-1}, our study documented approximately 67,000 oocysts $individual^{-1}$ day^{-1} for wild canids. Wild canids were also a significant source of *Giardia* deposition (2×10^6 cysts $individual^{-1}$ day^{-1}) compared to domestic dogs in our study (291,075.9 cysts $individual^{-1}$ day^{-1}).

Population-based environmental loading estimates differed from estimates based on individuals, demonstrating the need not only to identify the primary environmental sources of *Cryptosporidium* and *Giardia* but also to understand how mammalian populations infected with these protozoans distribute themselves on a watershed (6). For example, 8 million domestic dogs (3, 67) versus 250,000 to 750,000 coyotes (68) are estimated to reside in California. Domestic dogs may shed fewer *Giardia* cysts than wild canids, but when population estimates are incorporated, they are a much greater contributor to environmental loading than their wild counterparts. Unfortunately, very few abundance and distribution estimates for terrestrial animals in California exist, and loading estimates will improve as the population estimates become more accurate. For example, to our knowledge no estimates for gray foxes or opossums have been reported in the literature. The Virginia opossum is a nonnative species introduced to California by humans during the early 20th century (29), and population estimates for California are unknown. Fecal inputs from opossums to the coastal environment may be high, exposing native wildlife such as sea otters to potentially deadly pathogens (49, 58).

Most of the animals that were sampled during this study were shedding host-adapted (nonzoonotic) strains of *Cryptosporidium* and *Giardia*. Host-adapted genotypes are not typically considered a major public health risk because the vast majority of them have not been associated with human infection (77). However, some species or genotypes that preferentially infect one group of animals can opportunistically infect other animals or humans. To date, seven "host-specific" *Cryptosporidium* species (*C. hominis*, *C. meleagridis*, *C. andersoni*, *C. suis*, *C. muris*, *C. canis*, and *C. felis*) have confirmed zoonotic potential (39, 53, 78, 79). During this study, we identified *C. felis* from domestic cats and *C. andersoni* from beef cattle. Although the risk is probably minimal, domestic cats and beef cattle could serve as potential reservoirs for environmental contamination by zoonotic *Cryptosporidium* spp. in the Monterey Bay area.

During this study, domestic cats and gray foxes were shedding potentially zoonotic *Giardia* genotypes, including assemblages A and B. Prior reports have underscored the importance of cats as hosts of assemblages A, B, D, and F and have suggested significant human health risks (36, 59, 62, 63, 72). Genotyping information suggests that *Giardia* A and B assemblages harbored by red foxes (30) and gray foxes (66) are capable of infecting other animals and humans. Similarly, several genotypes obtained from sympatric domestic cats and gray foxes during the present study were identical, suggesting interspecific parasite transmission.

Here we report the first occurrence of *Giardia* assemblage E infection in felids (a domestic cat and a mountain lion). Assemblage E *Giardia* is typically reported from hoofed livestock such as beef and dairy cattle, goats, camels, and pigs (20). Recently, infection by assemblage E *Giardia* was reported in a human in Egypt (26) and a nonhuman primate in Africa (37); these areas exhibit considerable overlap between grazing cattle and humans and between cattle and primates, respectively. The samples from domestic cats and wild felids from this study were collected in rural areas where livestock, domestic animals, and wildlife species overlap. In the Monterey Bay area, mountain lions frequently prey upon domestic livestock, including cattle, goats, and sheep, and upon wild pigs. The sequences from both felids were identical to assemblage E genotypes isolated from beef cattle, suggesting that cattle could serve as potential reservoirs of *Giardia* for other domestic animals and wildlife.

This study demonstrates that broad application of environmental loading assessment and molecular genotyping techniques to a wide range of domestic and wild animals is necessary to facilitate effective watershed management and assessment of public health risks. Based on previous environmental loading estimates, much regulatory attention is being placed on the role that livestock play in contaminating watersheds with protozoan parasites such as *C. parvum* (6). During this study, fecal loading analysis revealed that infected beef cattle potentially contribute the greatest *Cryptosporidium* and *Giardia* loads relative to other host groups. Beef cattle, however, shed host-specific, minimally zoonotic *Cryptosporidium* and *G. duodenalis* genotypes, whereas wild canids shed potentially zoonotic genotypes, including *G. duodenalis* assemblages A and B. Thus, on an animal-by-animal basis,

beef cattle actually may pose less of a threat to surface water quality than wild canids. To ultimately protect water quality and minimize waterborne transmission of these parasites, it is necessary to focus on the roles that wildlife and domestic animals play in loading watersheds with these potentially pathogenic protozoa.

ACKNOWLEDGMENTS

This work was performed under the auspices of the Central Coast Long-term Environmental Assessment Network (CCLEAN), with funding provided by Grant 06-076-553 from the California State Water Board to the City of Watsonville.

Performance of this research was approved by the CDFG Office of Spill Prevention and Response and by the Institutional Animal Care and Use Committee at the University of California, Davis. Sea otter tagging and monitoring was permitted by a U.S. FWS Endangered Species Permit issued to MTT and by the Institutional Animal Care and Use Committee at the University of California, Santa Cruz.

We thank MWVCRC and UCD staff and volunteers, the Central Coast Regional Quality Control Board, Elkhorn Slough Foundation, California State Parks, Moss Landing Marine Laboratories, Monterey County Regional Park District, Monterey County SPCA, Monterey Bay Aquarium, USFWS, U.S. Geological Survey, Monterey County Animal Services, CDFG Wardens and Trappers, California State University Monterey Bay, City of Carmel, Moss Landing Harbor District, Moss Landing KOA, the Marine Mammal Center, and the U.S. Department of Agriculture—Forest Service for participating in this study.

We have no competing interests to declare and have all read and approved the final manuscript. Any use of trade, product, or firm names in this publication is for descriptive purposes only and does not imply endorsement by the U.S. Government.

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