

NOVEL AND CANINE GENOTYPES OF *GIARDIA DUODENALIS* IN HARBOR SEALS (*PHOCA VITULINA RICHARDSI*)

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ABSTRACT: Feces of harbor seals (*Phoca vitulina richardsi*) and hybrid Glaucous-winged/Western gulls (*Larus glaucescens/occidentalis*) from Washington State's inland marine waters were examined for *Giardia* spp. and *Cryptosporidium* spp. to determine whether genotypes carried by these wildlife species were the same as those that commonly infect humans and domestic animals. Using immunomagnetic separation followed by direct fluorescent antibody detection, *Giardia* spp. cysts were detected in 42% (41/97) of seal fecal samples. *Giardia* spp.–positive samples came from 90% (9/10) of the sites, and the prevalence of positive seal fecal samples differed significantly among study sites. Fecal samples collected from seal haulout sites with >400 animals were 4.7 times more likely to have *Giardia* spp. cysts than were samples collected at smaller haulout sites. In gulls, a single *Giardia* sp. cyst was detected in 4% (3/78) of fecal samples. *Cryptosporidium* spp. oocysts were not detected in any of the seals or gulls tested. Sequence analysis of a 398 base pair (bp) segment of *Giardia duodenalis* DNA at the glutamate dehydrogenase (GDH) locus suggested that 11 isolates originating from seals throughout the region were a novel genotype and 3 isolates obtained from a single site in south Puget Sound were the *G. duodenalis* canine genotype D. Real-time TaqMan polymerase chain reaction (PCR) amplification and subsequent sequencing of a 52 bp small subunit ribosomal DNA region from novel harbor seal genotype isolates showed sequence homology to canine genotypes C and D. Sequence analysis of the 52 bp small subunit ribosomal DNA products from the 3 canine genotype isolates from seals produced mixed sequences that could not be evaluated.

Species of *Giardia* and *Cryptosporidium*, protozoans once thought to be primarily associated with freshwater and terrestrial hosts, have been identified in a variety of wildlife species, including marine-foraging river otters (*Lontra canadensis*; Gaydos et al., 2007), California sea lions (*Zalophus californianus*; Deng et al., 2000), ringed seals (*Phoca hispida*; Olson et al., 1997; Fayer et al., 2004; Hughes-Hanks et al., 2005; Santin et al., 2005), north Atlantic right whales (*Eubalaena glacialis*), and bowhead whales (*Balaena mysticetus*) (Hughes-Hanks et al., 2005). Additionally, *Giardia* spp. have been identified in harp seals (*Phoca groenlandica*), grey seals (*Halichoerus grypus*), and 1 harbor seal (*Phoca vitulina*) from eastern coastal Canada (Measures and Olson, 1999), and *Cryptosporidium* spp. oocysts were identified in dugong (*Dugong dugon*; Hill et al., 1997). Very little has been done, however, to characterize the genotypes of *Giardia* spp. and *Cryptosporidium* spp. infecting marine wildlife.

Understanding the molecular epidemiology of these protozoans in marine wildlife is important for evaluating human health risks that could be associated with marine ecosystems. Oocysts of *Cryptosporidium parvum* can survive for at least a year 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* (synonymous with *G. lamblia* and *G. intestinalis*) cysts can persist in marine environments and are concentrated by bivalves such as mussels and clams (Graczyk et al., 2003). Washington State is the nation's largest producer of farmed shellfish (U.S. Department of Agriculture, 2006) and, depending on the species and genotypes carried by wildlife,

contamination of shellfish-growing areas by marine wildlife could present a threat to human health.

The molecular epidemiology of *Giardia* spp. and *Cryptosporidium* spp. also can provide insights as to whether marine wildlife species are being infected by protozoans of human or domestic animal origin. Human, companion animal, and agriculture-related fecal material is discharged, dumped, or carried in runoff into marine waters all over the world (Fayer et al., 2004). For example, in the inland marine waters shared by Washington State (United States) and British Columbia (Canada), untreated sewage effluent from an estimated 210,000 people living on the south end of Vancouver Island (Canada) is discharged from 2 marine outfalls, averaging 80,000 m³/day and 50,000 m³/day (Hodgins et al., 1998). Human and domestic animal feces from untreated sewage, failing septic systems, and storm water runoff have the potential to infect and impact marine wildlife species.

The shared inland marine waters of Washington and British Columbia (N48°30', W123°40') is a highly productive marine ecosystem. Nearly 7 million people reside on the shoreline of this ecosystem where humans, wildlife, and domestic animals share habitat and marine resources (Fraser et al., 2006). Harbor seals (*P. vitulina richardsi*) and hybrid Glaucous-winged/Western gulls (*L. glaucescens/occidentalis*) are some of the most common marine mammal and marine bird species found throughout this region (Angell and Balcomb, 1982; Jeffries et al., 2003). We tested seal and gull fecal samples from Washington's inland marine waters to determine whether they were infected with *Giardia* spp. and *Cryptosporidium* spp. and to molecularly characterize isolates from these common wildlife species.

MATERIALS AND METHODS

Between March and August 2005, fresh harbor seal fecal samples were collected from 10 seal haulout sites widely distributed throughout the Puget Sound region (Fig. 1). Fecal samples were individually collected and identified as harbor seal based on size, conformation, content, and location. Only samples less than 24-hr old were collected, as determined by location on haulout site, tidal heights over the last 24 hr,

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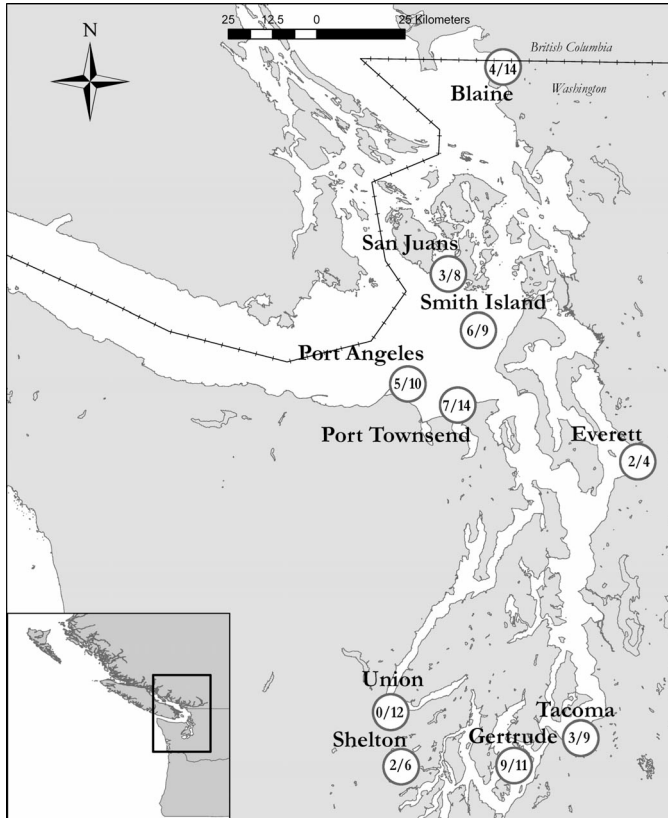


FIGURE 1. Locations of sampled harbor seal haulout sites throughout the Puget Sound region (circles) and the respective number of samples positive for *G. duodenalis* cysts over the number of fecal samples tested. The circles are 10-km diameter, representing likely harbor seal foraging distance from haulout.

and degree of desiccation relative to weather conditions. As many individual samples as could be found were collected from each site and refrigerated until tested. Gull samples were collected between February and May 2005. Using a net launcher, hybrid Glaucous-winged/Western gulls were captured at 10 sites proximal to seal haulout sites (Fig. 1). Because bird capture depended on the presence of adequate birds and a site suitable for detonation of a projectile net, distance between seal haulout and proximal gull capture site ranged from 1 to 10 km, with 1 site (Everett) 30 km from the seal haulout. Upon capture, gulls were placed in a clean cardboard holding box until they defecated, at which time the sample was collected and refrigerated.

Fresh fecal samples from harbor seals and gulls were screened for *Giardia* spp. and *Cryptosporidium* spp. using immunomagnetic separation, followed by direct fluorescent antibody (IMS-DFA) detection, with slight modification of the Pereira et al. (1999) protocols. Briefly, 5-g fecal samples were mixed with 50 ml 0.02% Tween 80, sieved through a tea strainer, centrifuged for 10 min at 1000 g, and centrifuged for 6 min at 700 g; the supernatant was then poured off to leave a 1:1 pellet/liquid ratio. Samples were resuspended, and those containing sand were allowed to settle for 2 min before aliquoting 1 ml of the suspension for IMS (DynaL Inc., Lake Success, New York) concentration per the manufacturer's instructions, including 2 acid wash steps. Each acid wash IMS product was dried onto a Merifluor slide well for at least 24 hr before DFA staining. Merifluor DFA staining involved incubating the samples with anti-*Giardia* and anti-*Cryptosporidium* spp. antibodies, as well as counterstain, for 30 min before enumerating apple green outlined cysts (10 × 14 μm) and oocysts (5 μm) using an Axioscop epifluorescent microscope (Karl Zeiss, New York, New York).

Positively labeled *Giardia* spp. cysts or *Cryptosporidium* spp. oocysts were scraped off the slidewell and washed for DNA extraction and amplification as described (Miller et al., 2005). *Giardia* sp. DNA from

IMS-DFA-positive samples was amplified using established primers for the GDH gene that was previously shown to amplify a variety of *G. duodenalis* genotypes (Read et al., 2004). Additionally, a new real-time TaqMan PCR system was designed by the Lucy Whittier Molecular and Diagnostic Core Facility (Davis, California) to target the small subunit ribosomal DNA (SSU rDNA) bp 270–320 region. The *Cryptosporidium* spp. primers for IMS-DFA-positive samples targeted the 18S rRNA gene (Morgan et al., 1997). Amplified products were sequenced and aligned using GeneDoc software (Nicholas et al., 1997) for comparison with GenBank reference genotypes.

Risk factors relating to sampling season and study site were evaluated for their association with detection of fecal protozoa in marine wildlife. Sample collection season was defined as spring (March and April), summer (June and July), and late summer (August). Human population abundance in the county associated with each harbor seal haulout site was categorized as low (up to 4,003 people), medium low (6,048–8,789 people), medium high (18,516–91,488 people), or high (>91,488 people), based on data from the 2000 U.S. Census (<http://quickfacts.census.gov/>). The extent of shoreline modified for human habitation for the county in which harbor seal haulouts occurred was categorized as low (<80% modification) or high (>80% modification) as determined by the Washington State ShoreZone Inventory (<http://www.sharesalmonstrategy.org/images/maps/shoreline.jpg>). Harbor seal haulout size was categorized as small (≤400 animals) or large (>400 animals), based on routine monitoring of haulout sites. Univariate statistics and the 1-sided Pearson's chi-square test of independence were used to assess correlation among all dependent and independent variables. Logistic regression techniques were used to assess the strength of association between putative risk factors and the outcome of detecting *G. duodenalis* in harbor seal feces, while adjusting for repeated sampling within sites by using robust standard errors clustered on sampling location (STATA SE 9.2, StataCorp LP, College Station, Texas). Variables were entered into the model through forward selection (likelihood ratio test, $P < 0.05$) and overall model fit was evaluated by the Hosmer-Lemeshow goodness-of-fit test. Odds ratios and 95% confidence intervals are reported.

RESULTS

Giardia spp. cysts were detected in 42% ($n = 41$) of the 97 harbor seal fecal samples tested, and positive samples originated from 90% (9/10) of the sites sampled (Fig. 1). The number of cysts detected per fecal sample ranged from 1 to >500, with a mean of 60 cysts per sample. Of the 78 gull fecal samples collected, 1 sample from Port Angeles, Port Townsend, and Gertrude Island each contained 1 *Giardia* sp. cyst. *Cryptosporidium* spp. oocysts were not detected in any seal or gull samples.

Sequence analysis of *G. duodenalis* DNA from the IMS-DFA slide scrapings provided molecular data to enhance our understanding of protozoal epidemiology in coastal wildlife populations. Two different *G. duodenalis* genotypes were detected. A novel *G. duodenalis* sequence (HS-1) was detected in 11 seal fecal samples collected at 5 different study sites and time points (Blaine [$n = 3$], Everett [$n = 2$], Gertrude Island [$n = 3$], Port Angeles [$n = 1$], and Port Townsend [$n = 2$]; Fig. 1). Figure 2 shows the harbor seal HS-1 sequence (GenBank DQ676483) differences along the 398 bp GDH gene segment compared to *G. duodenalis* genotype B (L40508), genotype C (U60985), genotype E (AY178740), and genotype D (U60986), with 87%–89% homology. More specifically, in the region from bp 76 to 473, the harbor seal GDH sequence differed consistently from the genotype B reference sequence at 49 individual bp locations. Using a new real-time TaqMan PCR SSU rDNA assay, *Giardia* sp. detection was confirmed in all GDH-positive samples and also in 2 GDH-negative samples that had been positive by IMS-DFA. Sequence analysis of the 52 bp SSU rDNA prod-

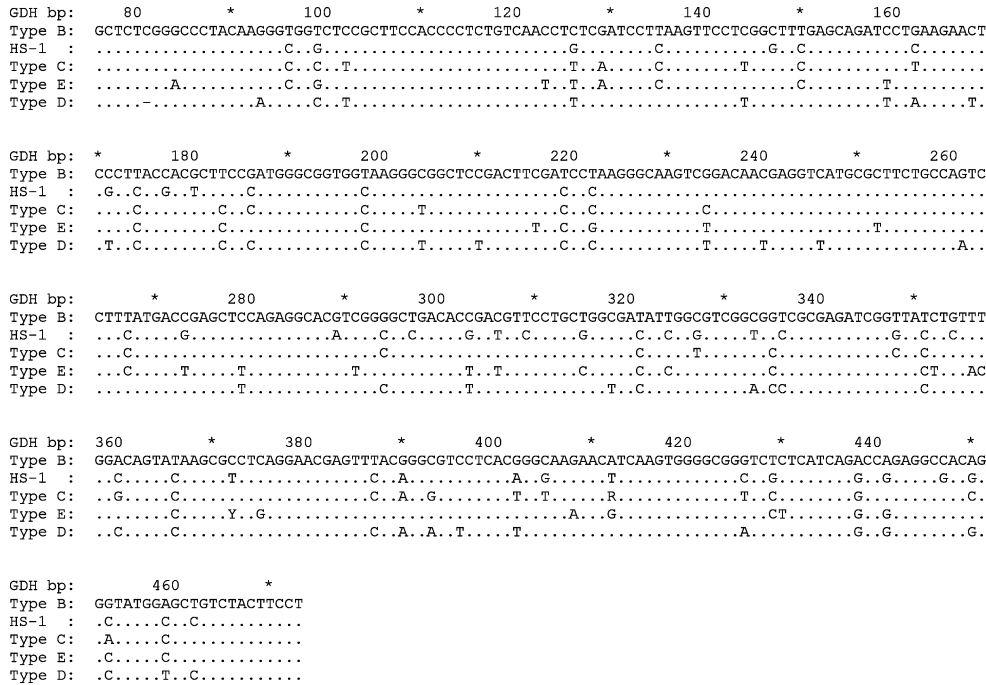


FIGURE 2. Sequence alignment of the novel harbor seal *G. duodenalis* GDH sequence (HS-1) compared to GenBank reference sequences for *G. duodenalis* genotypes B, C, D, and E. Genotype abbreviated as Type.

ucts revealed no difference between the seal HS-1 genotype and the canine genotypes C and D at the SSU rDNA locus (GenBank EU622507).

Additionally, GDH sequence analysis revealed 3 *G. duodenalis* canine genotype D isolates (GenBank DQ676482) that were detected from 1 study site in March 2005 (Gertrude Island; Fig. 1). All of these isolates were identical to the canine reference genotype D sequence (U60986) along the 386 bp region from bp 94–480 of the GDH gene. At the SSU rDNA locus (GenBank EU622507), however, sequencing results were mixed, preventing further genotype characterization.

The prevalence of *Giardia* sp.-positive seal fecal samples differed significantly among study sites (Pearson’s chi-square = 20.4, $P = 0.02$). Gertrude Island (82%) and Smith Island (67%) had the highest proportions of positive samples, while *Giardia* spp. cysts were not detected in any of the 12 samples collected at the Hood Canal site (Fig. 1). The prevalence of infection among samples at large haulout sites (61%) was higher than the prevalence at small haulout sites (26%) (Pearson’s chi-square = 12.0, $P = 0.001$). *Giardia* spp. prevalence was 58% at sites with more extensive shoreline modification, compared to 37% at sites with less shoreline modification (Pearson’s chi-square = 3.37, $P = 0.055$), but prevalence did not differ significantly among sites with different human population abundance. Prevalence also did not differ among the 3 seasons (spring, summer, and late summer) sampled in 2005.

A logistic regression model was fit for the risk factors (haulout size and shoreline modification) to assess the magnitude of their association with *Giardia* spp. cyst presence in fecal samples after adjusting for repeated sampling within the 10 haulout sites. Both shoreline modification and seal haulout size were significantly related to *Giardia* spp. cyst presence in the multivariate logistic regression model. Fecal samples collected from

large seal haulout sites (with >400 seals) were 4.7 times more likely to contain *Giardia* spp. cysts than were samples collected at small haulout sites (asymptotic OR 95% CI 2.1–10.5). Furthermore, for fecal samples collected at sites with similar haulout size, those collected at sites within counties where >80% of the shoreline had been anthropogenically modified were 2.7 times more likely to contain *Giardia* spp. cysts than were samples collected at sites with less shoreline modification (asymptotic OR 95% CI 1.3–5.4). The logistic model with both shoreline modification and seal haulout size demonstrated good overall fit (Hosmer-Lemeshow chi-square = 0.26, $P = 0.88$), suggesting that these risk factors explain most of the variation in *Giardia* spp. prevalence among seals sampled for this study.

DISCUSSION

Of the 6 recognized species of *Giardia*, *G. duodenalis* is the only one that infects humans, as well as wild and domestic animals (Wielenga and Thompson, 2007). Isolates of *G. duodenalis* are subdivided into genotypes that are based on host species, morphology, and molecular differences (Thompson et al., 2000). In this study, identification of cysts in 42% of fecal samples tested, with positives originating from 90% of sites sampled, suggests that harbor seal infection with *G. duodenalis* is common and widespread throughout the inland waters of Washington. The novel *G. duodenalis* genotype (HS-1) identified by molecular characterization of the GDH locus was the most common and geographically widespread genotype detected. It is interesting that while numerous bp differences were observed at the GDH locus between the HS-1 genotype and the reference genotypes, at the SSU rDNA locus, the HS-1 isolates were identical to the canine genotypes C and D. It is possible that the HS-1 genotype evolved from the canine genotype and

the 52 bp segment of the SSU rDNA amplified and sequenced was not variable enough to detect differences, as has been suggested by Wielenga and Thompson (2007).

When compared to the wide distribution of the HS-1 genotype in seals, the identification of 3 canine *G. duodenalis* genotype D isolates in seals from a single site (Gertrude Island, Pierce County, Washington) where 3 HS-1 genotypes also were identified suggests that seal infection with the canine genotype D likely represents an isolated or unique infection of seals with canine *G. duodenalis* genotype D. In contrast to the clean sequences obtained at the GDH locus, the SSU rDNA locus consistently provided mixed sequencing results that prevented further genotype characterization in the 3 canine *G. duodenalis* genotype D isolates from seals. This problem has been reported elsewhere (Wielenga and Thompson, 2007). The increased copy number of SSU rDNA as compared to GDH allows for increased sensitivity of detection using the SSU rDNA sequence, but also makes it more prone to mixed sequence results. The increased sensitivity of the SSU rDNA system is also shown in our results, as 2 of the IMS-DFA-positive samples that were negative by GDH PCR were positive by SSU rDNA PCR.

Both coyotes (*Canis latrans*) and domestic dogs have been identified as hosts for *G. duodenalis* genotype D (Monis et al., 1998; Trout et al., 2006), and both occur on Gertrude Island or neighboring McNeil Island, which are connected by a sand spit at low tide. Also, both animal species live on the mainland surrounding these islands, although data are not available for dog or coyote density in this region. Based on U.S. Federal Census (<http://quickfacts.census.gov/>), 700,820 people lived in Pierce County in 2000, at a density of 1,080 people per km², and it is likely that domestic dogs greatly outnumber coyotes. Regardless, while most seals in the Puget Sound area were infected with a novel *G. duodenalis* genotype, domestic dogs or coyotes defecating on or near the beach were likely responsible for infecting harbor seals with the canine *G. duodenalis* genotype, which is considered to have a limited host range (Hopkins et al., 1997; Thompson et al., 2000). Mixed canine (dogs and coyotes) and domestic dogs were the fourth and fifth highest source contributors in a microbial source tracking study of 27 sources that was conducted after high fecal coliform levels closed shellfish-growing areas in nearby Henderson Inlet, and one of the remediation recommendations was to institute educational campaigns to advocate proper disposal of domestic dog waste by owners (Samadpour et al., 2002). Supporting evidence suggests that canine feces deposited on or near the marine shoreline could be responsible for infecting harbor seals that haulout on Gertrude Island with a canine genotype of *G. duodenalis*.

Risk analysis for infection with *G. duodenalis* revealed that haulouts with >400 seals were much more likely to have positive samples than were haulouts with fewer seals. The haulout sites surveyed in this study likely represent independent sample groups, as seals tend to use the same haulout site repeatedly and the distance between sampled haulout sites is greater than harbor seal foraging distance. With use of radio transmitters, a study from this region demonstrated that haulout site fidelity was >75% and that individual harbor seals typically used haulout sites that were <5.6 km from foraging areas (Suryan and Harvey, 1998). For contagious pathogens with fecal-oral transmission, such as *G. duodenalis*, higher prevalence of infection

at locations with more individuals is expected because the probability of contacting an infected individual increases with animal density.

Shoreline modification was also related to the presence of *G. duodenalis*, as samples collected at haulout sites from counties where >80% of the shoreline was anthropogenically modified were more likely to contain *G. duodenalis* cysts than were samples collected at sites with less shoreline modification. The prevalence of *G. duodenalis* in samples found near heavily modified shoreline was 58%, compared to 37% in samples found near less-modified shorelines. If molecular evidence had suggested that all *G. duodenalis* that infected harbor seals in this region came from domestic animals, such as dogs, the association between shoreline modification and infection in seals could be explained by increased seal and domestic animal contact in sites with greater anthropogenic presence. This was not the case, however, as most seals in the inland waters of Washington were infected with the HS-1 genotype. This statistical association should be interpreted cautiously, as the processes that might promote *G. duodenalis* infection in seals in areas with extensively modified shoreline are unclear. While anthropogenic environmental changes could increase parasite transmission to humans (Patz et al., 2000), more work directed at testing this hypothesis in high-risk areas is necessary to understand the biological mechanisms underlying this apparent association.

The relatively few *Giardia* spp. cysts found in gulls (3 samples with 1 cyst each) could be because infection is rare in hybrid Glaucous-winged/Western gulls from this region. Alternatively, it is possible that gulls are infected with a species of *Giardia* that did not cross-react with the cyst wall antigen on the IMS kit and so was not detected. Similarly, our inability to detect *Cryptosporidium* spp. oocysts in any of the gull or seal samples tested could reflect a true absence of infection in the population or inadequate specificity or sensitivity of the IMS-DFA techniques to detect different *Cryptosporidium* spp. in this host, since the antibodies were originally developed against *C. parvum* oocysts. However, in our experience, the IMS and DFA protocols have been used successfully to detect oocysts from *C. felis*, *C. andersoni*, and *C. serpentis*, all non-*C. parvum* spp. It has also been our experience that IMS-DFA is a more sensitive oocyst detection technique compared with PCR (Miller et al., 2005, 2006), hence, the reason for our approach of screening and enumerating parasites with IMS-DFA, followed by slide scraping and gDNA sequence analysis for genotype characterization.

Giardia duodenalis infection prevalence of 42% in harbor seals from the Puget Sound region is the first report of *G. duodenalis* infection in harbor seals from the eastern Pacific Ocean and is within the range of what has been found in other pinniped species. Using different laboratory methods and smaller sample sizes, other work has estimated *Giardia* spp. prevalence to be 20% (Olson et al., 1997) and 64.5% (Hughes-Hanks et al., 2005) in ringed seals, and 50% (Measures and Olson, 1999) in harp seals. Despite the high prevalence of infection, it is not known whether infection with either the *G. duodenalis* HS-1 novel genotype or the canine D genotype causes enteric disease in harbor seals. Additionally, more work is needed to determine whether harbor seals from other regions are infected with this

novel *G. duodenalis* genotype, zoonotic genotypes known to infect humans, or terrestrial host genotypes.

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