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Authors: A.J. Appelbee, R.C.A. Thompson, L.M. Measures, M.E. Olson

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Giardia and *Cryptosporidium* in harp and hooded seals from the Gulf of St. Lawrence, Canada.

A.J. Appelbee[a], R.C.A. Thompson*[a], L.M. Measures[b] and M.E. Olson[c]

[a] World Health Organization Collaborating Centre for the Molecular Epidemiology of Parasitic Infections, School of Veterinary and Biomedical Sciences, Murdoch University, Murdoch, WA 6150, Australia

[b]Fisheries and Oceans Canada,, Maurice Lamontagne Institute, P.O. Box 1000, Mont-Joli, Québec, Canada

[c]Bow Valley Research Inc, Calgary, Alberta, Canada

* Corresponding author. Tel.: +61-8-9360-2466; Fax: +61-8-9310-4144. E-mail address: a.thompson@murdoch.edu.au (Andrew Thompson)

Abstract

Giardia and *Cryptosporidium* are protozoan parasites known to cause enteric disease in terrestrial wildlife species (mammals, reptiles and birds). Few surveys for *Giardia* and *Cryptosporidium* in marine wildlife species, such as pinnipeds, have been reported. The objective of this study was to determine the prevalence and genotype of *Giardia* and *Cryptosporidium* in two species of pinnipeds, harp seal *(Phoca groenlandica)* and hooded seal (*Cystophora cristata*), from the Gulf of St. Lawrence, Canada. Faecal samples were collected from pup and adult seals and examined for the presence of cysts of *Giardia* and oocysts of *Cryptosporidium* using microscopy and immunofluorescent staining. Tissues from the small intestine of adult seals were also collected and examined for infections using the

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polymerase chain reaction (PCR) technique. *Giardia* cysts were found in the faeces of 42% (16/38) of adult harp seals, but in none of the harp seal pups (0/20). Although *Giardia* cysts were not detected in faeces of adult hooded seals (0/10) using microscopy, 80% tested positive for Giardia using PCR of intestinal tissue indicative of a true replicating infection. Both harp and hooded seals harbored infections with the zoonotic strain, *Giardia duodenalis* Assemblage A, as determined using a nested PCR technique to amplify a small subunit ribosomal (SSU-rRNA) gene of *Giardia*. *Cryptosporidium* was not detected by microscopy, nor using the PCR technique on intestinal tissues from any of the 68 seals examined.

Keywords: *Giardia*, *Cryptosporidium*, seals, pinniped, zoonosis.

Introduction

Inadequate treatment and disposal of sewage, other effluents and terrestrial runoff into the marine environment from municipal, industrial, agricultural and shipping activities have resulted in contamination of the marine environment and, in some cases, have resulted in direct infection of some marine animals with various pathogens including parasites such as *Giardia*, *Cryptosporidium* and *Toxoplasma gondii* (See Fayer et al., 2004; Appelbee et al., 2005; Dixon et al., 2008, for reviews).

The Gulf of St. Lawrence in Atlantic Canada is an ideal area to study *Giardia* and *Cryptosporidium* in the marine environment as many species of marine mammals frequent the Gulf and *Giardia* and *Cryptosporidium* have been detected in the St. Lawrence ecosystem which includes the St. Lawrence River, the St. Lawrence Estuary and Gulf of St. Lawrence (Measures and Olson, 1999; Payment et al., 2000; Payment et al., 2001; Graczyk et al.,

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2001). Both parasites have a direct life cycle, producing environmentally resistant infective stages that initiate infection following ingection.

Measures and Olson (1999) observed cysts of *Giardia* in the rectal contents of adult harp seals from the Gulf of St. Lawrence, with a prevalence of 50% (15/30). Oocysts of *Cryptosporidium* were not detected in the same samples from that study, which included faeces from harp (N=47), grey (N=19) and harbour (N=8) seals, St. Lawrence beluga, *Delphinapterus leucas*, (N=11) and one bottlenose whale (*Hyperoodon ampullatus*) from the Gulf of St. Lawrence and St. Lawrence Estuary (Measures and Olson, unpublished data, see Measures and Olson, 1999 for host details). It is unknown whether seals in the St. Lawrence ecosystem are parasitized with *Giardia* that are replicating in seals, or whether seals are pseudo-parasitized, i.e. ingesting cysts from the environment and passing them through the intestine without excystation and replication.

Not only is the infection status of these marine mammals unclear, the species and genotypes of parasites that may be present in this population are unknown. Measures and Olson (1999) used microscopy with immunofluorescent staining and morphological comparison to identify the cysts as *G. duodenalis*. No molecular characterization was performed to confirm this observation nor to determine whether the strain of *G. duodenalis* was a zoonotic strain (Assemblage A or B) or a host-adapted strain, such as those identified in dogs, cats and livestock. Molecular characterization is essential in identifying the parasite in infections, as well as aiding in the elucidation of possible sources of contamination and routes of transmission.

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The objective of this study is to establish if *Giardia* cysts found in the faeces of harp and hooded seals indicates parasitic infection. To this end, a study was conducted to confirm parasitic infection with *Giardia* and *Cryptosporidium* and to determine the prevalence of these parasites in harp and hooded seals. To determine whether *Giardia* was undergoing excystation and replication in the intestine of harp and hooded seals, histological sections of the small intestine were analysed using light microscopy in order to detect trophozoites.

Materials and Methods

Harp (N=58) and hooded seals (N=10) were live captured or shot under a scientific permit issued by Fisheries and Oceans Canada and sampled during the winter of 2001 from breeding ice floes located west of the Magdalen Islands (47^{o} 23'N, 61^{o} 52'W) in the Gulf of St. Lawrence, Québec. Data from animals were stratified by species, sex and age class (adult, pup). All adults were sexually mature based on their presence on the breeding patch and all females had nursing pups (i.e. mother-pup pairs). Fresh faecal samples (1-5 g) were collected directly from the rectum of live-captured seals, placed in phosphate buffered saline (PBS) and stored at 4°C until analysed. Faeces were not collected from hooded seal pups. In addition to faeces, tissue from the small intestine (duodenum, jejunum and ileum) of dead harp (38) and hooded (10) seals was collected from all adult seals for histology and PCR analysis. Approximately 2 cm sections of small intestine were excised and fixed in 10% buffered formalin for histological analysis, or PBS and stored at -20°C for PCR.

Faecal samples were purified by centrifugation over a 1M sucrose cushion, then examined for the presence of *Giardia* cysts and *Cryptosporidium* oocysts utilizing fluorescein labelled monoclonal antibodies and microscopic examination as described previously (Olson et al.,

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1997a), with the exception that Aqua-Glo™ G/C Direct (Waterborne, Inc., New Orleans) was used enabling the simultaneous detection of *Giardia* and *Cryptosporidium*.

To determine the species and genotype of *Giardia* cysts detected in the sucrose-purified faecal samples, genomic DNA was isolated following a slightly modified protocol using cetyltrimethylammoniumbromide (CTAB) (Appelbee *et al*., 2003) prior to PCR analysis as described below.

Genomic DNA was also isolated from the jejunum, duodenum and ileum from all seals that were negative for *Giardia* or *Cryptosporidium* by microscopic examination of faeces (Table 1). A piece of small intestine (approximately 5 cm long) was opened longitudinally then vigorously vortexed in PBS for 1 minute before large pieces of tissue were removed with sterile tweezers. The remaining solution was then centrifuged at 900x*g* for 10 minutes at 4°C, the supernatant removed and the pellet re-suspended in approximately 1 mL of tissue lysis buffer (50 mM Tris pH 8.0, 500 mM NaCl, 1% SDS). Genomic DNA was extracted from a 500 µL aliquot of this suspension using the CTAB method described previously (Appelbee *et al*., 2003).

A two-step nested-PCR technique was utilized to amplify a 292 bp fragment of the small subunit ribosomal (SSU-rRNA) gene of *Giardia* (Appelbee et al., 2003) or a 448 bp fragment of the 70 Kda heat shock protein (HSP70) of *Cryptosporidium* (Morgan et al., 2001). To eliminate the possibility of PCR inhibition, duplicate PCR reactions were run for each sample at each locus, one mixture containing the test DNA and a second mixture containing the test DNA spiked with *Giardia* or *Cryptosporidium* DNA.

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To demonstrate parasitic infection in animals shown to be positive for *Giardia* by examination of faeces or PCR analysis of tissues from the small intestine, histological examination of tissues was conducted. Following dehydration in a graded series of ethanol, tissues from the small intestine were infiltrated and embedded using the JB-4 Embedding Kit® according to the manufacturer's instructions (Polysciences, Inc., Germany). Sections of approximately 1.5 μm thick were cut, stained with Lee's methylene blue and trophozoites of *Giardia* were observed at 400 X magnification and photographed.

RESULTS

The prevalence of *Giardia* and *Cryptosporidium* infections in harp and hooded seals was determined through microscopic analysis of faeces stained with fluorescein labelled monoclonal antibodies (Table 1). Of the sixty-eight faecal samples analysed, *Giardia* cysts were present in 39% (14/36) of adult female harp seals and two adult male harp seals, and cysts in all positive samples were identified as *G. duodenalis* Assemblage A by the described PCR technique. *Giardia* cysts were not detected in any of the faecal samples collected from harp seal pups (0/20) or adult hooded seals (0/10). *Cryptosporidium* oocysts were not observed in any faecal samples examined by microscopy.

As no *Giardia* cysts were found in faeces from the ten adult hooded seals examined, the PCR technique was used on their intestinal tissues, 38 from harp seals and 10 from hooded seals. Amplicons of *Giardia* were obtained from at least one of the intestinal sections from 80% (8/10) of the hooded seals. All PCR products were genetically sequenced and were identical (100%) to *G. duodenalis* Assemblage A (accession number AF199446). Similarly, harp and hooded seal intestinal tissues were examined for *Cryptosporidium* using the PCR technique. No amplification products of *Cryptosporidium* were observed in any of these samples. All

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148 faecal and intestinal samples spiked with positive control *Giardia* and *Cryptosporidium* DNA
149 were amplified at each locus indicating that PCR inhibition was not involved in negative
150 results for *Giardia* or *Cryptosporidium* using the PCR technique.
151
152 Replicating forms (trophozoites) of *Giardia* were observed in one or more histologic sections
153 of the duodenum, jejunum and ileum (Fig. 1) from each adult harp seal with *Giardia* cysts in
154 the faeces (n = 16) detected using microscopy and from hooded seals identified as infected
155 with *Giardia* using the PCR technique (n = 8). This confirms that *Giardia* excysted and
156 replicated in the small intestine of infected seals.
157
158 **DISCUSSION**
159 *Giardia* has been detected in six phocid species and one otariid in North America with
160 reported prevalences of up to 65% (Olson et al., 1997b; Measures and Olson, 1999; Deng et
161 al., 2000; Hughes-Hanks et al., 2005; Dixon et al., 2008; Gaydos et al., 2008). In the present
162 study, the prevalence of *Giardia* in adult harp seals, 42% (14/36 female, 2/2 male), is slightly
163 less than the previously reported prevalence of 50% (15/30) using the same method of faecal
164 analysis (Measures and Olson, 1999). Mammals infected with Giardia shed cysts
165 intermittently (O'Handley *et al*., 1999; Noordeen *et al*., 2001; Noordeen *et al*., 2002; Ralston
166 *et al*., 2003). As a consequence prevalence may be underestimated, especially if only one, or
167 a small faecal sample is analysed
168
169 In this and previous studies, Measures and Olson (1999) seal pups appear to be free of
170 *Giardia* infection as determined by microscopic examination of faecal specimens. These
171 animals may have been shedding cysts below the detection limit of the method, or pups may
172 have had sub-clinical infections. It is likely however, that seal pups are too young to develop

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clinical giardiasis. At the time of sampling, most pups were between one and twelve days old and still suckling.

Giardia cysts were not detected in faecal specimens from adult hooded seals, but using the PCR technique on tissue from the small intestine, 80% (8/10) of adult hooded seals were infected with *G. duodenalis*. Parasitic infection was confirmed by histological observation of trophozoites in intestinal tissues. Faecal samples from these animals may have been negative as a result of intermittent cyst shedding, or cyst shedding below the detection limit of the technique used in this study. Alternatively, infections may have been sub-clinical. A greater number of adult hooded seals would need to be sampled to determine if these differences seen between harp and hooded seals were due to the detection method or perhaps a difference in host-response to infection with *Giardia.*

The greater sensitivity of the PCR technique compared to faecal examination by microscopy has been reported by others (Erlandsen *et al*., 1990). The findings presented here illustrate the underestimation of prevalence of *Giardia* in the two phocid species examined in this study as well as that reported elsewhere in which only microscopic analysis of faecal samples was conducted. As shown in the present study and by others (McGlade et al., 2003; Amar et al., 2004) the PCR technique can also be a valuable diagnostic tool in the detection of infections with intermittent shedding or low numbers of cysts or oocysts in faecal samples. This variation in assay sensitivity was observed in these studies on marine mammals in the St. Lawrence. The prevalence of *Giardia* in the free-living adult harp and hooded seals was 42% and 0% respectively, based upon microscopic examination of the faeces. Analysis of small intestine mucosal scrapings by the more sensitive PCR method, however, showed that 80%

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198 of those adult hooded seals were in fact positive for *Giardia*. This difference in assay
199 sensitivity is supported by a recent blinded trial which showed *Cryptosporidium* and *Giardia*
200 spp. were detected 22 times more often by PCR than by conventional microscopic
201 examination of human faecal specimens (Amar *et al*., 2004). A similar study in cats also
202 showed PCR to be a more sensitive detection method. In this study, forty faecal samples
203 negative by microscopy were re-examined by PCR revealing 80% were, in fact, positive for
204 *Giardia* and 10% positive for *Cryptosporidium* (McGlade *et al*., 2003). These results
205 highlight how useful the application of PCR is as a diagnostic tool for the detection of
206 intermittent or low levels of parasites in faecal samples. PCR is also provides genotypic
207 analysis that affords insight into possible sources of transmission and contamination through
208 the monitoring of parasite genotypic variants in a geographic region.
209
210 Although *Giardia* cysts were reported from the faeces of marine mammals (Olson et al.,
211 1997b; Measures and Olson, 1999; Deng et al., 2000; Hughes-Hanks et al., 2005; Santin et
212 al., 2005), parasitic infection was not conclusively demonstrated. Unequivocal evidence of
213 parasitic infection would be the demonstration of replicating trophozoites on the mucosal
214 surface of the small intestine. Histological examination of the small intestine from adult harp
215 seals shedding cysts in the faeces, and that from adult hooded seals that were not apparently
216 shedding cysts demonstrate, for the first time, that these phocids can harbour parasitic
217 infections of *Giardia*, and that they were not simply passing ingested cysts without
218 undergoing excystation and replication (i.e. pseudoparasitism).
219
220 Histological examination of the mucosal surface of the small intestine can show both a true
221 infection, and when combined with PCR analysis, provides a highly sensitive diagnosis of
222 *Giardia* and *Cryptosporidium* infection. Since cysts and oocysts are often intermittently shed

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in the faeces of infected terrestrial mammals (Wolfe, 1992; Xiao and Herd, 1994; Fayer et al., 1998; O'Handley et al., 1999; Ralston et al., 2003), parasite prevalence is likely underestimated if data are based solely on microscopic examination of faecal samples.

Genetic analysis of the *Giardia* isolated from harp and hooded seals revealed that these phocid species harbour a single genotype of *Giardia* homologous to *Giardia duodenalis* Assemblage A. Assemblage A is thought to be of the greatest zoonotic risk, capable of infecting a wide variety of terrestrial animals including humans, livestock, domestic animals and wildlife (Thompson, 2004). Genetic characterization can provide insight into identification of possible sources of contamination and determine modes of transmission. The discovery and identification of this genotype in pelagic phocids supports the hypothesis that an anthropogenic source of infection may be contaminating the marine environment; either from insufficiently treated human sewage or agricultural runoff. Although the finding of similar genotypes in the marine and terrestrial environment is important, it is not conclusive evidence that zoonotic transmission is occurring between terrestrial and marine hosts.

High prevalence observed in all adult seals in the present study suggests that these marine mammals may have chronic giardiasis. High prevalence may also be a function of the season during which samples were collected. Many mammals have a periparturient rise in cyst shedding linked to birth and lactation (Xiao and Herd, 1994; Xiao et al., 1994; Castro-Hermida et al., 2005). This may also be occurring with harp and hooded seals, which were sampled during their breeding season that occurs in early to late March, respectively. Adult seals with short lactation periods (on average 12 days for harp seals and four days for hooded seals) expend a great deal of energy during lactation, with harp seals losing more than one quarter of their body weight by the time the pup is weaned (Lavigne and Kovacs, 1988). The

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hormonal, immunological, and physiological changes associated with pregnancy, parturition, lactation and the weight loss during lactation may cause a rise in cyst shedding thus accounting for the high prevalence in these animals.

Despite the high prevalence of *Giardia* in adult seals and the resulting contamination of ice floes with faeces containing infective cysts, pups do not appear to be infected with *Giardia*. Adult seals defecate on the ice and in the surrounding sea water and were observed with faecal material on their fur, particularly on the ventrum, likely acquired as they slide along the ice. The fur near the two abdominal mammary teats is often stained with faecal material and this may be a source of infection for nursing pups. Our negative results from examination of the faeces of harp seal pups is likely related to subclinical levels of infection, or protective immunity afforded by maternal antibodies acquired by nursing pups.

Oocysts of *Cryptosporidium* have been observed in California sea lion, dugong, bow-head whale, North Atlantic right whale and ringed seals, however, only isolates from the dugong and ringed seals have been genetically characterized at well recognised informative loci (Hill *et al*., 1997; Deng *et al*., 2000; Morgan *et al*., 2000; Hughes-Hanks *et al*., 2005; Santin *et al*., 2005). Analyses of these two isolates showed that infection with the terrestrially associated species, *C. hominis* and *C. muris*, as well as two novel seal-specific genotypes of *Cryptosporidium* were possible. These findings indicate the importance of genetically characterizing isolates found in the marine environment in order to identify the source of possible pathogen pollution from human activities. The apparent absence of *Cryptosporidium* in seals in the present work may require further study as microscopic detection of the intracellular protozoan and DNA isolation from the very stable oocysts can be difficult. In addition it would be useful to determine if seals are susceptible to infection with terrestrially

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273 derived strains of *Cryptosporidium* and *Giardia*. These findings highlight the importance of
274 genetically characterising isolates detected in the marine environment to aid in determining
275 the importance of pathogen pollution through human activities as a potential source of
276 infection.
277
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Table 1

Prevalence of *Giardia* and *Cryptosporidium* based on microscopic examination of faecal specimens

	Number Samples	*Cryptosporidium* Positive	*Giardia* Positive
Harp adult female	36	0	14 (38.8 %)
Harp adult male	2	0	2 (100 %)
Harp Pup male and female	20	0	0
Hooded adult female	5	0	0
Hooded adult male	5	0	0
Hooded pup male and female	0	0	0

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Figure 1. Representative photomicrographs of histologic sections of the small intestine of adult harp and hooded seals showing trophozoites of *Giardia*. Photograph A shows three trophozoites adhering to epithelial cells (100x). Photograph B shows a trophozoite at higher magnification (400x).

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