Genetic Characterization of Cryptosporidium Isolates From Ringed Seals (Phoca hispida) in Northern Quebec, Canada

Mónica Santín, Brent R. Dixon*, and Ronald Fayer†,

Environmental Microbial Safety Laboratory, Animal and Natural Resources Institute Agricultural Research Service, United States Department of Agriculture, Building 173, BARC-East, 10300 Baltimore Avenue, Beltsville, Maryland 20705; *Bureau of Microbial Hazards, Food Directorate, Health Canada, 4th floor West, Banting Research Centre, Tunney’s Pasture, Ottawa, Ontario, Canada K1A 0L2; †To whom correspondence should be addressed. e-mail: rfayer@anri.barc.usda.gov

ABSTRACT: This study reports the molecular characterization of Cryptosporidium spp. isolates identified from intestinal contents of ringed seals (Phoca hispida) from Nunavik (Quebec, Canada). Cryptosporidium spp. fragments of 18S rRNA, HSP-70, and actin loci were amplified by PCR from seal intestinal contents. PCR-positive specimens were sequenced and compared with other Cryptosporidium species and genotypes reported previously. Sequence analysis showed the presence of C. muris and 2 novel genotypes in ringed seals.

Cryptosporidium sp. is a protozoan parasite that has been identified as a causative agent of enteric disease in humans and other animals. Many mammalian species have been recognized as reservoirs of Cryptosporidium spp. (Fayer et al., 2000; Xiao et al., 2004). Using morphological criteria, host specificity, and DNA based studies, 13 species of Cryptosporidium have been recognized (Monis and Thompson, 2003; Xiao et al., 2004). Molecular studies have identified additional host-adapted genotypes of Cryptosporidium sp. (i.e., goose genotypes, deer genotype, duck genotype, pig genotypes I and II, skunk genotype, ferret genotype, muskrat genotypes I and II, C. canis fox genotype, and bovine B genotype) (Morgan et al., 1999; Fayer et al., 2000; Xiao et al., 2002, 2004; Enemark et al., 2003; Ryan et al., 2003; Jellison et al., 2004). Although Cryptosporidium spp. have been found in more than 150 species of mammals (Fayer et al., 2000), isolates from relatively few of these species have been genetically characterized. Therefore, it is likely that there are more host-adapted genotypes in mammals. Little is known about Cryptosporidium spp. infections in marine mammals (i.e., the potential sources of infection, the impact of infection on marine mammal health, or the possible role of these mammals in transmission of cryptosporidiosis to other animals or humans). Seals have been experimentally infected with C. parvum, but there are no reports of naturally acquired Cryptosporidium infection in seals. Cryptosporidium sp. has, however, been reported in naturally infected California sea lions (Deng et al., 2000). In this first report of naturally acquired Cryptosporidium sp. in seals, we have genetically analyzed Cryptosporidium sp. isolates to validate new host-adapted genotypes from ringed seals (Phoca hispida) using the 18S rRNA, HSP-70, and actin genes.

Intestinal contents were collected by local Inuit hunters during the spring and summer of 2001 and 2002 from 55 ringed seals and 5 bearded seals (Eringanus barbatus) in Nunavik, a region of Quebec, Canada, north of the 55th parallel. Seals are an important part of the Inuit diet, being the seal samples collected from Inuit hunters in this study for that purpose. Intestinal contents from 60 seals were examined for the presence of Cryptosporidium sp. by PCR using primers for the 18S rRNA gene. The hunters exposed the intestinal tract of each animal, compressed the entire intestinal contents into the rectum, and tied it off. The rectum was then excised and transported in a cooler to the Microbiology Laboratory at the Nunavik Research Center in Kuujjuaq. A few grams of the intestinal contents were suspended in PBS for molecular characterization studies.

Total DNA was extracted from each 400 µl of intestinal contents using a DNeasyTissue kit (Qiagen, Valencia, California) following organic extraction. After an overnight incubation at 55 C with proteinase K, the complete lysate was transferred to a Phase Lock Gel® (Eppendorf, Hamburg, Germany), 600 µl of phenol-chloroform-isoamylalcohol (25:24:1) (Invitrogen, Carlsbad, California) was added to each specimen, and the suspension was mixed well by repeated inversion. Spec-
imns were centrifuged at room temperature at 10,000 g for 10 min. The aqueous upper phase was pipetted to a new tube and 360 μl of Buffer AL (from the DNeasyTissue Kit) was added to each tube. The manufacturer's recommended DNA extraction protocol was then followed. To increase the quantity of recovered DNA, the nucleic acid was eluted in 100 μl of AE buffer (Elution Buffer from DNeasyTissue Kit).

Fragments of the 18S rRNA (~830 bp), HSP-70 (~325 bp), and actin (~1066 bp) genes were amplified by PCR as previously described (Xiao et al., 1999; Morgan et al., 2001; Sulaiman et al., 2002). DNA of C. parvum from experimentally infected calves was used as a positive control for the PCR. PCR products were analyzed on 1% agarose gel and visualized by ethidium bromide staining. PCR products were purified using Exonuclease I/Shrimp Alkaline Phosphatase (USB Corporation, Cleveland, Ohio). Purified products were sequenced in both directions using the same PCR primers in 10-μl reactions, Big Dye@ chemistries, and an ABI3100 sequencer analyzer (Applied Biosystems, Foster City, California). Each isolate was sequenced at least twice with independent PCR products.

The 18S RNA, HSP-70, and actin gene sequences were then compared with sequences obtained from other Cryptosporidium species and genotypes from GenBank. The phylogenetic analysis was conducted using sequences from Plasmodium falciparum 18S rRNA (GenBank M19172), HSP-70 (GenBank M19753), and actin (GenBank M19146) as out-groups. Sequences were aligned following the Clustal W algorithm included in the Megalign program (DNASTAR Inc., Madison, Wisconsin). The use of Clustal W determines that once a gap is inserted, it can be removed only by editing. Therefore, final alignment adjustments were performed manually to remove artificial gaps. Phylogenetic inference was performed by the neighbor-joining method described by Saitou and Nei (1987). Phylogenetic trees were conducted using MEGA version 2.1 (Kumar et al., 2001). Bootstrap analyses were conducted using 1,000 replications (sequence alignments can be obtained from the authors upon request).

The nucleotide sequences of the 18S rRNA, HSP-70, and actin gene sequences of the seal Cryptosporidium spp. isolates from this study have been deposited in GenBank under the accession numbersAY731234 through AY731240.

Intestinal contents from 10 ringed seals out of 55 examined were found positive for the 18S rRNA gene by PCR. None of the 5 bearded seals examined was positive by PCR. The partial 18S rRNA, HSP-70, and actin genes were successfully amplified in 2 or more independent PCRs in 10, 4, and 2 specimens, respectively (Table I). The 18S rRNA, HSP-70, and actin loci fragments were estimated to be approximately 800 bp, 300 bp, and 1,000 bp, respectively (data not shown). All PCR products were sequenced and 3 different types of Cryptosporidium sp. were identified from seals using the 18S rRNA gene. The first type of Cryptosporidium sp. was obtained from seals 3, 29, 51, and 57 (Cryptosporidium sp. seal 1); the second type from seals 13, 21, 31, 35, and 43 (Cryptosporidium sp. seal 2); and the third from seal 14 (Table I). For consistency and simplicity of identification the names Cryptosporidium sp. seal 1 and Cryptosporidium sp. seal 2 will be used throughout the remainder of this report.

When sequences of the 18S rRNA locus were compared with Cryptosporidium sp. sequence information obtained from GenBank, the sequence obtained from seal 14 had an identity of 99.8% (814 out of 816 bp were identical) with C. muris (GenBank AF0934498). However, 18S rRNA gene sequences from Cryptosporidium sp. Specimens from seals 1 and 2 had greater genetic differences with all previously reported

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>Seal gender and age class</th>
<th>Geographic region</th>
<th>Cryptosporidium species/genotype at 18S rRNA locus</th>
<th>Cryptosporidium species/genotype at HSP-70 locus</th>
<th>Cryptosporidium species/genotype at actin locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seal 3</td>
<td>Subadult male</td>
<td>Hudson Strait</td>
<td>Cryptosporidium sp. seal 1</td>
<td>ND*</td>
<td>ND</td>
</tr>
<tr>
<td>Seal 13</td>
<td>Adult female</td>
<td>Hudson Strait</td>
<td>Cryptosporidium sp. seal 2</td>
<td>Cryptosporidium sp. seal 2</td>
<td>Cryptosporidium sp. seal 2</td>
</tr>
<tr>
<td>Seal 14</td>
<td>Young male</td>
<td>Hudson Bay</td>
<td>C. muris</td>
<td>C. muris</td>
<td>ND</td>
</tr>
<tr>
<td>Seal 21</td>
<td>Subadult male</td>
<td>Hudson Strait</td>
<td>Cryptosporidium sp. seal 2</td>
<td>Cryptosporidium sp. seal 2</td>
<td>Cryptosporidium sp. seal 2</td>
</tr>
<tr>
<td>Seal 29</td>
<td>Adult female</td>
<td>Ungava Bay</td>
<td>Cryptosporidium sp. seal 1</td>
<td>Cryptosporidium sp. seal 1</td>
<td>ND</td>
</tr>
<tr>
<td>Seal 31</td>
<td>Male</td>
<td>Ungava Bay</td>
<td>Cryptosporidium sp. seal 2</td>
<td>Cryptosporidium sp. seal 2</td>
<td>ND</td>
</tr>
<tr>
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<td>Female</td>
<td>Ungava Bay</td>
<td>Cryptosporidium sp. seal 2</td>
<td>Cryptosporidium sp. seal 2</td>
<td>ND</td>
</tr>
<tr>
<td>Seal 43</td>
<td>Adult male</td>
<td>Hudson Bay</td>
<td>Cryptosporidium sp. seal 2</td>
<td>Cryptosporidium sp. seal 2</td>
<td>ND</td>
</tr>
<tr>
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<td>Ungava Bay</td>
<td>Cryptosporidium sp. seal 1</td>
<td>Cryptosporidium sp. seal 1</td>
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</tr>
<tr>
<td>Seal 57</td>
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<td>Hudson Bay</td>
<td>Cryptosporidium sp. seal 1</td>
<td>Cryptosporidium sp. seal 1</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND = not detected.
Cryptosporidium spp. sequences than differences reported between recognized Cryptosporidium species. For example, the sequence divergence between Cryptosporidium sp. genotypes in seals 1 and 2 and that of any other Cryptosporidium species or genotypes included in the analysis is greater at the 18S rRNA locus (more than 4%) than that between C. canis and C. parvum (former C. parvum bovine genotype or genotype 2) (2.3%), or between C. meleagridis and C. hominis (1.2%). Phylogenetic analysis of a partial fragment of the 18S rRNA (784 bp) was constructed based on neighbor-joining analysis to determine the relationship of the Cryptosporidium spp. isolated from seals in this study to that of other Cryptosporidium species and genotypes of which only partial sequences were available in GenBank (Fig. 1). This analysis placed the novel Cryptosporidium sp. genotypes in seals 1 and 2 together in a separate clade (Fig. 1), and placed the isolate from seal 14 with C. muris.

Partial sequences of the HSP-70 gene were obtained from 4 Cryptosporidium isolates (Table 1). One isolate (seal 29) was identified as Cryptosporidium sp. seal 1, 2 isolates (seals 13 and 21) were identified as Cryptosporidium sp. seal 2, and the fourth isolate (seal 14) was similar to C. muris using the sequence for the 18S rRNA gene. These sequences were compared with sequences from a range of Cryptosporidium species and genotypes obtained from GenBank. Cryptosporidium sp. isolate from seal 14 had an identity of 99.6% (283 out of 284 bp were identical) with C. muris (GenBank AF221543). There was a minor sequence variation (2 bp) between both Cryptosporidium sp. seal 2 genotype isolated from seals 13 and 21. Phylogenetic analysis of a partial fragment of the HSP-70 (284 bp) was constructed based on neighbor-joining analysis to determine the relationship of the seal isolates to Cryptosporidium species and genotypes of which only partial sequences were available in GenBank (Fig. 2). Phylogenetic analysis confirmed that Cryptosporidium sp. seal 1 and 2 were closely related genotypes, clustering together.

Partial actin gene sequence information was obtained from 2 isolates, identified before as Cryptosporidium sp. seal 1 and 2 genotypes using the 18S rRNA and HSP-70 sequence data (Table 1). These sequences were compared with sequences from a range of Cryptosporidium species and genotypes obtained from GenBank. Phylogenetic analysis of the actin gene (571 bp) was consistent with 18S rRNA and HSP-70 sequence analysis, with the Cryptosporidium sp. seal 1 and 2 genotypes forming a distinct cluster (Fig. 3).

Multilocus analysis of the Cryptosporidium sp. isolates from seals revealed C. muris and 2 novel genotypes. C. muris, a predominantly rodent species, is considered a concern to human health as an emerging zoonotic pathogen (Xiao et al., 2004). It has been identified in Kenya, Peru, and Thailand in healthy persons and in those with HIV infection (Gatei et al., 2002; Tiangtip and Jongwutiwes, 2002; Palmer et al., 2003). Because the ringed seal is the most common seal in the Arctic and an important part of the Inuit diet, the presence of C. muris infection in seals should be considered a potential public health risk. Consumption of seals by native people can pose risks due to the zoonotic diseases carried by these marine mammals, including Giardia sp. and Cryptosporidium sp. (Olson et al., 1997). Our results highlight the potential for zoonotic transmission of Cryptosporidium sp. from seals to the Inuit people who consume them. Because the intestines of ringed seals are simply dried before being consumed, this may be a source of foodborne transmission in Nunavik. Future work will involve genotyping Cryptosporidium sp. isolates from human fecal specimens collected in Nunavik, to provide some insight regarding prevalence of human infection and the possible relationship to consumption of uncooked seal intestines. In contrast, Cryptosporidium sp. seal 1 and Cryptosporidium sp. seal 2 are genotypes identified only in seals. These genotypes have genetic variation at the 3 loci characterized, which was greater than or equivalent to that used to discriminate between currently recognized Cryptosporidium species. For classification of the genotypes as distinct species of Cryptosporidium, biological differences, including host specificity, must be demonstrated. Therefore, in the present study, these genotypes were placed in a novel group that represents a new species within the Cryptosporidium complex.
C. hominis (AF382337)
C. parvum bovine (AF382338)
C. wrairi (AF382348)
C. meleagris (AF382351)
C. suis (AF382344)
C. saurophilum (AF382349)
C. canis (AF382340)
C. felis (AF382347)
Cryptosporidium sp. seal 2
Cryptosporidium sp. seal 1
Bovine genotype B
Deer genotype (AY120928)
Deer-like genotype
C. baileyi (AF382346)
C. serpentis (AF382353)
C. andersoni (AF382352)
C. muris (AF382350)
Plasmodium (M19146)

Figure 3. Phylogenetic relationships among Cryptosporidium species and genotypes inferred by neighbor-joining analysis using a fragment of the actin gene. Values on branches are percent bootstrapping using 1,000 replicates. The GenBank accession numbers of each Cryptosporidium species or genotype are shown in parentheses.

isolate, designated Cryptosporidium sp. seal 1 and 2, represent 2 new genotypes of Cryptosporidium, and new taxa for these novel genotypes must await the establishment of distinct biological data.

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LITERATURE CITED


