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PREVALENCE OF MICROSPORIDIA, *CRYPTOSPORIDIUM* SPP., AND *GIARDIA* SPP. IN BEAVERS (*CASTOR CANADENSIS*) IN MASSACHUSETTS

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Abstract: Feces from 62 beavers (*Castor canadensis*) in Massachusetts were examined by fluorescence microscopy (IFA) and polymerase chain reaction (PCR) for Microsporidia species, *Cryptosporidium* spp., and *Giardia* spp. between January 2002 and December 2004. PCR-positive specimens were further examined by gene sequencing. Protist parasites were detected in 6.4% of the beavers. All were subadults and kits. Microsporidia species were not detected. *Giardia* spp. was detected by IFA from four beavers; *Cryptosporidium* spp. was also detected by IFA from two of these beavers. However, gene sequence data for the ssrRNA gene from these two *Cryptosporidium* spp.–positive beavers were inconclusive in identifying the species. Nucleotide sequences of the TPI, ssrRNA, and β-giardin genes for *Giardia* spp. (deposited in GenBank) indicated that the four beavers were excreting *Giardia duodenalis* Assemblage B, the zoonotic genotype representing a potential source of waterborne *Giardia* spp. cysts.

Key words: Beaver, genotype, molecular, survey, waterborne, zoonotic.

INTRODUCTION

The beaver (*Castor canadensis*) is distributed broadly throughout North America. They reside in ponds and streams that humans sometimes use for recreation or sources of drinking water, and consequently have been implicated as sources of waterborne disease. Waterborne pathogens of public health concern include some species and genotypes of Microsporidia, *Giardia*, and *Cryptosporidium*, but the role of the beaver as a source of these organisms is not known, is not firmly established, or has been implicated from circumstantial data. The best evidence that beavers serve as a source of waterborne parasites comes from transmission studies in which *Giardia* spp. cysts from humans were used experimentally to infect beavers, and those from beavers were used to infect humans experimentally (Table 1). Studies of intestinal protozoa in beavers have relied almost exclusively on microscopy (Table 2), much the same as surveys that have relied heavily on microscopic methods for the detection of spores, oocysts, and cysts of these pathogens in other wildlife. Although microscopy has been helpful in identifying many organisms at the generic or higher taxonomic levels, neither morphologic features nor application of immunofluorescence reagents have proven sufficient for identification or differentiation of the multiple species and strains of Microsporidia, *Giardia*, and *Cryptosporidium*, each of which parasitize a specific range of hosts. Highly sensitive and specific molecular methods have proven helpful for parasite detection in two ways: when parasite numbers are so low in some specimens that microscopic methods fail to detect them, and when morphologically identical organisms can be distinguished only by identifying genetic differences. Therefore, in addition to the classical and more routinely used microscopic methods, and because of the potential importance of beavers as sources of waterborne pathogens, the present study was conducted by molecular methods to determine the identity of any species of Microsporidia, *Giardia*, and *Cryptosporidium* excreted by beavers.

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MATERIALS AND METHODS

Feces from 62 beavers live-trapped and then released in northeastern, central, and western Massachusetts (Fig. 1) were shipped to the USDA laboratory in Beltsville, MD. Feces were collected in spring and fall in 2002, 2003, and 2004. Three age groups were considered (adults, subadults, and kits). The present study reports analyses from 27 adults (15 males and 12 females), 31 subadults (22 males and 9 females), and 4 kits (2 males and 2 females).

Table 1. Experimental transmission of *Giardia* spp. cysts between humans and beavers.

Source	Recipient	Number positive/ number recipients	Dose	Identification method	Citation
Human	Beaver	2/2	1.00×10^4	Microscopy	6
Beaver	Human	2/3	Unknown	Microscopy	
Human	Beaver	Positive[a]	Unknown	Microscopy	19
Human	Beaver	6/8	5.00×10^5	Microscopy	12
Human	Beaver	12/20	5.00×10^2 to 5.00×10^5	Microscopy	11

[a] Neither the number positive nor the number of recipients were provided.

Fifteen grams of feces were placed into a 50-ml tube and thoroughly mixed with 35 ml dH_2O. The fecal suspension was passed through a 45-μm screen, collected into a second 50-ml tube, and adjusted to a final volume of 50 ml with dH_2O. The tubes were centrifuged at 1,500 g for 15 min, the supernatant discarded and the fecal pellet resuspended in 25 ml dH_2O. Twenty-five milliters of

Table 2. Prevalence of *Giardia duodenalis* or *Giardia* sp. cysts from beavers in North America identified microscopically by morphology or immunofluorescence, or by molecular techniques.

Location	Number examined	Prevalence (% positive)	Microscopy	Molecular	Flow cytometry	Citation
Colorado	244	18.0	×			6
Washington	7	42.7	×			10
	173	6.3	×			15
Washington	177	6.8	×			
	179	19.0	×			
Alberta, Canada	58	3.5	×			30
British Columbia, Canada	299	19.5	×			18
Washington	313	24.3	×			24
Colorado			×			22
Minnesota	89	10.1	×			11
Minnesota	41	4.9	×			12
Massachusetts	3	33.3	×			19
	6	0	×			
Maine	138	15.9	×			13
Massachusetts	9	11.1	×			
Minnesota	87	56.4	×			
New Hampshire	259	39.0	×			
New York	96	30.3	×			
Vermont	73	6.8	×			
British Columbia, Canada	7	57.1	×			23
	7	0	×			
Kansas	63	0	×			21
Ontario, Canada	94[a]	14.9				8
	94[a]	7.5	×		×	8
	94[a]	9.4	×			8
Texas	100[b]	33.0	×			9
Alberta, Canada	334[c]	8.7	×	×		16
Maryland	7[d]	100.0				28

[a] Of the 94 beaver fecal samples examined, conventional microscopy identified 7.5%, immunofluorescence microscopy identified 9.4%, and flow cytometry identified 14.9% as positive.

[b] Of the 100 beavers examined, immunofluorescence microscopy for cysts detected 30 positive beavers and trichrome staining of duodenal mucoid samples detected 26 positive beavers.

[c] All specimens were examined by immunofluorescence microscopy.

[d] All seven specimens examined by molecular methods were preselected.

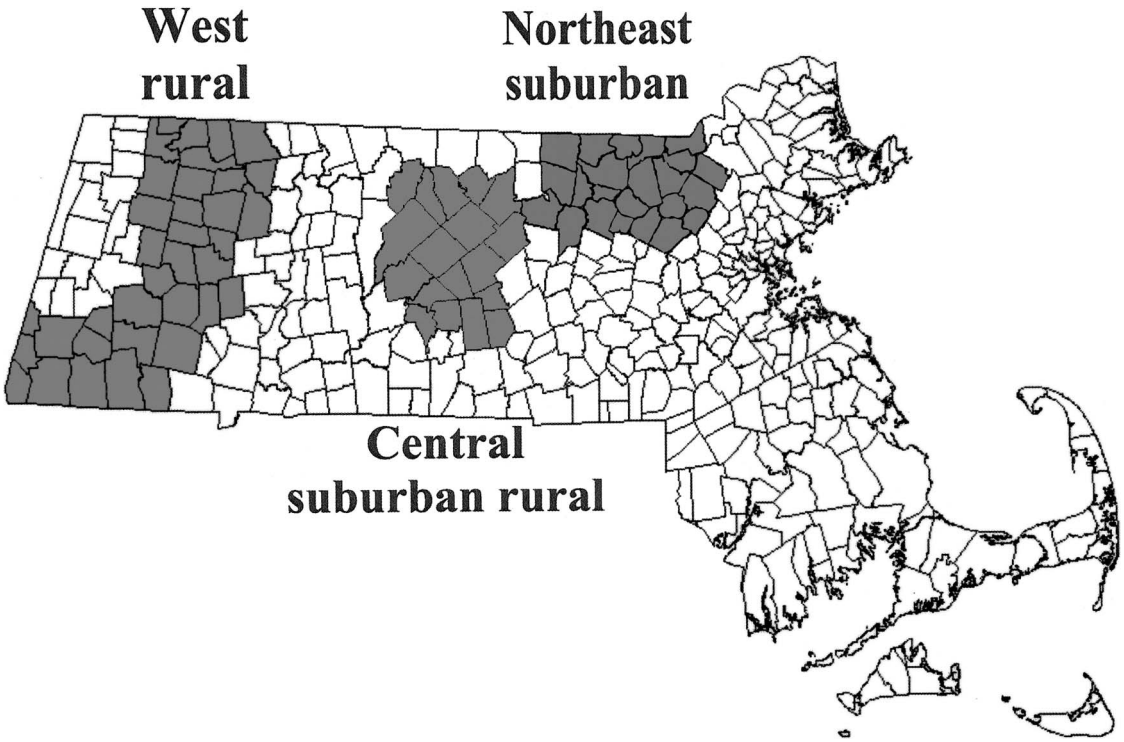

Figure 1. Map of 351 towns in Massachusetts. Each shaded area represents sites where beavers were trapped.

CsCl (1.4 g/ml) was added to each tube and the samples were thoroughly mixed. The samples were subjected to a second centrifugation at 250 g for 20 min. Following the second centrifugation, the top 4 ml of supernatant was aspirated from each sample and transferred into a 15-ml tube. Deionized H_2O was added to all tubes to bring the final volume to 15 ml. Samples were washed twice with dH_2O and the final pellet was suspended in 500 μl of dH_2O. Portions of this 500-μl suspension were used for immunofluorescence analysis or molecular analysis, as described below.

For the immunofluorescence analysis of species of *Cryptosporidium* and *Giardia*, a 100-μl aliquot of the fecal suspension was transferred to a microcentrifuge tube and washed once with dH_2O. The pellet was suspended in 25 μl of premixed MeriFluor™ reagents (Meridian Diagnostics, Cincinnati, Ohio, USA). Premixed MeriFluor™ reagents were prepared as follows. MeriFluor test reagent and counterstain were mixed with 3 ml sterile PBS. Two microliters of the feces–stain suspension was transferred to one well of a three-well slide (Celline, HTC, Portsmouth, New Hampshire, USA); a coverslip was placed on the slide and slides were examined at 400× with the use of a Zeiss Axioskop microscope equipped with epifluorescence and an FITC-Texas Red dual-wavelength filter (Chroma Technology Corporation, Rockingham, Vermont, USA).

For the immunofluorescence analysis of Microsporidia 15 μl of suspended pellet was aspirated, pipetted into a poly-L-lysine-coated, 11.5-mm-diameter ring on a glass microscope slide (Erie Scientific, Portsmouth, NH), stained with Calcofluor white (Becton Dickinson and Company, Sparks, Maryland, USA), and examined by fluorescence microscopy (Carl Zeiss Microimaging Inc., Thornwood, New York, USA).

For the molecular analysis, total DNA was extracted from each CsCl-cleaned fecal sample using a DNeasyTissue Kit (Qiagen, Valencia, California, USA) with a slightly modified protocol. The protocol, described below, utilized reagents provided by the manufacturer. A total of 50 μl of processed feces were suspended in 180 μl of ATL buffer and thoroughly mixed by vortexing. To this suspension, 20 μl of Proteinase K (20 mg/ml) was added, and the sample was thoroughly mixed. Following an overnight incubation of the mixture at 55°C, 200 μl of AL buffer was added. The remaining protocol followed manufacturer's instructions with one exception. To increase the quantity of recovered

DNA, the nucleic acid was eluted in 100 μl of AE buffer.

For *Giardia* spp., fragments of the ssrRNA (~292 bp), β-giardin (~750 bp), and TPI (~500 bp) genes were amplified by PCR as previously described.[5,17,28] For *Cryptosporidium* spp. a fragment of the ssrRNA gene (~800 bp) was amplified by PCR as previously described.[32] For species of Microsporidia, generic primers that amplify a fragment of the ssrRNA gene (~292 bp) as well as primers specific for *Enterocytozoon bieneusi* that amplify the ITS region as well as a portion of the flanking large and small subunit ribosomal RNA genes were used.[4,14] PCR products were analyzed on 1% agarose gel and visualized by ethidium bromide staining.

PCR positives were purified with the use of EXO-SAP enzyme (USB Corporation, Cleveland, Ohio, USA). Purified products were sequenced with the same PCR primers used for the original amplification in 10-μl reactions, Big Dye™ chemistries, and an ABI3100 sequencer analyzer (Applied Biosystems, Foster City, California, USA). Each sample was sequenced in both directions. Sequence chromatograms from each strand were aligned and inspected with the use of Lasergene software (DNASTAR, Inc., Madison, Wisconsin, USA). All PCR-positive samples were sequenced. To determine the *Giardia* sp. genotype, each sequence for all three gene fragments was independently compared to GenBank sequences of *Giardia* spp. genotypes.

RESULTS

Spores of Microsporidia were not detected by fluorescence microscopy of Calcofluor-stained fecal smears. The PCR method, using both generic primers for Microsporidia and specific primers for *E. bieneusi*, also failed to detect species of Microsporidia in fecal specimens.

Cysts of *Giardia* spp. were detected by immunofluorescence microscopy in feces from four subadult and kit beavers, and their presence was confirmed by PCR (Table 3). Gene sequence data for these PCR products indicated that the four beavers were excreting the *G. duodenalis* genotype equivalent to the zoonotic Assemblage B. Nucleotide sequences of the TPI, 16s rRNA, and β-giardin genes of *G. duodenalis* isolates from beavers in the current study, representing Assemblage B were deposited in GenBank under accession numbers DQ789112 through DQ789116.

Based on immunofluorescence microscopy, oocysts of *Cryptosporidium* spp. were identified in the feces from two (numbers 23 and 25) of the four *Giardia* spp. positive beavers (Table 3). However, when specimens from these beavers were examined by PCR, using primers for the ssrRNA gene, they were negative (Table 3).

DISCUSSION

Microsporidia are intracellular parasites consisting of more than 1,200 species in 143 genera that infect invertebrate and vertebrate hosts.[7] At least six genera including 15 species have been reported to infect humans, primarily the immunocompromised. The most common microsporidian that infects humans is *E. bieneusi*, but little is known of reservoirs or routes of infection.[7] However, recently, *E. bieneusi* was isolated from domesticated animals and wildlife including cattle (*Bos taurus*), pigs (*Sus scrofa*), dogs (*Canis familiaris*), a cat (*Felis domesticus*), beavers (*Castor canadensis*), muskrats (*Ondatra zibethica*), otters (*Lutra canadensis*), raccoons (*Procyon lotor*), and foxes (*Vulpes vulpes*).[20,26,29] Of 100 beavers trapped in Maryland, 14 were found positive for *E. bieneusi* by nucleotide sequence analysis of the ITS region of the rRNA gene.[29] Five of these 14 isolates were identical to genotypes isolated from humans, 5 were identical to genotypes isolated from pigs, and 5 were unique to beavers. In contrast, none of the beavers in the present study were found infected with species of Microsporidia. Because beavers have been examined for species of Microsporidia only in Massachusetts (present study) and Maryland[29] too few an-

Table 3. Beavers found positive for *Giardia* Spp. and *Cryptosporidium* Spp. in the present study.

Beaver number	Date collected	Area	Colony	Age	Sex	IFA	PCR	*Giardia* assemblage
4	4/25/02	NE	Groton 1	Subadult	F	+ *Giardia*	+ *Giardia*	Assemblage B
23	10/23/02	W	Eugene	Kit	F	+ *Giardia*	+ *Giardia*	Assemblage B
			Moran 1			+ *Crypto*	− *Crypto*	
25	11/09/02	W	Eugene	Kit	F	+ *Giardia*	+ *Giardia*	Assemblage B
			Moran 2			+ *Crypto*	− *Crypto*	
59	10/05/04	NE	Groton 2	Kit	M	+ *Giardia*	+ *Giardia*	Assemblage B

imals have been examined to provide meaningful data on the prevalence, age and sex distribution, sources, and other factors to characterize these infections.

Beavers have been thought to be a major source of *Giardia* spp.–contaminated drinking water for reasons both obvious and deductive: Beavers are found associated with water and some are infected with *Giardia* spp. (Table 2), they have been experimentally infected with *Giardia* spp. cysts derived from humans (Table 1), and beavers excreting *Giardia* spp. cysts have been found in the vicinity of contaminated drinking water supplies. However, despite efforts by many investigators of waterborne outbreaks of giardiasis, the source of *Giardia* spp. cysts in the water has never been irrecusably determined. For many years, morphology and cross-transmission studies were the only methods available for identifying cysts and trophozoites from various hosts.[11] Immunofluorescence microscopy facilitated recognition of cysts, but this technique had a major drawback. Antibodies to the cyst wall were not species or subspecies specific, and therefore, because most cysts appeared similar in size and shape, they were indistinguishable.[2] Isoenzyme patterns of isolates provided a refined method to distinguish isolates and place them in zymodemes,[3,25] but this method was both time consuming and required a great deal of expertise to interpret the intricate patterns. Molecular methods have now provided a universally accepted tool for facilitating the identification of *Giardia* spp. isolates at the genetic level.[28] The present study has identified *Giardia* spp. cysts based on immunofluorescence microscopy, and based on sequencing three different genes, TPI, ssrRNA, and β-giardin, indicating that four young beavers were excreting cysts of the zoonotic assemblage B of *G duodenalis*. In another study, beavers in Texas were examined over a period of 2 years and identified as juveniles or adults, and as males or females.[9] However, the species of *Giardia* was not determined and neither sex nor age differences were found to be related to infection.[9]

Cryptosporidium, a protozoan parasite responsible for numerous drinking water and recreational water outbreaks of diarrhea worldwide, has been reported to infect over 150 species of animals.[31] Of 16 species and over 30 genotypes, each with a limited host range, *C. parvum*, a zoonotic pathogen, is the species reported most often and with the widest range of hosts. In a study involving 481 fur-bearing mammals (beaver, fox, muskrat, otter, and raccoon) trapped in Maryland, 8% were found infected by *Cryptosporidium* spp. utilizing RFLP analysis of PCR, but none of the 87 beavers were found infected.[33] Of 19 European beavers (*Castor fiber*) examined in Poland, oocysts were detected in two Ziehl-Neelsen stained specimens by bright-field microscopy and immunofluorescence microscopy,[1] but the species were not determined. Although the present report is the first to document the presence of *Cryptosporidium* spp. in *C. canadensis* in North America by immunofluorescence microscopy, molecular methods were unable to provide the data necessary to determine the species or genotype. It is interesting to note that all four infected beavers were young animals. Similarly, a multiyear prevalence study of cryptosporidiosis in dairy cattle has found a much higher rate of infection in calves than in more mature animals.[27]

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