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Genotypes of *Cryptosporidium* Species Infecting Fur-Bearing Mammals Differ from Those of Species Infecting Humans

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Of 471 specimens examined from foxes, raccoons, muskrats, otters, and beavers living in wetlands adjacent to the Chesapeake Bay, 36 were positive for five types of *Cryptosporidium*, including the *C. canis* dog and fox genotypes, *Cryptosporidium* muskrat genotypes I and II, and *Cryptosporidium* skunk genotype. Thus, fur-bearing mammals in watersheds excreted host-adapted *Cryptosporidium* oocysts that are not known to be of significant public health importance.

The enteric parasites in the genus *Cryptosporidium* can be transmitted through ingestion of contaminated water (5). However, the sources of contamination are not clearly identified. *Cryptosporidium* spp. have been reported to infect a wide range of wild mammals (15). Among them, wild rodents have received particular attention, and it has been suggested that they may serve as reservoirs of *Cryptosporidium* infection for domestic animals and humans (1–6, 13–15, 18). Since the 1993 cryptosporidiosis outbreak in Milwaukee, Wis., the water industry in the United States has been striving to provide *Cryptosporidium*-free drinking water through stringent treatment practices and source water protection. Although there has been speculation that wild mammals serve as potential sources of watershed contamination with *Cryptosporidium* oocysts infectious for humans (1–3, 7), the actual role of wildlife in the contamination of source water with human-pathogenic *Cryptosporidium* spp. remains unknown.

Results of recent studies indicate a strong host adaptation for *Cryptosporidium* (22). Eight species of *Cryptosporidium* have been identified as pathogens in humans: *C. parvum*, *C. hominis*, *C. meleagridis*, *C. felis*, *C. canis*, *C. muris*, and *Cryptosporidium* pig and cervine species (8–12, 20, 21). Of these, *C. hominis*, *C. parvum*, and *C. meleagridis* have been found most frequently, whereas the others have been identified mostly in clinical case reports involving a few persons. Thus, unless wild mammals can be shown to be a source of these three species, they do not represent a significant risk as a source of water contamination affecting humans under normal circumstances. The present study was conducted to determine if *Cryptosporidium* infections are present in wild mammals (beavers, muskrats, otters, raccoons, and foxes) living in Chesapeake Bay watersheds and, if so, to determine the prevalence and species of *Cryptosporidium* by molecular methods. Results of the study provide the first genetically based data on the role of wildlife in *Cryptosporidium* contamination in watersheds.

Wildlife fecal specimen collection and genomic DNA extraction. A total of 471 fecal specimens were collected during January 2001 and January 2002 from 87 beavers, 237 muskrats, 20 otters, 51 raccoons, and 76 foxes trapped in the Caroline (Marshy Hope Creek, Federalsburg), Charles (Clifton Creek, Newburg), Dorchester (Hunting Creek, Hurlock), and Talbot (Choptank River, Easton) counties of Maryland. With few exceptions, most of the trapped animals were older than 12 months. Details of the animal sources were described previously in studies of *Enterocytozoon* spp. and *Giardia* spp. in these animals (16, 17). After removal of fecal debris from 15 g of feces from each animal by sieving followed by density gradient centrifugation over cesium chloride, 200 μl of purified parasite suspension was washed twice with distilled water by centrifugation at 12,000 × *g* for 15 min as previously described (16). The oocyst walls were lysed with 1 M KOH, and genomic DNA was extracted as previously described with a QIAamp DNA stool mini kit (QIAGEN Inc., Valencia, Calif.) (22).

SSU rRNA PCR-RFLP analysis. The species and genotypic nature of *Cryptosporidium* in each fecal specimen was determined by a previously described PCR-restriction fragment length polymorphism (RFLP) method based on the small-subunit (SSU) rRNA gene (20, 21). DNA from each specimen was analyzed three times by a nested PCR using 0.5, 1.0, or 2.0 μl of DNA as templates. The secondary PCR products (10 μl) of about 830 bp were digested with SspI (New England BioLabs, Beverly, Mass.) and VspI (GIBCO BRL, Grand Island, N.Y.). *Cryptosporidium* species and genotypes were determined on the basis of banding patterns in agarose electrophoresis. Positive (DNA of *C. serpentis*) and negative (no templates) controls were used in each PCR run.

DNA sequence and phylogenetic analyses. To confirm the RFLP results, all secondary PCR products were sequenced with an ABI 3100 genetic analyzer (Applied Biosystems, Foster City, Calif.). The nucleotide sequences obtained were aligned with each other and those from the GenBank database with the program ClustalX (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/).

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TABLE 1. *Cryptosporidium* species and genotypes in wild mammals in watersheds in the Chesapeake Bay area

Animal	No. of SSU rRNA-positive specimens/total no. of specimens (%)	RFLP pattern (no. of specimens)	Species and genotype(s) (no. of positive specimens)
Beaver	0/87		
Fox	6/76 (7.9)	*C. canis* (5) muskrat genotype (1)	*C. canis* fox genotype (4), *C. canis* dog genotype (1), *Cryptosporidium* muskrat genotype I (1)
Muskrat	28/237 (11.8)	*Cryptosporidium* muskrat genotype (22), new *Cryptosporidium* genotype (4), muskrat genotype + new genotype (2)	*Cryptosporidium* muskrat genotype I (22), *Cryptosporidium* muskrat genotype II (4), m uskrat genotype I + muskrat genotype II (2)
Otter	0/20 (0)		
Raccoon	2/51 (3.9)	*Cryptosporidium* skunk genotype (2)	*Cryptosporidium* skunk genotype (2)
Total	36/471 (7.6)	4 RFLP patterns	5 *Cryptosporidium* spp.

A neighbor-joining phylogenetic tree was generated with TREECON version 1.3b (http://www.psb.rug.ac.be/bioinformatics/psb/Userman/treeconw.html), based on evolutionary distances calculated with the Kimura two-parameter model. The confidence of grouping was accessed by bootstrapping, using 1,000 replicates.

Prevalence of *Cryptosporidium* spp. in wildlife. Fecal specimens from 471 wild mammals were analyzed for *Cryptosporidium* spp. by PCR. *Cryptosporidium* spp. were detected in 36 animals (8%), including 28 of 237 muskrats (12%), 6 of 76 foxes (8%), 2 of 51 raccoons (4%), none of 87 beavers, and none of 20 otters (Table 1). Infected animals, of which 17 were females and 19 were males, ranged from 12 to 36 months.

***Cryptosporidium* genotypes in wildlife.** RFLP analyses of the PCR products with restriction enzymes SspI and VspI showed four banding patterns among positive specimens (Fig. 1). Most of the muskrat specimens had RFLP banding patterns identical to those of the previously described *Cryptosporidium* muskrat genotype, with two bands visible after both SspI and VspI digestion (lanes 1 and 2 in Fig. 1). Some muskrats had a PCR product very similar to that of the muskrat genotype, but the sizes of the two SspI bands were smaller (lanes 3 and 4 in Fig. 1). Specimens from two muskrats (specimens 3579 and 5538), however, had both RFLP banding patterns. In contrast, the two positive specimens from raccoons had an RFLP pattern identical to that of the previously described *Cryptosporidium* skunk genotype (lanes 5 and 6 in Fig. 1), whereas most of the positive fox specimens had the RFLP pattern for *C. canis*. One fox specimen had the RFLP pattern of the *Cryptosporidium* muskrat genotype.

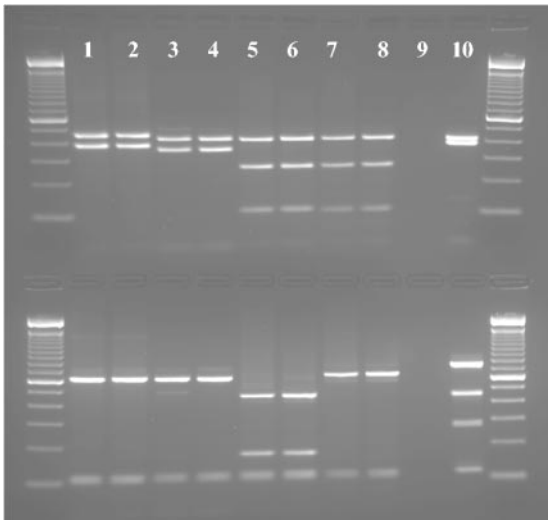

FIG. 1. Presence of four *Cryptosporidium* genotypes in fur-bearing wild mammals in the Chesapeake Bay area as indicated by RFLP analyses of PCR products of the SSU rRNA gene with restriction enzymes SspI (the upper lanes) and VspI (the lower lanes). Lanes 1 and 2, muskrat genotype I (specimens 5526 and 5559); lanes 3 and 4, muskrat genotype II (specimens 3568 and 3665); lanes 5 and 6, skunk genotype (specimens 6006 and 6001); lanes 7 and 8, *C. canis* (specimens 6008 and 5977); lane 9, blank; and lane 10, positive control (*C. serpentis*). Molecular size markers are 100-bp ladders.

DNA sequencing analyses of the PCR products showed the presence of five *Cryptosporidium* spp., four of which have been reported before (22). The 25 specimens with the RFLP pattern of the *Cryptosporidium* muskrat genotype generated SSU rRNA sequences typical of the genotype; 24 were from muskrats and one was from a fox. The SSU rRNA sequences from four fox specimens were identical to the *C. canis* fox genotype, but the sequence of one fox specimen was identical to that of the *C. canis* dog genotype. The SSU rRNA sequences from the two raccoons were of the *Cryptosporidium* skunk genotype. As expected, SSU rRNA sequences from six muskrat specimens belonged to a new *Cryptosporidium* genotype. To differentiate the two genotypes in muskrats, the previously named *Cryptosporidium* muskrat genotype was renamed muskrat genotype I and the new genotype was named genotype II. DNA sequence analysis confirmed that two muskrat specimens (3579 and 5538) had both *Cryptosporidium* muskrat genotypes I and II.

Genetic uniqueness of *Cryptosporidium* muskrat genotype II. New *Cryptosporidium* muskrat genotype II showed significant differences from the SSU rRNA sequences from other known *Cryptosporidium* spp. or genotypes. The difference in the nucleotide sequences of muskrat genotype II and muskrat genotype I was about 5%. A similar level of difference was found between muskrat genotype II and the two *C. canis* genotypes. The genetic distance between muskrat genotype II and the skunk genotype was 1.4%. This distance was still significantly greater than the differences between some other established *Cryptosporidium* species, such as between *C. muris* and *C. andersoni* and between *C. parvum* and *C. wrairi* (22). The

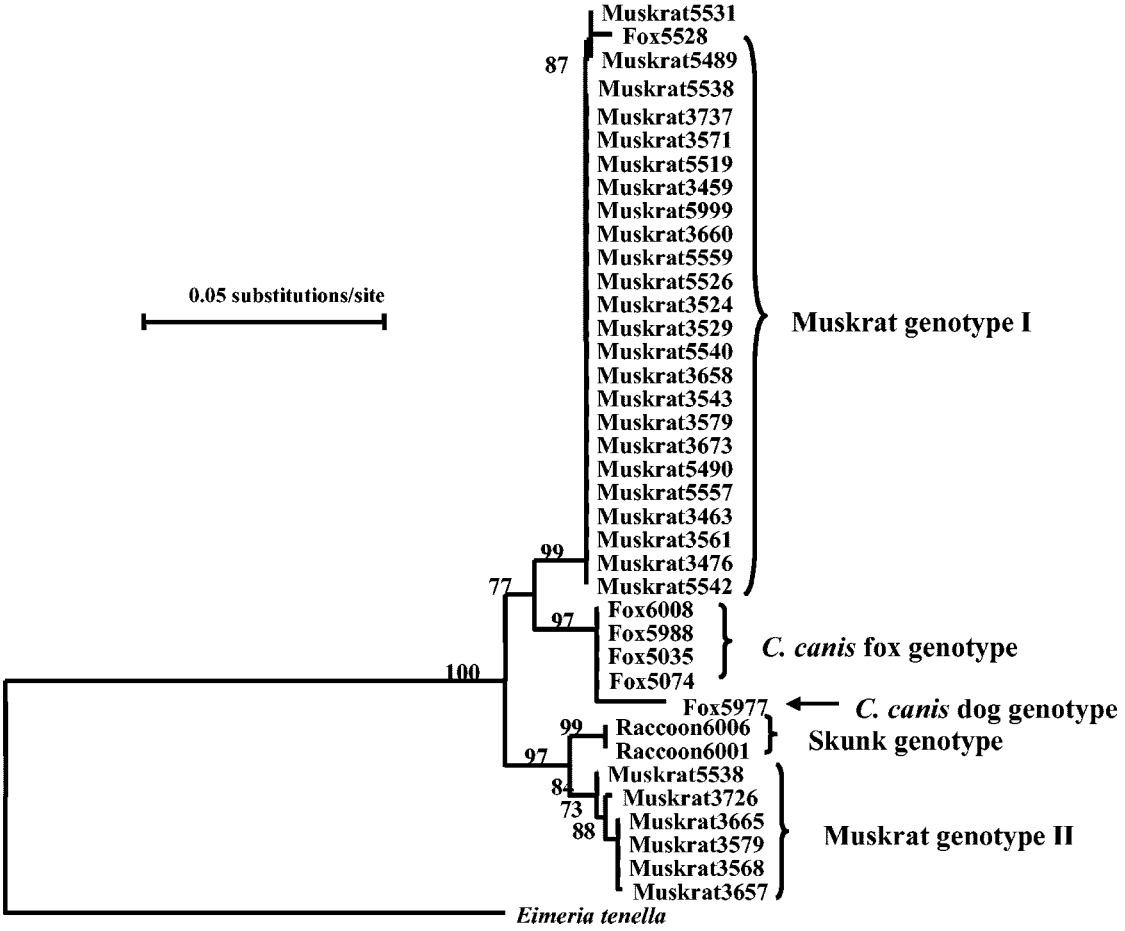

FIG. 2. Relationship of five wildlife *Cryptosporidium* spp. inferred by a neighbor-joining analysis of SSU rRNA sequences. The Kimura two-parameter model was used in distance calculation. Numbers on branches are percent bootstrap values from 1,000 resamplings.

genetic uniqueness of the *Cryptosporidium* muskrat genotype II was also reflected in the phylogenetic analysis; all isolates of this genotype formed a distinct cluster. Again, muskrat genotype II was more closely related to the skunk genotype than to other genotypes found in this study (Fig. 2).

Public health significance of *Cryptosporidium* spp. in wild mammals. Results of this study support previous findings on the host-specific nature of *Cryptosporidium* spp. (22). In general, each of the four *Cryptosporidium*-positive wildlife species in this study had its own host-specific *Cryptosporidium* genotype. Muskrats were infected only with *Cryptosporidium* muskrat genotypes I and II, and foxes were infected mostly with the *C. canis* fox genotype. Both positive raccoons had the *Cryptosporidium* skunk genotype, which was previously described to occur only in a few skunks (22). The only exception to host specificity of the parasites in this study is the finding of muskrat genotype I in one fox. It is not known, however, whether this rare finding represents established infection or merely passage of ingested *Cryptosporidium* oocysts, as coprophagia is common in foxes.

From feces of 471 animals examined in this study, the *C. canis* dog genotype was the only known human pathogen found, and this was found in less than 3% of all the *Cryptosporidium*-positive specimens. Findings from this study have clearly demonstrated for the first time that nearly all *Cryptosporidium* oocysts from a large number of diverse species of fur-bearing wild mammals are host-adapted *Cryptosporidium* species and genotypes that have never been found in humans or farm animals. These findings are in agreement with a previous report in which only wildlife *Cryptosporidium* genotypes were identified in runoff (storm water) from rural nonagricultural areas (19). The most common *Cryptosporidium* parasite found in fur-bearing mammals in this study, muskrat genotype I, is also one of the most common genotypes (W7) found in storm water in the previous study (19). Therefore, adult, wild, fur-bearing animals in wetlands can be a source of *Cryptosporidium* oocysts in watersheds, but this contamination poses little risk to public health or to livestock.

Nucleotide sequence accession numbers. The nucleotide sequences of the SSU rRNA gene of the *Cryptosporidium* muskrat genotype II have been deposited in GenBank under accession numbers AY545546 to AY545548.

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