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# *CRYPTOSPORIDIUM* SPP. FROM SMALL MAMMALS IN THE NEW YORK CITY WATERSHED

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ABSTRACT: The objective of this study was to assess the potential role that wildlife plays in environmental degradation of watersheds through the contamination of the water supply with zoonotic genotypes of *Cryptosporidium*. *Cryptosporidium* isolates recovered from wildlife in the New York City (NYC) watershed were examined to determine genotype using a polymerase chain reaction protocol targeting the 18-Small Subunit (SSU) rRNA locus. Seventy-seven DNA samples recovered from 12 wildlife host species captured in the NYC watershed were amplified and sequenced. Data on risk factors associated with the perpetuation of these genotypes also were collected and analyzed. Although many genotypes appeared to be host-specific, 38% of the samples examined were identified as *Cryptosporidium parvum*, indicating the presence of zoonotic *Cryptosporidium.* Adult animals were more likely to shed the zoonotic strains of *Cryptosporidium* spp. Animals captured in the fall and winter were more likely to be infected with *C. parvum* than those captured in spring and summer.

*Key words:* *Cryptosporidium*, *Cryptosporidium parvum*, epidemiology, polymerase chain reaction, watersheds, wildlife.

## INTRODUCTION

*Cryptosporidium* is a parasitic protozoan of public health significance (Fayer, 2004). The threat to immunocompromised individuals (Sorvillo et al., 1994) coupled with economic impacts associated with large-scale outbreaks (Neumann et al., 2005) has emphasized the importance of this potentially zoonotic parasite. Infection occurs via the fecal-oral route; this is facilitated by environmentally resistant oocysts that can survive for extended periods of time even under harsh conditions (Robertson et al., 1992). Waterborne transmission is believed to be the major pathway of infection involved in several large outbreaks (Rose et al., 2002), and surveys of surface water (LeChevallier et al., 1991) and soil (Barwick et al., 2003) indicate that oocysts are commonly found in the environment. Because of the resistance of *Cryptosporidium* to standard water treatment practices, epidemiologic research related to the risk of watershed contamination is needed.

Cryptosporidia are obligate parasites. A host is required to produce and release infectious oocysts (Sinski and Behnke, 2004), and knowledge of host systems is a preliminary step in understanding and controlling sources of environmental pollution. Vertebrate hosts of *Cryptosporidium* species have been broadly characterized in the literature into three categories: humans, domestic animals, and wildlife (Heitman et al., 2002; Xiao et al., 2002; Caccio et al., 2005). Of these potential sources, wildlife has received the least attention and the risk posed by these populations to public health is not fully understood (Appelbee et al., 2005).

Molecular evidence indicates that humans are primarily infected with two species: *Cryptosporidium hominis* and *Cryptosporidium parvum* (Leoni et al., 2006). The former species appears to be specific to human and nonhuman primates (Morgan-Ryan et al., 2002), whereas *C. parvum* is found in many mammalian species (Fayer, 2004). Many potential hosts of *C. parvum* are commonly found in watershed ecosystems, including cattle (Santin et al., 2004), deer (Perz and Le Blancq, 2001), and voles (Bednarska et al., 2003).

Surveys of wildlife have detected *Cryptosporidium* infection in many species (Chalmers et al., 1997; Torres et al., 2000); however, only a few studies have characterized the isolates found in these hosts (Perz and Le Blancq, 2001; Zhou et al., 2004). An understanding of host-parasite ecology is an essential component for public health risk assessments, and to address public health and livestock concerns accurately, it is important that epidemiologic-based investigations of this protozoan identify the full range of potential hosts, including wildlife. With this objective in mind, a molecular epidemiologic study was conducted to elucidate the genotypes of *Cryptosporidium* that had been diagnosed as *C. parvum* using microscopic and immunologic assays, and to identify the ecologic factors associated with the presence of these genotypes.

## MATERIALS AND METHODS

All specimens evaluated in this study were collected from the New York City watershed as part of a cross-sectional study on wildlife (Ziegler et al., in press). Briefly, 327 *Cryptosporidium*-positive samples were collected from a wide range of wildlife species consisting primarily of rodents. Fecal samples from each animal were first examined in the Parasitology Section of the Animal Health Diagnostic Center for the presence of *Cryptosporidium* using a centrifugation concentration flotation method (Georgi and Georgi, 1990) and a *Cryptosporidium*-specific antigen capture enzyme-linked immunosorbent assay (ELISA; ProSpect *Cryptosporidium* Microplate Assay, Alexon-Trend, Inc., Ramsey, Minnesota, USA.; Chapman et al., 1990). Samples that were diagnosed as *Cryptosporidium*-positive by either method were stored at −20 C until they were processed for genetic analysis.

### Molecular analysis of samples

DNA was extracted from fecal swabs and/or intestinal scrapings preserved in ELISA buffer, using the bead-beating protocol described previously (Lindergard et al., 2003). The DNA extract was stored at −20 C until polymerase chain reaction (PCR) amplification was performed. A nested PCR protocol was used to target a conserved region of the 18-Small Subunit (SSU) rRNA gene approximately 830 base pairs in length. The first base of the two external primers, 5′-GATAA CCGTGGT AATTCTAGAGCTA-3′, and 5′-TAAGGTGC TGAAGGAGTAAGG-3′, corresponds to position 1,629 (forward) and 2,520 (reverse) of the complete ribosomal DNA sequence (GenBank accession number L16996) (Le Blancq et al., 1997). The reverse external primer corresponds to CPB-DIAGR, as described in Perz and Le Blancq (2001). The internal primers, 5′-GAAGGGTTGTATTTATTAGATAAAG-GAAC-3′, and 5′-AAGGAGTAAGGAA-CAACCTCCA-3′, match those used by Xiao et al. (1999) with slight modification to the forward primer.

The primary reaction (20 μl total volume) consisted of 1 μl of 1:10 diluted DNA solution added to a mixture of 1× PCR buffer ($NH_4SO_4$, Mbi Fermentas, Hanover, Maryland, USA), 0.2 μM of external primers, 6 mM $MgCl_2$, 200 μM of each dNTP, and 1.0 U *Taq* DNA polymerase. For the secondary reaction, 1 μl of product from the primary reaction was added to a 19-μl volume of 1× PCR buffer ($NH_4SO_4$, Mbi Fermentas), 0.2 μM of internal primers, 3 mM $MgCl_2$, 200 μM of each dNTP, and 1.0 U *Taq* DNA polymerase. Identical thermocycler conditions were used for both reactions: 35 cycles of 96 C for 45 sec (to denature), 55 C for 45 sec (to anneal), and 72 C for 1 min (to extend). Successful amplification of DNA fragments was confirmed by running 6 μl of PCR product in a 1% agarose gel with controls and a standard 100-base pair ladder.

All PCR products were treated with Exonuclease I/Shrimp Alkaline Phosphatase (ExoSAP-ITTM; USB Corporation, Cleveland, Ohio, USA) to purify DNA fragments prior to sequencing. Amplicons were sequenced with an Automated 3730 DNA Analyzer (Applied Biosystems, Foster City, California, USA), using the Big Dye® Terminator Sequencing Kit protocol (Applied Biosystems), internal primers described above, and AmpliTaq®-FS DNA Polymerase (Roche Molecular Systems, Inc., Branchburg, New Jersey, USA). Each fragment was sequenced in the forward and reverse directions and contigs were assembled using Sequecher software (Gene Codes Corporation, Ann Arbor, Michigan, USA).

Individual isolates were subjected to a BLAST query to determine their similarities to previously reported sequences and additional reference sequences acquired from GenBank. Ninety-five sequences were aligned using ClustalW (Chenna et al., 2003) with default parameters of MEGA 3.1 software (Kumar et al., 2004). The neighbor-joining

method, using the Kimura two-parameter model with pairwise deletion, was used to build the phylogenetic tree. *Cryptosporidium andersoni* (GenBank AB089285) and *Cryptosporidium muris* (GenBank AF026388) were used as out-groups for the created dendogram.

### Statistical analysis

Environmental factors including land use, habitat, and season and host-related factors, such as age and sex, were analyzed to examine putative associations with a particular zoonotic *Cryptosporidium* genotype. Factors that were found to be significantly associated with a particular genotype in the bivariate association were considered for the multivariate analysis to assess the significance of association of each factor while simultaneously controlling for other factors. All statistical analysis was performed using the PROC LOGISTIC function in SAS® software (SAS software, Version 9.1, SAS Institute Inc., Cary, North Carolina, USA).

## RESULTS

Seventy-seven *Cryptosporidium* isolates were recovered from several small mammal hosts (Table 1). Thirty-six of the isolates examined exhibited high similarity to previously described sequences of *Cryptosporidium* accessioned to GenBank (Table 1). Isolates recovered from two opossums (*Didelphis virginiana*) were identified as the marsupial genotype (GenBank AY120902) commonly found in this host species. *Cryptosporidium* from a house mouse (*Mus musculus*) was identified as the "mouse genotype" (GenBank AF112571). Two isolates, one from a raccoon (*Procyon lotor*) and one from a grey squirrel (*Sciurus carolinensis*), matched with a sequence designated as the "skunk genotype" (GenBank Acc # AY120903). An isolate of *Cryptosporidium baileyi* (GenBank Acc # AF093495), normally associated with birds, was recovered from a chipmunk (*Tamias striatus*). One isolate from a deer mouse (*Peromyscus maniculatus*), five isolates from the meadow vole (*Microtus pennsylvanicus*), and one isolate from a southern red-backed vole (*Clethrionomys gapperi*) shared a genotype similar to those isolated from muskrats, known as the Muskrat II genotype (GenBank AY545547). Six isolates from three different rodent species—*Peromyscus leucopus, Peromyscus maniculatus,* and *Clethrionomys gapperi*—were determined to be *C. parvum*.

There were 17 isolates from a number of wildlife hosts that matched several sequences recovered from environmental samples recovered from storm-water events in New York State. A unique isolate from a short-tailed shrew (*Blarina brevicauda*) was identical to isolate W5 (GenBank AY737594). Another isolate from a southern red-backed vole was similar to the environmental isolate W12 (GenBank AY737558). Environmental isolates W3 (GenBank AY737591), W4 (GenBank AY737593), and W17 (GenBank AY737573) were associated with isolates that formed three host-associated clusters designated as the *Peromyscus* II genotype, Sciuridae II genotype, and Sciuridae I genotype, respectively.

Thirty-three isolates formed five host-associated clusters. Within each cluster there was limited variation, although they remain distinct from other types (Table 2). There were two distinct clusters for the genus *Peromyscus*, designated *Peromyscus* I and *Peromyscus* II genotypes. Each cluster contained both species of *Peromyscus* found in New York State: the deer mouse and the white-footed mouse. The majority of these mice isolates clustered within *Peromyscus* I. A second pair of unique clusters was detected from members of the family Sciuridae, designated Sciuridae I and Sciuridae II genotypes. Isolates in the Sciuridae I cluster were recovered from one red squirrel (*Tamiasciurus hudsonicus*) and five chipmunks. Isolates in the Sciuridae II cluster were recovered from one red squirrel, four chipmunks, and a grey squirrel. A fifth cluster, formed around vole hosts, was made up of three meadow voles and four southern red-backed voles.

### Isolates of novel sequences

The isolates that formed the two clusters, *Peromyscus* I and Vole I, exhibited close relationships between known isolates; however, they appeared distinct enough to

TABLE 1. Genotypes of *Cryptosporidium* isolates from samples previously determined to be positive for *Cryptosporidium* spp. by flotation and/or enzyme-linked immunosorbent assay (Ziegler et al., 2007), examined by polymerase chain reaction using an 18-Small Subunit (SSU) rRNA locus.

| Species | No. | Genotypes |
|---|---|---|
| Opossum (*Didelphis virginiana*) | 2 | Marsupial genotype |
| Short-tailed shrew (*Blarina brevicauda*) | 2 | *Cryptosporidium* species[a] |
| | 1 | *Cryptosporidium* species |
| Big brown bat (*Eptesicus fuscus*) | 2 | *Cryptosporidium* species |
| Chipmunk (*Tamias striatus*) | 5 | Sciuridae I[b] |
| | 3 | Sciuridae II[c] |
| | 1 | *Cryptosporidium baylei* |
| Gray squirrel (*Sciurus carolinensis*) | 1 | *Cryptosporidium* species |
| | 1 | Sciuridae II[c] |
| | 1 | Skunk genotype |
| Red squirrel (*Tamiasciurus hudsonicus*) | 2 | *Cryptosporidium* species |
| | 1 | Sciuridae I[b] |
| | 2 | Sciuridae II[c] |
| White-footed mouse (*Peromyscus leucopus*) | 3 | *Cryptosporidium parvum* |
| | 6 | *Peromyscus* cluster I |
| | 1 | *Peromyscus* cluster II[d] |
| | 7 | *Cryptosporidium* species |
| Deer mouse (*Peromyscus maniculatus*) | 2 | *Cryptosporidium parvum* |
| | 6 | *Peromyscus* cluster I |
| | 2 | *Peromyscus* cluster II[d] |
| | 1 | Muskrat II genotype |
| | 5 | *Cryptosporidium* species |
| Red-backed vole (*Clethrionomys gapperi*) | 1 | *Cryptosporidium parvum* |
| | 4 | Vole cluster |
| | 1 | Muskrat II genotype |
| | 1 | *Cryptosporidium* species |
| | 1 | *Cryptosporidium* species[e] |
| Meadow vole (*Microtus pennsylvanicus*) | 5 | Muskrat II genotype |
| | 4 | Vole cluster |
| House mouse (*Mus musculus*) | 1 | *Cryptosporidium parvum*—mouse type |
| Porcupine (*Erethizon dorsatum*) | 1 | *Cryptosporidium* species |
| Raccoon (*Procyon lotor*) | 1 | Skunk genotype |

[a] W5 (GenBank Acc # AY737594); isolate was highly similar to environmental isolates recovered from storm water events (Jiang et al., 2005).

[b] Isolate W17 (GenBank Acc # AY737573); isolate was highly similar to environmental isolates recovered from storm water events (Jiang et al., 2005).

[c] Isolate W4 (GenBank Acc # AY737593); isolate was highly similar to environmental isolates recovered from storm water events (Jiang et al., 2005).

[d] Isolate W3 (GenBank AY737591); isolate was highly similar to environmental isolates recovered from storm water events (Jiang et al., 2005).

[e] Isolate W12 (GenBank AY737558); isolate was highly similar to environmental isolates recovered from storm water events (Jiang et al., 2005).

represent novel and perhaps host-specific species. There were also 14 isolates, recovered from an assortment of wildlife hosts, in which the phylogenetic relationships were randomly distributed in the upper portion of the dendogram (Fig. 1). The high variation of these genotypes coupled with limited numbers of the hosts they were isolated from suggests these should also be considered novel genotypes.

#### Generalist isolates

Approximately one-third (24) of the isolates were clustered within the upper branch of the tree and were treated as potentially zoonotic in nature. The signif-

TABLE 2. The sequence of four novel genotypes found in rodent species in New York State.

| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Peromyscus_I | AAC | CAA | TTT | AAT | TTG | G-T | GAT | TCA | TAA | TAA | CTT | TAC | GGA | TCA | CAT | TTA | TAT | GTG | ACA | TAT | CAT | TCA | AGT | TTC | TGA | CCT | ATC | AGC | [ 84] |
| Peromyscus_II | ... | ... | .-G | CTC | ... | .-. | ..C | ... | ... | ... | ... | ... | ... | ... | ... | .C- | .T. | ... | ..G | ..C | ... | ... | ... | ... | ... | ... | ... | ... | [ 84] |
| Vole | ... | ... | .A. | T.A | ... | .-. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..T | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | [ 84] |
| Sciuridae_I | ... | ... | ..A | TT. | ... | .-. | ..C | ... | ... | ... | ... | ... | ... | ... | ... | ..T | G.. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | [ 84] |
| Sciuridae_II | ... | ... | .A. | ... | ... | .G. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | [ 84] |
| Peromyscus_I | TTT | AGA | CGG | TAG | GGT | ATT | GGC | CTA | CCG | TGG | CAA | TGA | CGG | GTA | ACG | GGG | AAT | TAG | GGT | TCG | ATT | CCG | GAG | AGG | GAG | CCT | GAG | AAA | [168] |
| Peromyscus_II | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | [168] |
| Vole | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | [168] |
| Sciuridae_I | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | [168] |
| Sciuridae_II | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | [168] |
| Peromyscus_I | CGG | CTA | CCA | CAT | CTA | AGG | AAG | GCA | GCA | GGC | GCG | CAA | ATT | ACC | CAA | TCC | TAA | TAC | AGG | GAG | GTA | GTG | ACA | AGA | AAT | AAC | AAT | ACA | [252] |
| Peromyscus_II | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | [252] |
| Vole | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | [252] |
| Sciuridae_I | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | [252] |
| Sciuridae_II | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | [252] |
| Peromyscus_I | GGA | CTT | TAA | ATA | GTT | TTG | TAA | TTG | GAA | TGA | GTT | AAG | TAT | AAA | CCC | CTT | TAC | AAG | TAT | CAA | TTG | GAG | GGC | AAG | TCT | GGT | GCC | AGC | [336] |
| Peromyscus_II | ... | ... | A-- | T.. | ... | ... | ... | ... | ... | ... | ... | G.. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | [336] |
| Vole | ... | ... | --. | T.. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | [336] |
| Sciuridae_I | ... | ... | .-- | T.G | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | [336] |
| Sciuridae_II | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | [336] |
| Peromyscus_I | AGC | CGC | GGT | AAT | TCC | AGC | TCC | AAT | AGC | GTA | TAT | TAA | AGT | TGT | TGC | AGT | TAA | AAA | GCT | CGT | AGT | TGG | ATT | TCT | GTT | AAT | AAT | TTA | [420] |
| Peromyscus_II | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | .C. | ... | .T. | C.. | [420] |
| Vole | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | [420] |
| Sciuridae_I | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | [420] |
| Sciuridae_II | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | [420] |
| Peromyscus_I | TAT | ATA | ATA | TTT | TTA | TTR | ATA | TTT | ATA | TAA | TAT | TAA | CAT | AAT | TCA | TAT | TAC | TAA | WTT | TTT | ATT | AGT | ATA | TGA | AAT | TTT | ACT | TTG | [504] |
| Peromyscus_II | .G. | .A. | TGC | ... | .-- | --- | GCC | ... | .C. | .GT | .G. | ..G | .C. | TCC | C.G | ... | ... | .G- | --- | --. | T.C | ... | ..G | C.G | .-. | ... | ... | ... | [504] |
| Vole | ... | ... | ... | ... | .-. | A.G | ... | ... | ... | ... | ... | ... | ... | ... | ... | C.. | ... | ..- | --. | A.. | ... | ... | ..G | ... | ... | ... | ... | ... | [504] |
| Sciuridae_I | ... | ... | ... | ... | .G. | .GA | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..- | -.A | ... | T.. | ... | ... | ... | ... | ... | ... | ... | [504] |
| Sciuridae_II | ... | ... | ... | ... | .-. | ..A | ... | ... | ... | ..G | ... | ... | ... | ... | ... | ... | ... | ..- | -.A | ... | TA. | ... | ... | ... | ... | ... | ... | ... | [504] |
| Peromyscus_I | AGA | AAA | TTA | GAG | TGC | TTA | AAG | CAG | GCA | TTT | GCC | TTG | AAT | ACT | CCA | GCA | TGG | AAT | AAT | ATA | AAA | GAT | TTT | TAT | CTT | TTT | TAT | TGG | [588] |
| Peromyscus_II | ... | ... | ... | ... | ... | ... | ... | ... | ..T | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..T | ... | ... | ... | ... | ... | .C. | ... | ... | [588] |
| Vole | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..G | ... | ... | ... | ... | ... | ... | ... | ... | [588] |
| Sciuridae_I | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..A | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..T | ... | ... | ... | ... | ... | .C. | ... | ... | [588] |
| Sciuridae_II | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..A | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | [588] |
| Peromyscus_I | TTC | TAA | GAT | AAA | AAT | AAT | GAT | TAA | TAG | GGA | CAG | TTG | GGG | GCA | TTT | GTA | TTT | AAC | AGT | TAG | AGG | TGA | AAT | TCT | TAG | ATT | TGT | TAA | [672] |
| Peromyscus_II | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | C.. | ... | ... | ... | ... | ... | ... | ... | ... | [672] |
| Vole | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | C.. | ... | ... | ... | ... | ... | ... | ... | ... | [672] |
| Sciuridae_I | ... | ... | ... | ..G | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | C.. | ... | ... | ... | ... | ... | ... | ... | ... | [672] |
| Sciuridae_II | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | C.. | ... | ... | ... | ... | ... | ... | ... | ... | [672] |
| Peromyscus_I | AGA | CAA | ACT | AGT | GCG | AAA | GCA | TTT | GCC | AAG | GAT | GTT | TTC | ATT | AAT | CAA | GAA | CGA | AAG | TTA | GGG | GAT | CGA | AGA | CGA | TCA | GAT | ACC | [756] |
| Peromyscus_II | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | [756] |
| Vole | ... | ... | ... | .A. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | [756] |
| Sciuridae_I | ... | ... | ... | .A. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | [756] |
| Sciuridae_II | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | [756] |

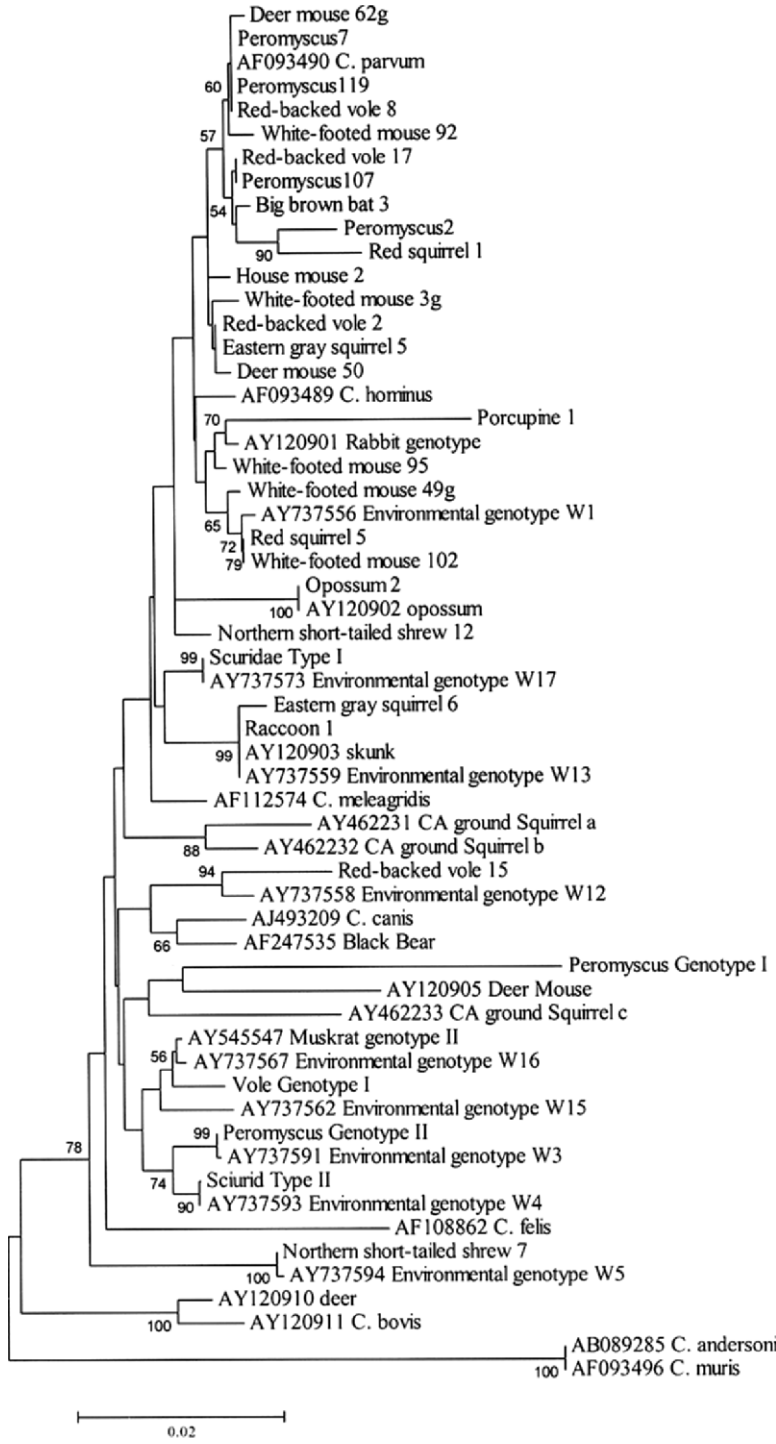


FIGURE 1. A phylogentic dendogram representing the relationships of identified *Cryptosporidium* genotypes from wildlife with known sequences. Using the Kimura two-parameter neighbor-joining and bootstrap (500 replicates) with pairwise deletion.

TABLE 3. Results of bivariate analysis of hypothesized risk factors associated with the likelihood *Cryptosporidium parvum* infection among wildlife hosts in the New York City watershed. Significant factors only.

| Factor | Regression coefficient | Standard error | Raw odds ratio | 90% confidence interval |
|---|---|---|---|---|
| Habitat | | | | |
| Woodland | −1.6739 | 0.7608 | 0.188 | 0.05, 0.66 |
| Commensal | 0 | | 1 | |
| Age | | | | |
| Adult | 1.24 | 0.4996 | 3.44 | 1.51, 7.83 |
| Immature | 0 | | 1 | |
| Season | | | | |
| Summer | −1.5041 | 0.6124 | | |
| Spring | −1.7047 | 0.7953 | 0.182 | 0.05, 0.67 |
| Fall | 0 | | 1 | |

TABLE 4. Ecologic risk factors associated with the likelihood of a host-specific genotype versus *Cryptosporidium parvum* infection among wildlife hosts in the New York City watershed in the multivariate analysis.

| Factor | Regression coefficient | Standard error | Adjusted odds ratio | 90% confidence interval |
|---|---|---|---|---|
| Age | | | | |
| Adult | 1.356 | 0.569 | 3.88 | 1.52, 9.89 |
| Immature | 0.0 | | 1.00 | |
| Season | | | | |
| Spring and summer | −1.783 | 0.580 | 0.20 | 0.07, 0.44 |
| Fall and winter | 0.0 | | 1.0 | |
| Intercept | 0.227 | 0.568 | | |

icance of association between genotype clusters with ecologic and host-associated factors was investigated. The genotypes were grouped into two classes: generalists (identified in more than one species of wildlife) and specialists (host-specific). No significant associations were found between land use ($P=0.63$) and the likelihood of a generalist genotype. The likelihood of a zoonotic genotype of *Cryptosporidium* varied significantly by the habitat, the season of the year, and the age of the animal (Table 3). Isolates recovered from wildlife collected in commensal habitats were more likely to be generalist genotypes when compared to wildlife collected in woodland habitats ($P=0.053$). Isolates recovered from wildlife in the fall were five times more likely to be *C. parvum* when compared to isolates recovered in the summer. Age was significant ($P=0.025$); adult animals were 3.4 times more likely to be infected with a generalist genotype than were immature animals.

The three significant factors—season, age, and habitat—were further examined using multivariate analysis. Only two factors were associated with the likelihood of shedding the zoonotic genotype of *Cryptosporidium* spp.: age of the animal and the season of the year. Seasons were collapsed into two categories: winter was combined with fall, and spring with summer. Older animals were four times more likely to shed the generalist genotype in comparison to immature ones when we controlled for the season of collection (Table 4). Animals captured in the summer or spring were five times less likely to shed the zoonotic genotype compared to animals captured in fall or winter.

## DISCUSSION

This study was conducted to identify the genotypes of *Cryptosporidium* found in wild mammals in the New York City watershed. The ability of some members of the genus to infect multiple host species, including humans, has been established. There are currently seven species recognized as being zoonotic, although the majority of human cases consist of two species: *C. hominis* and *C. parvum* (Caccio, 2005). The former species exhibits an anthroponotic (human-to-human) cycle whereas the latter exhibits a zoonotic (animal-to-human) cycle (Peng et al., 1997). Many multihost parasites maintain three life cycles, which also include a sylvatic (animal-to-animal) cycle seen typically

among wildlife populations (Patz et al., 2000). Overlap of the three life cycles may facilitate the perpetuation of viable pathogens in the environment. Determining whether wildlife harbor zoonotic isolates of *Cryptosporidium* is necessary to assess the potential risk these populations pose to public health regarding the contamination of water supply systems.

There was great diversity among the isolates recovered from the 13 host species. The majority of the isolates examined clustered relative to the taxonomy of the host. The nature of this relationship would suggest host-specific genotypes. However, the specificity of these parasites seems to be associated at a broader taxonomic level rather than being specific to individual species. In some cases, such as with the sciurid species, there are overlaps in the niche they inhabit where cross-species transmission of the parasite might occur. However, the voles and muskrats, in subfamily Arvicolinae, which are found in very different ecologic niches, appear to share the same *Cryptosporidium* genotype. Additionally, the sciurids and deer mice were infected by two distinct genotypes, the Sciuridae I and II and *Peromyscus* I and II, respectively. These findings, which are in agreement with previous studies characterizing *Cryptosporidium* isolates from wildlife, suggest that many species are infected by host-specific strains. Some studies show clustering of isolates at broader taxonomic levels. A study of furbearing mammals in the Chesapeake Bay watershed identified *Cryptosporidium canis*, a type found in domestic dogs, in foxes; raccoons were infected with another carnivore genotype previously reported in skunks (Zhou et al., 2004). A survey of *Cryptosporidium* genotypes found in zoo animals in the Czech Republic described the cervid genotype from a variety of cervid hosts (Ryan et al., 2003). Other studies have shown multiple genotypes isolated from host groups. A study of the eastern grey kangaroo in Australia classified three types of isolates specific to a range of marsupials (Power et al., 2004). Atwil et al. (2004) found three distinct genotypes in a population of California ground squirrels; although none of those isolates were recovered in this study it is possible that other species of squirrels within the California region are infected with the Sbey03a-c genotypes.

Several *Cryptosporidium* isolates identified with rodents in this study matched closely with isolates recovered from stormwater runoff within the same region (Jiang et al., 2005), supporting the authors' assumptions regarding wildlife as sources of these genotypes. Although the number of host species found with these genotypes is too limited to label them as the definitive sources, these findings highlight two points. First, the small size of rodent hosts and the amount fecal output of individuals underscore the importance of the host population size on the levels of contamination in the environment. Secondly, although some hosts such as deer and deer mice are found distributed throughout the environment, others such as voles and house mice are restricted within by habitat constraints. The habitat features of watershed drainages may influence the number of *Cryptosporidium* genotypes found in storm runoff.

Six isolates recovered were identified as *C. parvum*; all hosts were rodents and included five deer mice and one red-backed vole. The identification of zoonotic isolates of *Cryptosporidium* has been previously reported from house mice (Morgan et al., 1999) and Eastern chipmunks (Perz and Le Blancq, 2001). As the exchange of these pathogens between cattle and wildlife have become of increasing interest, the issue of pseudoinfection in either population is an important factor to consider. One of the limitations of many of the diagnostic methods currently employed in *Cryptosporidium* research is the ability to discriminate between active versus transient infection; this includes many molecular methods such as PCR (Ziegler et al., 2007). Although not applied in this study, confirmation of true *C. parvum* infection has been attained

through histologic examination of the host intestines (Graczyk and Cranfield, 1998). However, infectivity studies have demonstrated the potential for cross-transmission exists between rodents and cattle (Donskow et al., 2005). Rodents, because of their close proximity to humans and livestock, pose a potential threat as maintenance reservoir for *Cryptosporidium*. Although the prevalence of these genotypes among infected wildlife is unclear, host species that do shed these zoonotic genotypes in the watershed ecosystem could become a source for cattle, which produce large amounts of manure and by picking up the infection amplify the risk by more than 5,000 times.

There were 23 other *Cryptosporidium* isolates that were not completely classified genotypically in this study and their zoonotic potential remains to be elucidated in the future. These unique isolates were included as zoonotic (generalists) in the risk factor analysis for two reasons. First, the high degree of heterogeneity among these unidentified isolates which could be attributed to their multihost nature. Parasitic generalists may exhibit a greater genotypic diversity (Read and Taylor, 2001). Secondly, the taxonomy of *Cryptosporidium* is confounded by a number of factors such as a lack of standardization when defining species (Xiao et al., 2002).

Analyses based on the assumption that these genotypes are potentially zoonotic compared to the host specialist clusters demonstrated sensible associations with the putative risk factors. The two habitat types, commensal and woodland, serve as proxies for interactions between wildlife, livestock, and humans (Ziegler et al., 2007). There was significant association between the proportions of generalist *Cryptosporidium* and hosts found in commensal habitats (buildings, barnyards, and residential areas) when compared to the woodland habitat. The associations between generalist isolates and age and season showed a correlated pattern. The higher association in fall and winter than in spring and summer may be linked to the breeding cycles of many of the wildlife species in the study. The majority of animals tend to give birth in the warmer time of year and later in the fall many young begin to disperse as adults. Additionally, within the study region there is seasonal calving during fall on farms, which potentially contaminates the environment with zoonotic isolates.

The potential association between several putative risk factors and the likelihood of zoonotic genotype of *C. parvum* was investigated to identify factors that either exacerbate or modify the risk to wildlife species. It is important to consider wildlife within an ecologic context before their role as a source of zoonotic *Cryptosporidium* is determined. Sylvatic cycles of zoonotic *Cryptosporidium* may require close proximity to agricultural practice such as dairy farms. No reports of previous studies have been found that investigate this risk in watersheds where the predominant population is dairy cattle. Atwill et al. (2001) investigated the risk associated with shedding *C. parvum* by the California ground squirrel in a watershed where the predominant population was beef cattle. The variations of a novel genotype found in this population of squirrels were different from the sciurid genotypes in this study, and no isolates of *C. parvum* were recovered. The difference in concentration of dairy versus beef herds within a given area could account for the absence of zoonotic *Cryptosporidium*.

As a result of the reports that humans and cattle have a shared susceptibility to *C. parvum*, livestock populations have been extensively surveyed (Olson et al., 2004). Genotyping isolates from cattle has shown that in addition to zoonotic strains these species are also infected with the host-specific protozoa *C. andersoni* and *Cryptosporidium bovis* (Santin et al., 2004; Fayer et al., 2006). Wildlife populations have not been as extensively studied as cattle populations; thus, our under-

standing of genotypic diversity of *Cryptosporidium* in these species is limited. The findings of this study showed that, similarly to cattle, several wildlife species are infected with both host-specific genotypes and generalist strains of *Cryptosporidium.* A greater effort that includes molecular techniques and host ecology is needed in order to characterize the risk that wildlife populations pose to the contamination of watershed ecosystems.

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