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Phylogenetic Analysis Implicates Birds as a Source of *Cryptosporidium* spp. Oocysts in Agricultural Watersheds

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Cryptosporidium parvum and *C. hominis* are protozoan parasites responsible for cryptosporidiosis, an acute gastrointestinal illness that can be life-threatening for immunocompromised persons. Sources and genotypes of *Cryptosporidium* oocysts were investigated in two agricultural areas within the Wachusett Reservoir watershed, a drinking water source for Boston, Massachusetts. Two brooks (denoted Brook SF and Brook JF, respectively), each downgradient from a dairy farm, were chosen as sample sites. For one year, Brooks SF and JF were sampled monthly; oocysts were detected in 6 (50%) out of 12 samples from Brook JF, and no oocysts were detected in Brook SF. Oocyst genotypes from agricultural surface waters were compared to oocyst genotypes from Genbank, as well as fecal samples of cattle and birds, using phylogenetic analysis of a hypervariable region of the 18S rRNA gene by both neighbor-joining and parsimony methods. Results show extensive heterogeneity among *Cryptosporidium* spp. 18S rRNA sequences, and also suggest that birds are an oocyst source in this watershed. Principal components analysis showed oocyst presence correlating strongly with seasonal factors, and oocysts in surface waters were only detected in the summer through late fall, co-incident with the presence of migratory birds in this watershed. If birds are confirmed to be an important source of oocysts infectious to humans, the data suggest that protection of raw drinking water supplies in some agricultural areas may depend upon management and control of resident and migratory bird populations.

Introduction

Cryptosporidium parvum and *C. hominis* are protozoan parasites responsible for cryptosporidiosis, an acute gastrointestinal illness that can be life-threatening for immunocompromised persons. Although *C. parvum* and *C. hominis* are the species most often associated with human cryptosporidiosis, multiple species of *Cryptosporidium* are recognized (*1*), and some of these other *Cryptosporidium* species have also been associated with illness among at-risk individuals (*2*−*4*). Oocysts are spread from host to host via fecal−oral transmission, and outbreaks have been associated with ingestion of contaminated food and water and with exposure to contaminated recreational water (*5*−*12*). Although host-adapted genotypes of *Cryptosporidium* spp. have been identified (*13*), the reported host-range of oocyst genotypes, as well as the genetic diversity of *Cryptosporidium* spp. oocysts, continues to broaden as more environmental studies are performed. Thus, genotyping oocysts recovered from surface water supplies does not provide absolute answers with respect to the sources of waterborne oocysts but can provide information on likely oocyst sources when combined with (i) seasonal detection of oocysts in water supplies, (ii) knowledge of the seasonal presence of local host animals, and (iii) identification of oocyst genotypes associated with the host animals present in the watershed.

Agriculture is widely recognized as a source of *Cryptosporidium* spp. oocysts in the environment. Heitman et al. (*14*) studied *Cryptosporidium* spp. in wildlife, sewage, and agricultural sources and found the highest *Cryptosporidium* spp. concentrations from agricultural sources. Agricultural runoff has been identified as the source of oocysts in a number of waterborne outbreaks of cryptosporidiosis (*5*), and a foodborne cryptosporidiosis outbreak from fresh-pressed apple cider was attributed to contamination of apples with fecal material from an infected calf on a farm (*6*). Thus, identification of oocyst sources in agricultural watersheds will aid in improved environmental management to safeguard food and water supplies from oocyst contamination.

The goal of the present study was to assess the sources and genotypes of *Cryptosporidium* oocysts in two agricultural areas within the Wachusett Reservoir watershed, an important drinking water source for Boston, Massachusetts. Two brooks, each downgradient from a dairy farm, were chosen as sample sites. *Cryptosporidium* spp. phylogeny based on the 18S rRNA gene has been described (*15*), and molecular characterization of the 18S rRNA gene has been applied to previous environmental studies (*14*, *16*−*20*). In the present study, a hypervariable region of the 18S rRNA gene of oocysts recovered from both fecal and surface water samples was sequenced and used in a phylogenetic analysis to identify the genotypes and likely sources of oocysts.

Materials and Methods

Oocysts. GCH1 *Cryptosporidium parvum* oocysts were a kind gift of Giovanni Widmer at Tufts University School of Veterinary Medicine in North Grafton, Massachusetts.

Site Selection and Sample Collection. Two dairy farms in the Wachusett Reservoir watershed, Farms SF and JF, were chosen as sample sites. Farms SF and JF are located upgradient from small brooks, designated Brook SF and Brook JF, respectively. Surface water samples were collected monthly from Brooks SF and JF for one year beginning June 2001 and ending May 2002. One additional water sample from Brook SF in March 1999, collected during a previous study (*19*), was included in this analysis. Fecal samples collected during previous studies but included in this analysis include an adult cow on Farm SF (*19*), a composite manure pit sample on Farm JF (*19*), and two geese (one each from Illinois and New York) (*20*).

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TABLE 1. Summary of Brook JF Samples that were Positive for *Cryptosporidium* spp

date	volume filtered (L)	nested PCR	number of clones analyzed by *Not*I/*Nde*I digestion	number of clones sequenced	number of genotypes identified[a]	sample ID[b]
Jun. 2001	103	+	12	5	2	JF#1 (Jun. 2001), JF#2 (Jun. 2001)
Jul. 2001	103	+/ND[c]				
Aug. 2001	109	+	6	6	1	JF#3 (Aug. 2001)
Oct. 2001	98	+/ND				
Nov. 2001	102	+	7	3	3	JF#4 (Nov. 2001), JF#5 (Nov. 2001), JF#6 (Nov. 2001)
Apr. 2002	96	+/ND				

[a] When multiple clones were sequenced with less than 1% difference, a consensus sequence was used for the phylogenetic analysis. [b] Label used to denote the genetic sequence of the sample in Figure 1. [c] ND = sample was positive by nested PCR but the genetic sequence was not determined.

Surface waters were filtered through Gelman Envirochek Sampling Capsules (Pall Gelman Sciences, Inc., Ann Arbor, MI) at 1−2 L min^{-1} according to manufacturer's recommendations. Water was filtered from a depth below the water surface of approximately 0.1 m; care was taken to filter from the middle of the water column and to avoid disturbance of bottom sediments. Filtration continued for 1 h or until the backpressure exceeded the filter rating (207 kiloPascal, or 30 lb/in^2 [psi]). On average, 55 L of water were filtered from Brook SF (SD = 32 L) and 96 L of water were filtered from Brook JF (SD = 13 L). Filters were transported to the laboratory on ice, and samples were eluted according to manufacturer's recommendations within 24 h of sample collection. Eluted solids were resuspended in 10 mL of laboratory-grade water (Milli-Q System; Millipore Corp., Bedford, MA) for each 0.5 mL of solids, stored at 4 °C, and processed within 24 h.

Immunomagnetic Separation of Oocysts. Oocysts were purified from water samples using immunomagnetic separation (IMS) with the Crypto-Scan IMS kit (ImmuCell, Portland, ME) as previously described (*19*). Positive and negative IMS controls were processed with each set of field samples. Positive IMS controls consisted of 9.5 mL of laboratory-grade water and 500 μL of a 10^4 oocyst mL^{-1} suspension; negative IMS controls consisted of 10 mL of laboratory-grade water.

Genomic DNA Extraction. Oocyst DNA was extracted from the IMS products with phenol chloroform and precipitated with ethanol as previously described (*19*). Positive and negative DNA extraction controls were included with each set of field samples as previously described (*19*).

Nested PCR Assay. Nested PCR amplification of a hypervariable region of the 18S rRNA gene (approximately 434-bp in length) was performed as previously described (*20*) using nested PCR primers designed by Johnson et al. (*21*). Positive and negative PCR controls were included with each set of water or fecal samples as previously described (*20*).

Cloning. Secondary PCR products positive for *Cryptosporidium* spp. were cloned into the pGEM-T Easy Vector System (Promega Corporation, Madison, WI) and used to transform XL-1 Blue *E. coli* cells (Stratagene, La Jolla, CA) as previously described (*20*). Restriction digestion with *Nde*I was performed to identify any heterogeneity among the clones (*19*, *20*).

Sequencing. Representative clones of the secondary PCR products were sequenced on an ABI Prism 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA) using a Big Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (PE Applied Biosystems). If multiple *Nde*I digestion patterns existed among clones from a given sample, at least one clone of each digestion pattern was sequenced. At least three clones for each positive sample were sequenced (Table 1), and data were confirmed by sequencing both strands of each clone. When multiple clones were sequenced with less than 1% difference, the consensus sequence was used in the phylogenetic analysis.

Phylogenetic Analysis. Sequences were aligned manually, based on the secondary structure of the 18S rRNA, using GCG software (Genetics Computer Group, Madison, WI). Sequence regions of variable length, or positions which otherwise could not be aligned with certainty, were eliminated from the phylogenetic analyses. Phylogenetic Analysis Using Parsimony (PAUP), beta version 4.0 (*22*), was used to create both neighbor-joining and parsimony trees from the GCG alignments. Construction of neighbor-joining trees was based on the evolutionary distances between different isolates calculated by the Kimura two-parameter analysis. Construction of parsimony trees was performed with a heuristic search using default parameters. Statistical support for the resulting trees was tested using 1000 pseudoreplicates of the bootstrap test; only values above 50% were reported, and bootstrap values greater than 70% were considered significant (*23*).

Water Quality Data Collection and Analysis. During surface water collection, water temperature (°C), dissolved oxygen (mg/L and % saturation), pH, specific conductivity (μS/cm), and turbidity (ntu) were recorded. Temperature, dissolved oxygen, pH, and specific conductivity were measured with Minisonde probes and a Surveyor 4 (Hydrolab Corporation, Austin, TX). Turbidity was measured with a DRT-15CE Portable Turbidimeter (HF Scientific, Inc., Fort Myers, FL). Principal components analysis, performed with StatView software (SAS Institute, Cary, NC), was used for factor extraction to describe interrelationships among *Cryptosporidium* contamination and the recorded parameters. Samples were assigned a value of 1 (positive for *Cryptosporidium* spp. oocysts) or 0 (negative for *Cryptosporidium* spp. oocysts).

Results

Prevalence of Oocyst Contamination. Brook JF was sampled 12 times during the year, and 6 samples (50%) were positive for *Cryptosporidium* spp. by nested PCR (Table 1). Three of the 6 positive samples were successfully cloned and sequenced. Multiple 18S rRNA sequences were detected in two of the Brook JF samples (Jun. 2001 and Nov. 2001), indicating a mixed population of *Cryptosporidium* genotypes in these waters. Diversity (in terms of % base pair difference) between the two sequences identified in the June 2001 sample (JF#1 and JF#2) was 2.4% (10 out of 425 nucleotides). Diversity among the sequences identified in the November 2001 sample was (i) 4.2% (18 out of 432 nucleotides) between JF#4 and JF#5, (ii) 3.5% (15 out of 432 nucleotides) between JF#4 and JF#6, and (iii) 1.6% (7 out of 432 nucleotides) between JF#5 and JF#6. Brook SF was sampled 12 times throughout the year and was never observed to be positive for *Cryptosporidium* spp. oocysts.

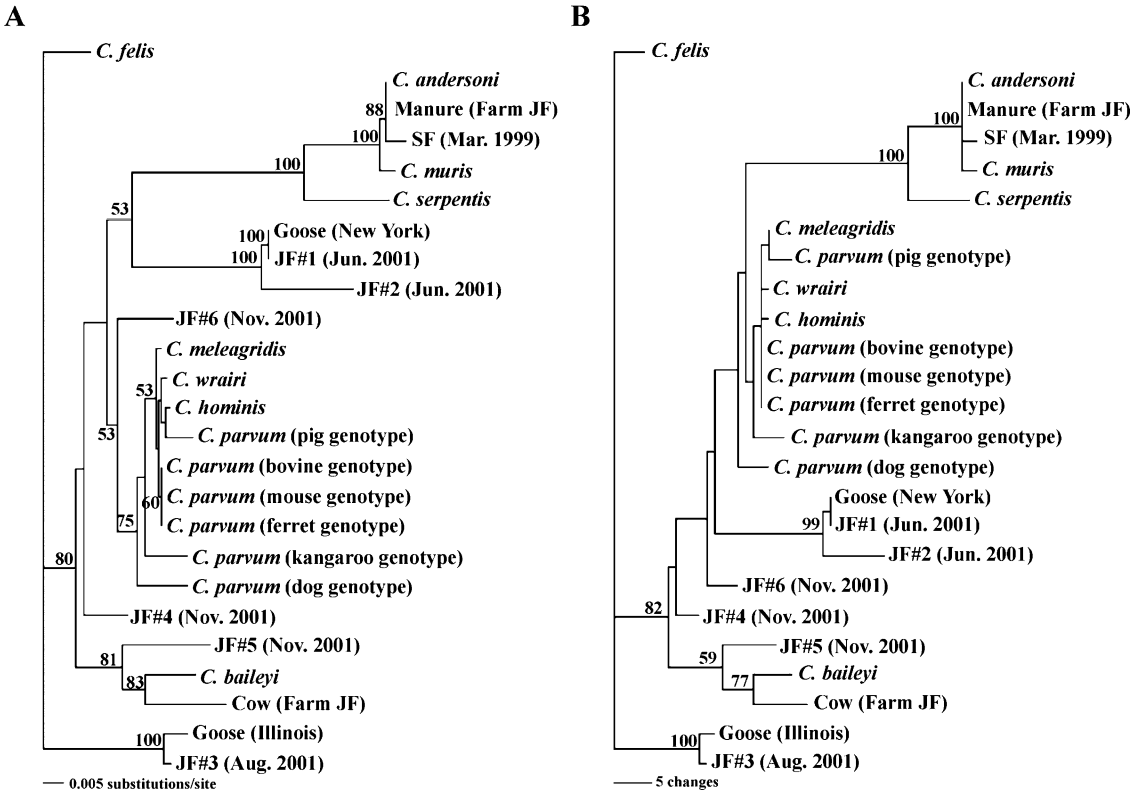

FIGURE 1. (A) Neighbor-joining and (B) parsimony trees based on a hypervariable region of the 18S rRNA gene (corresponding to nucleotides 601−1035 of *C. parvum* L16996 in GenBank). GenBank accession numbers are AB089285 (*C. andersoni*), L19068 (*C. baileyi*), AF112575 (*C. felis*), AF112574 (*C. meleagridis*), AB089284 (*C. muris*), AF093489 (*C. hominis*), AF093493 (*C. parvum* bovine genotype), AF112571 (*C. parvum* mouse genotype), AF112572 (*C. parvum* ferret genotype), AF115377 (*C. parvum* pig genotype), AF112576 (*C. parvum* dog genotype), AF112570 (*C. parvum* kangaroo genotype), AF093499 (*C. serpentis*), and U11440 (*C. wrairi*), AY324641 (Goose, New York), AY324638 (Goose, Illinois), EF060289−EF060294 (JF #1−6, respectively), EF060295 (Cow, Farm JF), EF060296 (Manure, Farm JF), and EF060297 (SF Mar. 1999). Samples collected from previous studies include Manure (Farm JF) (*19*), Cow (Farm JF) (*19*), SF (Mar. 1999) (*19*), Goose (New York) (*20*), and Goose (Illinois) (*20*). Bootstrap values greater than 50% are indicated in bold at their respective nodes.

Phylogenetic Analysis. Phylogenetic trees constructed by both neighbor-joining and parsimony methods (Figure 1) show that sequences from Brook SF and the manure pit on Farm JF (*19*) form a single clade with *C. andersoni* and *C. muris* in 100% of bootstrap replicates. Although it is not possible to infer precise relationships between evolutionary distance estimates and degree of taxonomic differentiation, the sequence from the manure pit was indistinguishable from *C. andersoni* (evolutionary distance = 0.000) and the sequence from Brook SF was closest to *C. andersoni* (with an evolutionary distance of 0.005, identical to that between *C. hominis* and *C. meleagridis*). The evolutionary distances between known species and genotypes of *Cryptosporidium* are provided in Table 2 to provide a baseline for comparison with the environmental genotypes detected in this study.

The sequence from the adult cow on Farm JF (*19*) formed a clade with *C. baileyi* with significant bootstrap values in both the neighbor-joining (83%) and parsimony (77%) phylogenetic analyses (Figure 1). The cow-derived sequence was most closely related to *C. baileyi* with an evolutionary distance of 0.031, comparable to the evolutionary distance between *C. felis* and *C. parvum* (Table 2).

Sequences JF#1 and JF#2 recovered from Brook JF in June 2001 did not form a clade with any existing taxonomic group, yet they grouped with a sequence recovered from a Canada goose in New York (*20*) with significant bootstrap values by both neighbor-joining (100%) and parsimony (99%) analyses (Figure 1). JF#1 was indistinguishable from the goose-derived sequence (evolutionary distance = 0.000), but the similarity between the goose-derived sequence and JF#2 (evolutionary distance = 0.023) was comparable to that between *C. hominis* and *C. parvum* dog genotype (Figure 2). The evolutionary distances between JF#1 and (i) *C. baileyi* (0.069) and (ii) *C. meleagridis* (0.047), respectively, were the same or larger than the evolutionary distance between *C. baileyi* and *C. meleagridis* (Table 2).

Similarly, the sequences JF#4, JF#5, and JF#6 from Brook JF in November 2001 did not form a well-supported clade with any existing taxonomic group (Figure 1). JF#4 and JF#6 were most closely related to *C. wrairi* and *C. meleagridis* with equal evolutionary distances of 0.028 and 0.023, respectively. JF#5 was most closely related to *C. baileyi* with an evolutionary distance of 0.039, identical to the distance between *C. andersoni* and *C. serpentis* (Table 2).

The sequence JF#3 recovered from Brook JF in August 2001 formed a well-supported independent clade with a *Cryptosporidium* spp. sequence recovered from a goose in Illinois (*20*) with bootstrap values of 100% by both neighbor-joining and parsimony analyses (Figure 1). The evolutionary distance between the goose-derived sequence and JF#3 was 0.008, identical to the distance between *C. hominis* and *C. parvum* pig genotype (Table 2). The evolutionary distance between JF#3 and all other sequences in the analysis ranged from 0.042 to 0.102, on par with the distances between biologically distinct species of *Cryptosporidium* (Table 2).

Water Quality Correlations. Surface water temperatures (Figure 2) showed the expected seasonality, ranging from a low of near 0 °C in January to a high of 18 °C in July. *Cryptosporidium* spp. oocysts were detected in Brook JF only during the warmer months and were absent from samples collected at water temperatures less than 6 °C.

TABLE 2. Kimura Two-Parameter Distance Matrix (Substitutions Per Site)[a]

	C. felis	*C. andersoni*	*C. muris*	*C. serpentis*	*C. baileyi*	*C. meleagridis*	*C. wrairi*	*C. hominis*	*C. parvum* (bovine)	*C. parvum* (mouse)	*C. parvum* (ferret)	*C. parvum* (pig)	*C. parvum* (dog)
C. felis	–												
C. andersoni	0.099	–											
C. muris	0.102	0.005	–										
C. serpentis	0.088	0.039	0.041	–									
C. baileyi	0.052	0.085	0.082	0.085	–								
C. meleagridis	0.036	0.074	0.077	0.077	0.047	–							
C. wrairi	0.042	0.076	0.080	0.079	0.049	0.005	–						
C. hominis	0.036	0.079	0.083	0.082	0.052	0.005	0.005	–					
***C. parvum* (bovine)**	0.039	0.076	0.080	0.080	0.049	0.003	0.003	0.003	–				
***C. parvum* (mouse)**	0.039	0.076	0.080	0.080	0.049	0.003	0.003	0.003	0.000	–			
***C. parvum* (ferret)**	0.039	0.076	0.080	0.080	0.049	0.003	0.003	0.003	0.000	0.000	–		
***C. parvum* (pig)**	0.034	0.079	0.082	0.082	0.052	0.008	0.008	0.008	0.010	0.010	0.010	–	
***C. parvum* (dog)**	0.047	0.088	0.085	0.091	0.052	0.015	0.020	0.020	0.018	0.018	0.018	0.023	–
***C. parvum* (kangaroo)**	0.036	0.076	0.080	0.091	0.052	0.015	0.015	0.015	0.013	0.013	0.013	0.023	0.026

[a] GenBank accession numbers are AF112575 (*C. felis*), AB089285 (*C. andersoni*), AB089284 (*C. muris*), AF093499 (*C. serpentis*), L19068 (*C. baileyi*), AF112574 (*C. meleagridis*), U11440 (*C. wrairi*), AF093489 (*C. hominis*), AF093493 (*C. parvum* bovine genotype), AF112571 (*C. parvum* mouse genotype), AF112572 (*C. parvum* ferret genotype), AF115377 (*C. parvum* pig genotype), AF112576 (*C. parvum* dog genotype), and AF112570 (*C. parvum* kangaroo genotype).

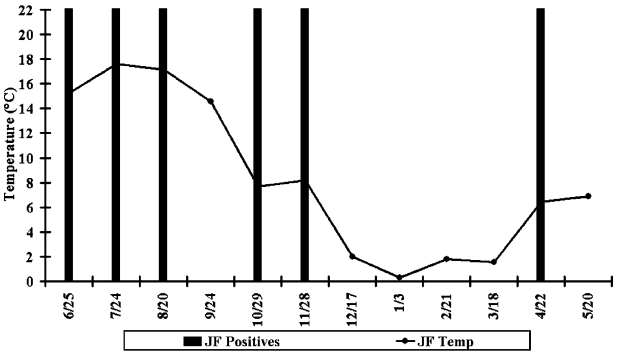

FIGURE 2. JF water temperature plotted against sample date to illustrate seasonal trends. Solid line is water temperature data for JF. Dark bars indicate dates when JF was positive for *Cryptosporidium* spp.

TABLE 3. Oblique Factor Extractions Derived from Principal Components Analysis

	F1[a] (C)[b]	F2 (S & I)[c–e]
oocysts[f]	0.04	**0.74**
volume filtered (L)	**0.80**	0.08
temperature (°C)	−**0.90**	**1.04**
pH	−0.31	**1.05**
specific conductivity (μS/cm)	−0.21	−**0.76**
turbidity (ntu)	−**0.89**	0.08
dissolved oxygen (mg/L)	**1.12**	−0.38

[a] Factors (F1 and F2) were derived for the combined data set of Brook JF and Brook SF. Values in the table (i.e., factor loadings) show the correlation of each variable with each factor (values close to 0 indicate low correlation, values close to 1 indicate high correlation). High factor loadings (above 0.50) are shown in bold. Factors were assigned descriptive titles (C, S, or I) based upon the variables with highest factor loadings. [b] C = Cleanliness factor. Samples with a large filtered volume, low turbidity, and high dissolved oxygen imply high-quality, clean surface water. Note that because volume filtered was determined on the basis of filter backpressure, large filtered volume is likely a surrogate for suspended solids and hence turbidity. [c] S = Seasonal factor. Samples of high temperature and high pH are indicative of late spring and summer seasons. [d] I = Ionic strength factor. Samples of higher specific conductivity imply, in the absence of large qualitative changes in ionic composition, waters of higher ionic strength. [e] S&I = Seasonal and Ionic strength factor. Factor has high loadings from temperature, pH, and specific conductivity. [f] Presence or absence of oocysts. For factor analysis, "oocysts" variable was assigned a value of 1 (present) or 0 (absent).

Results of the principal components analysis are shown in Table 3. Two factors were extracted that were named "sample cleanliness" (F1) and a combination of "season and ionic strength" (F2). Oocyst presence contributed significantly to F2, the season and ionic strength factor, only.

Discussion

Environmental samples of *Cryptosporidium* spp. oocysts have been shown to exhibit extensive heterogeneity among 18S rRNA gene sequences (*16*, *17*, *24*). The majority of the sequences recovered from the present study could not be assigned with confidence to any previously well-defined taxonomic group. Although biological and phenotypic data are needed to make conclusive identifications, the phylogenetic analysis suggests that these sequences may represent novel genotypes or perhaps even uncharacterized species of *Cryptosporidium*. Additional data will be needed to characterize the full extent of this heterogeneity and aid in the interpretation of oocyst sources and species.

These data do, however, provide insight into the dynamics of *Cryptosporidium* spp. in agricultural watersheds. Cattle are susceptible to infection with *C. parvum* and *C. andersoni*

(*25*−*29*), and *C. andersoni* 18S rDNA was detected in the manure pit on Farm JF. More surprising, however, was the identification of a novel 18S rRNA gene sequence from an adult cow on Farm JF. The evolutionary distance of this 18S rRNA gene sequence from known *Cryptosporidium* species suggests that it represents a previously uncharacterized species, although additional morphological and biological data are needed to confirm a taxonomic designation. This finding further suggests that cows may act as mechanical or biological vectors of *Cryptosporidium* species other than *C. parvum*, *C. muris*, and *C. andersoni*; the significance of such potentially novel species for human health requires further investigation.

It is also suggested that birds may be a source of *Cryptosporidium* spp. oocysts in this watershed, given the phylogenetic relationships inferred between oocysts recovered from Brook JF and those recovered from geese, as well as the fact that the cow-derived 18S rRNA gene sequence was most similar to that of *C. baileyi*, which is known to infect birds. The extent to which birds can promote *Cryptosporidium* spp. oocyst transmission is becoming increasingly recognized. Traditionally, *C. baileyi* and *C. meleagridis* were the only two *Cryptosporidium* species known to infect birds, but more recent studies have proposed two novel species of *Cryptosporidium*, *C. galli* and *C. blagburni*, in finches (*30*, *31*) and have shown that Canada geese shed oocysts with a much broader range of 18S rRNA genotypes than previously characterized (*20*). *Cryptosporidium* spp. oocysts have also been recovered from the feces of gulls (*32*), and a report by Graczyk et al. (*33*) of zoonotic *C. parvum* in Canada geese showed that birds can be carriers of infectious oocysts. The fact that the infectivity of *C. parvum* oocysts for neonatal BALB/c mice has been demonstrated to be retained upon intestinal passage through ducks (*34*) and geese (*33*, *35*) further supports the hypothesis that birds can be vectors of infectious oocysts in the environment.

The link between birds and agricultural watersheds is plausible. Canada geese are grazers and often feed in large open areas typical of farms (*36*). Geese and other birds have been observed in the watersheds of the current study; and, in an agricultural region near the Chesapeake Bay, geese were actually observed to wander behind cattle and pick up undigested corn from their feces (*33*). The potential impact of geese on surface water quality in agricultural areas of the Wachusett Reservoir watershed is further supported by the water quality data. Results of the factor analysis (Table 3) show oocyst presence correlating strongly with seasonal factors: oocyst presence (0.74) shows a strong positive correlation with temperature (1.04) in F2. The largest bird populations in this northern watershed are found in the warm summer months, co-incident with the observed trend of oocyst contamination of agriculture-influenced surface waters during warmer water temperatures (Figure 2).

The seasonal pattern of oocyst detection in Brook JF does not correspond with previously reported seasonal trends of cattle shedding, further supporting the hypothesis that birds rather than cattle are exerting the more significant influence on *Cryptosporidium* presence in the surface waters of these watersheds. Wade et al. (*37*) found no seasonal pattern of *C. parvum* or *C. andersoni* infection of dairy cattle, and Bodley-Tickell et al. (*38*) found oocysts in surface waters draining a livestock farm throughout the year, suggesting that cattle can shed *Cryptosporidium* spp. oocysts year-round. Oocyst shedding by dairy herds was found to be higher in the winter than in the summer (*39*), however, and maximum concentrations in surface waters draining a livestock farm were found during the autumn and winter (*38*). By contrast, *Cryptosporidium* spp. were detected in Brook JF from summer through late fall, and no oocysts were detected in the winter or early spring. The seasonal pattern observed in Brook JF is also in contrast to the detection of oocysts in wildlife-influenced surface waters in the late fall through early spring (*19*). This variability of seasonal pattern in surface waters susceptible to oocyst contamination from different sources suggests that source dynamics have a role in the presence of *Cryptosporidium* spp. in surface waters. Migratory Canada geese are present in this northern agricultural watershed during the warmer summer months and fly south for the colder winter months, coinciding with the seasonal detection of *Cryptosporidium* spp. oocysts in Brook JF.

Further sampling from adult cattle and calves on Farms SF and JF is needed to more conclusively identify birds, and not domesticated agricultural animals, as the important source of oocysts detected in this agricultural watershed. (More exhaustive sampling was not possible in the current study due to farmers' concerns about the ramifications of oocyst detection in their animal herds.) Given the close proximity of cattle and birds in the large open areas typical of farms, the potential for cross-transmission of oocysts between birds and cattle is very real and confounds the ability to identify the primary oocyst source. However, when the genotyping and seasonal data are considered as a whole, results from this study suggest that birds are a source of *Cryptosporidium* spp. oocysts in this agricultural watershed. The heterogeneity of *Cryptosporidium* 18S rRNA genotypes recovered from birds has been found to be extensive, however, and oocysts recovered from farms or agriculture-influenced surface waters cannot be presumed infectious to humans without further molecular and biological characterization. However, if birds are confirmed to be an important source of oocysts infectious to humans, such knowledge could aid in developing agricultural watershed management practices that could help to reduce *Cryptosporidium* spp. concentrations and protect against waterborne cryptosporidiosis.

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