JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 2003, p. 5254–5257 Vol. 41, No. 11
0095-1137/03/$08.00+0 DOI: 10.1128/JCM.41.11.5254–5257.2003

Molecular Surveillance of *Cryptosporidium* spp. in Raw Wastewater in Milwaukee: Implications for Understanding Outbreak Occurrence and Transmission Dynamics

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Received 5 May 2003/Returned for modification 16 July 2003/Accepted 20 August 2003

Six *Cryptosporidium* spp. were found in 50 of 179 Milwaukee wastewater samples collected weekly over a year. Of the eight subtypes of *Cryptosporidium hominis* and *Cryptosporidium parvum* present, allele Ib was found in 14 of 16 samples, and its sequence was identical to that of the subtype in human samples from the 1993 Milwaukee outbreak of cryptosporidiosis.

Two *Cryptosporidium* spp., *Cryptosporidium hominis* (previously known as the *Cryptosporidium parvum* human genotype or genotype 1) and *C. parvum* (previously know as *C. parvum* bovine genotype or genotype 2), are responsible for cryptosporidiosis outbreaks (3, 5). *C. hominis* is found almost exclusively in humans, while *C. parvum* is found in humans and domestic ruminants (5). *C. hominis* has been responsible for more outbreaks than *C. parvum*, even in countries where *C. parvum* is the predominant *Cryptosporidium* parasite in humans (3, 5). Recently, DNA sequence characterizations of several genes have identified intragenetic variations in *C. hominis* and *C. parvum*. These tools have been used effectively in the investigation of several outbreaks. One such subtyping tool is based on sequence analysis of the 60-kDa glycoprotein (GP60), which divides *C. hominis* and *C. parvum* into several allelic groups, each of which consists of multiple subtypes (1, 2, 4, 6, 7). One of the allele families, Ib, was responsible for the waterborne outbreaks of cryptosporidiosis in Milwaukee, Wis., which caused illness in over 400,000 people (6).

The distribution of *Cryptosporidium* species was previously characterized in 49 wastewater samples in Milwaukee (9). *Cryptosporidium andersoni* was identified as the most common parasite in wastewater, followed by *Cryptosporidium muris*, *C. hominis*, *C. parvum*, *Cryptosporidium canis*, *Cryptosporidium felis*, and the *Cryptosporidium* cervid genotype (W4). In the present study, we examined the seasonal distribution of *Cryptosporidium* species in raw Milwaukee wastewater and subtyped *C. hominis* and *C. parvum* isolates from wastewater to assess current cryptosporidiosis transmission in Milwaukee and its relationship to the 1993 outbreak.

Wastewater sample collection and processing. A total of 179 raw wastewater samples were obtained from the Jones Island Wastewater Treatment Plant in Milwaukee from August 2000 to July 2001 with an average of two to four samples per week (no more than one sample per day). Fifteen more samples were also obtained in March 2002. Each sample was a composite from the three separate siphons that delivered influent to the treatment plant: domestic sewage, combined domestic and industrial sewage, and wastewater from the deep tunnel, which was comprised of infiltration and inflow during dry-weather periods and storm water-diluted sewage during periods of precipitation. For each siphon, a small quantity (10 to 15 ml) was automatically drawn every 15 min for 24 h, and the three collections were mixed together in proportion to the volumes delivered by the three systems. Only 50 ml of the 24-h composite wastewater was analyzed for each sample. Samples were concentrated by centrifuging at 1,000 × *g* for 10 min. *Cryptosporidium* oocysts were further purified by immunomagnetic separation, using magnetic beads coated with an anti-*Cryptosporidium* monoclonal antibody (Dynal, Inc., Lake Success, N.Y.).

PCR-RFLP analysis. DNA was extracted from the immunomagnetic separation concentrates obtained above were used in DNA extraction without detachment of *Cryptosporidium* oocysts from beads (8, 9). *Cryptosporidium* species in DNA from wastewater were determined by a previously described small-subunit (SSU)-rRNA-based PCR-restriction fragment length polymorphism (PCR-RFLP) (8, 9). Each sample was analyzed at least three times by nested PCR using 0.5, 1.0, or 2.0 μl of DNA as the template. Positive (*Cryptosporidium serpentis* DNA) and negative controls were included in each PCR run. For the differentiation of *Cryptosporidium* species or genotypes, 10 μl of the secondary PCR product was subjected to restriction digestions by *Ssp*I (New England BioLabs, Beverly, Mass.) and *Vsp*I (GIBCO BRL, Grand Island, N.Y.). If *C. muris* or *C. andersoni* was present, they were differentiated from each other by restriction digestion with *Dde*I (New England BioLabs). Species and genotypes were determined by banding patterns in electrophoresis with 2% agarose gels (8, 9). Unusual *Cryptosporidium* parasites were confirmed by DNA sequencing of the PCR products.

Subtyping. For subtyping, the GP60 gene was amplified by nested PCR with the primer sets 5′-ATAGTCTCGCTGTATTC-3′ and 5′-GCAGAGGAACCAGCATC-3′ in the primary PCR and 5′-TCCGCTGTATTCTCAGCC-3′ and 5′-GAGATATATCTTGGTGCG-3′ in the secondary PCR (1, 7). The

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TABLE 1. *Cryptosporidium* species and subtypes in raw wastewater in Milwaukee

Month	No. of samples	No. (%) positive	Species or genotype[a]	GP60 allele[a]
July 2000	12	4 (33.3)	*C. andersoni* (1), *C. hominis* (2), cervid genotype (1), *C. parvum* (1)	Ia (1), Ie (1)
Aug.	8	0 (0)		
Sept.	12	4 (33.3)	*C. hominis* (3), *C. muris* (1), cervid genotype (1)	Ib (1)
Oct.	15	5 (33.3)	*C. andersoni* (4), *C. hominis* (2), *C. parvum* (1)	Ia (1), Ib (1), Ie (1)
Nov.	12	2 (16.7)	*C. andersoni* (2), *C. hominis* (1)	Ib (1)
Dec.	13	8 (61.5)	*C. andersoni* (4), *C. hominis* (2), *C. muris* (2), cervid genotype (W4), (2), *C. parvum* (1)	Ia (1), Ib (2), IIa (1)
Jan. 2001	14	2 (14.3)	*C. andersoni* (1), *C. hominis* (1)	Ib (1)
Feb.	12	2 (16.7)	*C. andersoni* (1), mouse genotype (1)	
Mar.	12	2 (16.7)	*C. andersoni* (1), *C. hominis* (1)	Ib (1)
Apr.	15	6 (40)	*C. andersoni* (4), *C. hominis* (2)	Ib (2)
May	12	3 (25)	*C. andersoni* (2), *C. hominis* (1), *C. parvum* (1)	Ia (1), Ib (1), IIa (1)
June	12	1 (8.3)	*C. andersoni* (1)	
July	15	4 (26.7)	*C. andersoni* (1), *C. hominis* (2), *C. parvum* (1), cervid genotype (2)	Ib (2)
March 2002	15	7 (46.7)	*C. hominis* (7), *C. muris* (1), *C. andersoni* (1)	Ib (2)
Total	179	50 (27.9)	6 species/genotypes	4 alleles

[a] Numbers in parentheses are numbers of samples positive for each species or subtype allele.

secondary PCR amplicons were sequenced in both directions on an ABI 3100 genetic analyzer (Applied Biosystems, Foster City, Calif.). Two PCR products per sample were sequenced to confirm the accuracy of diagnosis. Nucleotide sequences were aligned with known *Cryptosporidium* GP60 sequences, and a neighbor-joining tree was constructed based on evolutionary distances calculated by the Kimura two-parameter model with 1,000 bootstrap samplings (8, 9).

***Cryptosporidium* species or genotypes in wastewater.** A total of 179 wastewater samples were examined in this study, of which 50 were positive for *Cryptosporidium* by the SSU-rRNA-based PCR-RFLP technique. Restriction analysis of PCR products revealed the presence of six types of *Cryptosporidium* parasites in these wastewater samples (Table 1): *C. hominis*, *C. parvum*, *C. andersoni*, *C. muris*, the *Cryptosporidium* mouse genotype, and the *Cryptosporidium* cervid genotype. *C. hominis* was the most commonly detected parasite overall (24 samples, or 13.4% of the total samples). The order of prevalence for the remaining *Cryptosporidium* parasites was *C. andersoni* (12.8%), the *Cryptosporidium* cervid genotype (3.3%), *C. parvum* (2.8%), *C. muris* (2.2%), and the *Cryptosporidium* mouse genotype (0.6%). Among the 50 positive samples, 5 showed the concurrent presence of two or more *Cryptosporidium* parasites: 4 of these samples had two types of *Cryptosporidium* (samples 2466 and 2474 had *C. hominis* and *C. andersoni*, 2623 had *C. muris* and *C. hominis*, and 2683 had the *Cryptosporidium* cervid genotype and *C. muris*), and 1 (sample 4229) had three types of *Cryptosporidium* (*C. hominis*, *C. parvum*, and the *Cryptosporidium* cervid genotype).

Subtypes of *C. hominis and C. parvum* in wastewater. Wastewater samples positive for *C. hominis* (24 isolates) and *C. parvum* (5 isolates) were subtyped by GP60 locus analysis, and 16 isolates were positive for GP60 amplification. The reduced detection rate by GP60 PCR was probably due to the single-copy nature of the target gene (instead of five copies for the SSU-rRNA gene) and low numbers of oocysts in water samples. The obtained nucleotide sequences were compared with the known subtype sequences and those present in four stool samples from the 1993 Milwaukee outbreak. Phylogenetic analysis of GP60 sequences obtained suggested the presence of four allelic groups (eight subtypes) in 16 wastewater samples: Ia (four subtypes), Ib (one subtype), Ie (two subtypes), and IIa (one subtype) (Fig. 1). In contrast, there was only one subtype allele (Ib) in human stool samples from the 1993 Milwaukee outbreak. Fourteen of the wastewater samples had allele Ib, and all allele Ib sequences were identical to sequences obtained from four stool samples collected during the 1993 outbreak (Fig. 1). Most of the samples (i.e., 12) had only one subtype allele: Ib (11 samples) or Ia (1 sample). However, two samples had two subtypes (Ia and Ie in sample 2127; Ib and Ie in sample 2466), and two samples had three subtypes (Ia, Ib, and IIa in both 2636 and 4006) (Fig. 1).

Public health significance. Results of this study support a previous finding of the complexity of *Cryptosporidium* in raw urban wastewater (9). Six *Cryptosporidium* species or types were found in raw wastewater samples from Milwaukee, with *C. hominis* and *C. andersoni* as the most common. Because these host-adapted parasites occur in humans (*C. hominis* and *C. parvum*), farm animals (*C. andersoni* and *C. parvum*), rodents (*C. muris* and the *Cryptosporidium* mouse genotype), and deer (the *Cryptosporidium* cervid genotype), these results confirmed the previous conclusion that humans, slaughtered farm animals, rodents, and deer all contributed to *Cryptosporidium* contamination in wastewater. In the previous study conducted in Milwaukee, a few samples were found to be positive for *C. canis* and *C. felis*, which were not found in this study.

Results of subtype analysis further support the complexity of human-pathogenic *Cryptosporidium* in wastewater. Four subtype alleles of *C. hominis* and *C. parvum* were found in Milwaukee wastewater: Ia, Ib, Ie, and IIa. Subtype alleles Ia, Ib, and Ie belonged to *C. hominis* and thus were likely of human origin, whereas IIa belonged to *C. parvum* and probably originated from cattle as well as humans. Nevertheless, allele Ib was the predominant subtype of *C. hominis* in Milwaukee wastewater samples. All parasites in this group in the wastewater had identical GP60 sequences and belonged to the subtype involved in the 1993 Milwaukee outbreak.

The frequent detection of *C. hominis* in Milwaukee waste-

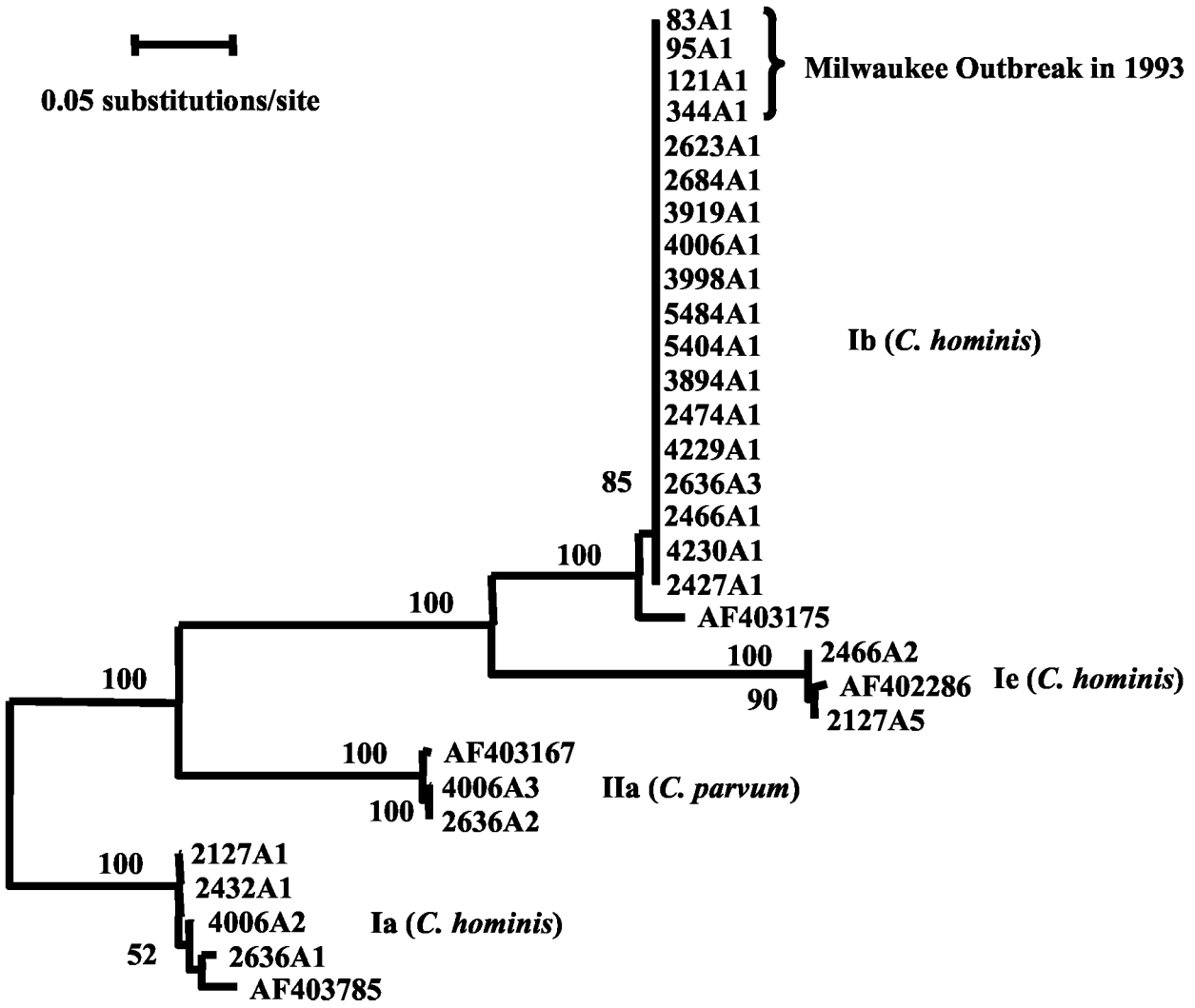

FIG. 1. Relationship among *C. parvum* and *C. hominis* subtypes inferred by neighbor-joining analysis of GP60 nucleotide sequences. The Kimura two-parameter model was used in distance calculation. Numbers on branches are percent bootstrap values from 1,000 resamplings.

water all year round indicates that there is stable transmission of human cryptosporidiosis in Milwaukee in the absence of an outbreak. The high-frequency detection of the allele Ib parasite, which was probably responsible for the 1993 outbreak, suggests that this parasite is probably quite virulent or infectious and has been circulating in humans in the Milwaukee area at least since the outbreak. Even though there are many subtypes of *C. hominis* and *C. parvum* in humans, quite a few food-borne and waterborne outbreaks of cryptosporidiosis in the United States and Northern Ireland are caused by allele Ib parasites (1, 6).

In summary, results of this study suggest that molecular tools can be very useful in assessing the human-infective potential of *Cryptosporidium* oocysts in wastewater and the source of *Cryptosporidium* oocyst contamination. The distribution of *Cryptosporidium* species indicates that even in urban areas, a significant proportion of *Cryptosporidium* oocysts in wastewater are probably not from humans and are of no significant public health concern. Continuous monitoring of *Cryptosporidium* species and subtypes in urban wastewater can be an effective tool in postoutbreak surveillance and may provide useful information for understanding *Cryptosporidium* transmission in humans and the occurrence of waterborne outbreaks of cryptosporidiosis.

Nucleotide sequence accession numbers. Nucleotide sequences were submitted to the GenBank database under accession numbers AY262027 to AY262034.

This study was supported in part by a research grant from the AWWA Research Foundation.

We thank Jeff MacDonald of the Milwaukee Metropolitan Sewerage District and Birhane Dashew for their assistance in sample collection.

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