Molecular Characterization of Cryptosporidium Oocysts in Samples of Raw Surface Water and Wastewater

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Consumption of contaminated water has been implicated as a major source of Cryptosporidium infection in various outbreak investigations and case control studies (22, 24). Surveys conducted in several regions of the United States revealed the presence of Cryptosporidium oocysts in 67 to 100% of wastewaters, 24 to 100% of surface waters, and 17 to 26.8% of drinking waters (11–13, 24). The identity and human-infective potential of these waterborne oocysts are not known, although it is likely that not all oocysts are from human-infecting Cryptosporidium species. Likewise, the source of oocyst contamination is not clear. Farm animal and human sewage discharges are generally considered the major sources of surface water contamination with C. parvum (15). Because Cryptosporidium infection is common in wildlife, it is conceivable that wildlife contamination in water can also be a source of Cryptosporidium oocysts in water (24).

Currently, Cryptosporidium oocysts in environmental samples are identified largely by an immunofluorescent assay after concentration by methods such as the ICR method or method 1622/1623. Because the immunofluorescent assay detects oocysts of most Cryptosporidium spp., the species distribution of Cryptosporidium parasites in environmental samples cannot be assessed. Although many surface water samples contain Cryptosporidium oocysts, it is unlikely that all of these oocysts are from human-pathogenic species or genotypes, because only five genotypes of Cryptosporidium parasites (the C. parvum human, bovine, and dog genotypes, C. meleagrisidis, and C. felis) have been explicitly found in humans so far (17, 18, 32). Information on the source of C. parvum contamination is necessary for effective evaluation and selection of management practices to reduce Cryptosporidium contamination of surface water and the risk of cryptosporidiosis. Thus, identification of oocysts to species and strain levels is of public health importance.

The existence of host-adapted Cryptosporidium spp. and C. parvum genotypes makes it possible to develop species differentiation and genotyping tools to determine whether the Cryptosporidium oocysts found in water are from human-infective species and to track the source of Cryptosporidium oocyst contamination in water (16, 32). One such tool, the small-subunit (SSU) rRNA-based nested PCR-restriction fragment length polymorphism (RFLP) method, has been successfully used by us to differentiate Cryptosporidium species and C. parvum genotypes in clinical samples and storm water (29–31). In this study, we evaluated the use of this technique for detection and characterization of Cryptosporidium oocysts in samples of raw surface water and wastewater.

MATERIALS AND METHODS

Water samples and sample processing. Samples of raw surface water and wastewater were used in this study. Most of the surface water samples were collected from the Milwaukee region of Lake Michigan, from rivers in Illinois, and from the Maryland portion of the Chesapeake Bay area. A few samples, however, were collected from rivers in Iowa, Missouri, and Texas (see Table 1). These samples were collected during 1999 and the first half of 2000. Samples from the Chesapeake Bay area were collected from sites located near wastewater discharges (samples from Choptank River, Severn River, and Miles River) or beef cattle farms (samples from Wye River, St. George’s Creek, and Wicomico River), which were adjacent to the river. The allowable amount of waste discharge was 3.6 million gallons per day (MGD) at the Choptank River discharge site, 7.5 MGD at the Severn River discharge site, and 0.3 MGD at the Miles River discharge site. One sample from each of the sites was taken during the spring (May), summer (August), and fall (October) of 1999 to avoid seasonal fluctuations in oocyst contamination. Water samples were always taken down-
TABLE 1. Cryptosporidium genotypes in samples of surface water from various locations

<table>
<thead>
<tr>
<th>Sample location</th>
<th>Vol filtered (liters)</th>
<th>Total no. of samples</th>
<th>No. of positive samples</th>
<th>Species and/or genotypes(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lake Michigan, Linwood, Wis.</td>
<td>50.3–60</td>
<td>8</td>
<td>0</td>
<td>C. parvum human genotype and C. baileyi (1), C. andersoni (1)</td>
</tr>
<tr>
<td>Lake Michigan, San Benito, Wis.</td>
<td>10</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Lake Michigan, Howard, Wis.</td>
<td>50.0–63.1</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Aurora, Ill.</td>
<td>10</td>
<td>7</td>
<td>2</td>
<td>C. parvum human genotype and C. baileyi (1), C. andersoni (1)</td>
</tr>
<tr>
<td>Decatur, Ill.</td>
<td>10</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Iowa City, IOWA</td>
<td>20.8–24.2</td>
<td>2</td>
<td>1</td>
<td>C. andersoni (1)</td>
</tr>
<tr>
<td>Kansas City, Mo.</td>
<td>4.6–10.0</td>
<td>3</td>
<td>1</td>
<td>C. andersoni (1)</td>
</tr>
<tr>
<td>St. Louis, Mo.</td>
<td>10</td>
<td>1</td>
<td>1</td>
<td>C. andersoni (1)</td>
</tr>
<tr>
<td>San Benito, Tex.</td>
<td>3.0–10.0</td>
<td>5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Potomac River, Washington, D.C.</td>
<td>10</td>
<td>1</td>
<td>1</td>
<td>C. andersoni (1)</td>
</tr>
<tr>
<td>Chesapeake Bay, Choptank River, Cambridge, Md.</td>
<td>50</td>
<td>3</td>
<td>3</td>
<td>C. parvum human and bovine genotypes (2)</td>
</tr>
<tr>
<td>Chesapeake Bay, Severn River, Annapolis, Md.</td>
<td>50</td>
<td>3</td>
<td>3</td>
<td>C. parvum human and bovine genotypes (3)</td>
</tr>
<tr>
<td>Chesapeake Bay, mouth of Severn River, Annapolis</td>
<td>50</td>
<td>3</td>
<td>3</td>
<td>C. parvum human and bovine genotypes (3)</td>
</tr>
<tr>
<td>South, Md.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chesapeake Bay, Miles River, St. Michael’s, Md.</td>
<td>50</td>
<td>3</td>
<td>3</td>
<td>C. parvum bovine genotype (2), C. parvum human and bovine genotypes (1)</td>
</tr>
<tr>
<td>Chesapeake Bay, St. George’s Creek, Lumberland, Md.</td>
<td>50</td>
<td>3</td>
<td>3</td>
<td>C. parvum bovine genotype (3)</td>
</tr>
<tr>
<td>Chesapeake Bay, Wicomico River, Mt. Vernon, Md.</td>
<td>50</td>
<td>3</td>
<td>2</td>
<td>C. parvum bovine genotype (2)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses are numbers of samples positive for each genotype or species.

Results

A total of 55 surface water samples and 49 wastewater samples were used in this study. Examination of IMS concentrates by PCR analysis indicated that 25 of the surface water samples and 12 of the wastewater samples were positive for Cryptosporidium. When the surface water samples were examined, none of the 13 samples from Lake Michigan contained Cryptosporidium, but 19 of 21 samples from rivers in the Chesapeake Bay area were positive for Cryptosporidium (Tables 1 and 2).

Restriction analysis of secondary PCR products with SpI and VspI revealed the presence of C. parvum human and bovine genotypes, C. baileyi, and C. andersoni-C. muris in surface water samples and C. parvum human, bovine, and dog genotypes, C. felis, C. andersoni-C. muris, and an unknown Cryptosporidium genotype in wastewater samples (Fig. 1A). C. andersoni and C. muris were differentiated from each other by DdeI digestion of the secondary PCR products. The PCR products of C. andersoni yielded bands at 20, 156, 186, and 470 bp, and three bands were visible on an agarose gel. In contrast, the
PCR products of *C. muris* yielded bands at 20, 156, 186, 224, and 247 bp, and four bands were visible (Fig. 1B). Sequence analysis of all of the PCR products yielded DNA sequences identical to those which we previously obtained from humans or animals infected with *C. parvum* human, bovine, and dog genotypes, *C. felis*, *C. andersoni*, *C. muris*, and *C. baileyi* (data not shown). The unknown *Cryptosporidium* genotype was identical to a *Cryptosporidium* wildlife genotype (W3) which we previously identified in storm water (31).

*C. parvum*, (both human and bovine genotypes) was the predominant *Cryptosporidium* sp. found in surface water; 10 samples contained the *C. parvum* human genotype, and 19 samples contained the *C. parvum* bovine genotype. *C. andersoni* was also detected at a moderate frequency (five samples) in surface water samples. With the exception of one sample, the *C. parvum* human genotype was found only in surface water samples from the Chesapeake Bay area, along with the *C. parvum* bovine genotype (Table 1). In contrast, *C. andersoni* was the major *Cryptosporidium* sp. found in wastewater, occurring in eight samples (Table 2). Many surface water and wastewater samples contained more than one *Cryptosporidium* genotype; this was especially true of surface water samples from rivers in the Chesapeake Bay area (Tables 1 and 2).

**FIG. 1.** Genotyping of *Cryptosporidium* oocysts in water with a SSU rRNA-based PCR-RFLP technique. (A) Differentiation of *Cryptosporidium* spp. and *C. parvum* genotypes by digestion of the secondary PCR products with *Ssp*I (upper panel) and *Vsp*I (lower panel). Lane 1, *C. parvum* human genotype (sample 574); lane 2, *C. parvum* bovine genotype (sample 5F); lanes 3 and 4, *C. parvum* human and bovine genotypes (samples 1F and 2F); lane 5, *C. andersoni* (sample 104); lane 6, *C. muris* (sample 194); lane 7, *C. parvum* bovine genotype and *C. andersoni* (sample 163). (B) Differentiation of *C. andersoni* from *C. muris* by digestion of the secondary PCR products with *Dde*I. Lanes 1 through 4 and 6 through 8, *C. andersoni* (samples 104, 99, 98, 641, 192, 224, and 225); lane 5, *C. muris* (sample 194).

**TABLE 1.** *Cryptosporidium* genotypes in samples of raw wastewater from a wastewater treatment plant in Milwaukee

<table>
<thead>
<tr>
<th>Month</th>
<th>Sample vol (ml)</th>
<th>Total no. of samples</th>
<th>No. of positive samples</th>
<th>Species and/or genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>April</td>
<td>10</td>
<td>13</td>
<td>2</td>
<td><em>C. andersoni</em> and <em>C. muris</em> (1), <em>C. andersoni</em> and <em>C. parvum</em> bovine genotype (1)</td>
</tr>
<tr>
<td>May</td>
<td>10</td>
<td>15</td>
<td>2</td>
<td><em>C. andersoni</em> (1), <em>C. muris</em> (1)</td>
</tr>
<tr>
<td>June</td>
<td>10</td>
<td>12</td>
<td>6</td>
<td><em>C. andersoni</em> (3), <em>C. parvum</em> dog genotype (1), <em>C. felis</em> (1), <em>C. parvum</em> human genotype and <em>C. andersoni</em> (1)</td>
</tr>
<tr>
<td>July</td>
<td>50</td>
<td>9</td>
<td>2</td>
<td><em>C. andersoni</em> (1), unknown genotype (1)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses are numbers of samples positive for each genotype or species.

**DISCUSSION**

Numerous attempts have been made to apply PCR techniques to detection of *Cryptosporidium* oocysts in water samples (1–3, 6, 10, 14, 19–21, 23, 25, 26, 28). In most of these studies the workers used water seeded with *Cryptosporidium* oocysts, and various degrees of success were reported. One major obstacle is the presence of PCR inhibitors in water, which are coextracted with DNA and inhibit PCR amplification of the target DNA. This has greatly reduced the sensitivity of PCR detection of oocysts in various water samples. The PCR inhibitors can be removed by IMS (8, 9). This practice led to successful detection of *Cryptosporidium* oocysts in water samples from the 1993 outbreak in Milwaukee, Wis., by a *Cryptosporidium* genus-specific PCR technique (9) and to genotyping of *C. parvum* parasites in surface and filter backwash water samples by an integrated cell culture-PCR technique (3).

Results of the present study indicate that in conjunction with IMS, the SSU rRNA-based PCR-RFLP technique which we previously developed for differentiating *Cryptosporidium* spp. and *C. parvum* genotypes in clinical samples has the specificity and sensitivity needed for analysis of *Cryptosporidium* oocysts in water samples.

Eight *Cryptosporidium* parasites that commonly occur in humans, farm animals, pets, or wildlife were found in surface water and wastewater samples used in this study. The high frequency of detection of the *C. parvum* human and bovine genotypes and *C. andersoni* (a gastric *Cryptosporidium* parasite of juvenile and adult cattle) is congruent with the previous theory that humans and farm animals are two major sources of *Cryptosporidium* oocyst contamination in surface water at locations where this type of contamination potentially occurs (15, 22). This is in contrast with *Cryptosporidium* parasites in storm runoff water from a feral area, in which there is a high frequency of *Cryptosporidium* genotypes from wildlife (31). There may be geographic differences in *Cryptosporidium* oocyst con-
tamination of surface water, because the Cryptosporidium oocyst detection rate for river water samples from the Chesapeake Bay area was much higher than those for samples from other areas, and no Cryptosporidium oocysts were detected in water from Lake Michigan. This was expected, because the sampling sites in the Chesapeake Bay area were located near potential sources of contamination of water with Cryptosporidium oocysts (i.e., wastewater discharges and runoff from large commercial cattle farms) (5). Three of six rivers examined in this region frequently contained C. parvum human genotype oocysts, a finding congruent with these sampling sites’ locations near wastewater discharges.

Although Cryptosporidium was not detected in surface water from the Milwaukee portion of Lake Michigan and only 10 to 50 ml of wastewater was examined for each sample, Cryptosporidium oocysts were detected in raw wastewater from Milwaukee at a moderate frequency. The high rate of detection of C. andersoni in wastewater was probably the result of effluents from cattle slaughterhouses in the city. This hypothesis is supported by the fact that mature cattle are more likely to be infected with C. andersoni than with the C. parvum bovine genotype, which was detected in wastewater at a much lower frequency. The biggest slaughterhouse in the city processes 1,800 beef cattle daily and drains its contents into the city sewage system after satisfying city specifications (amount of fat, size of meat chunks, etc.). The slaughterhouse is less than 5 miles upstream of the Jones Island wastewater treatment plant, where we collected composite samples.

Likewise, the C. muris oocysts in wastewater were probably from rodents, which are expected to be present in a wastewater distribution system in abundance. Urban runoff may also have been a contributing factor in Cryptosporidium oocysts in wastewater, because the C. parvum dog genotype, C. felis, and an unknown Cryptosporidium genotype from wildlife were also detected in wastewater at low frequencies. The low rate of detection of the C. parvum human genotype in wastewater from a major metropolitan area is surprising, because it is likely that a major component of the wastewater in this area is human sewage. However, sampling was done during the period from April to July, when the incidence of human cryptosporidiosis is generally low.

In summary, the results of this study show the usefulness of the SSU rRNA-based PCR-RFLP technique for differentiating Cryptosporidium spp. and the C. parvum genotype and for tracking Cryptosporidium contamination sources in water. Extensive genotyping of water samples from various matrices (source water, finished water, wastewater, river storm water, combined sewer overflow) and environmental settings (feral, rural, urban, recreational) is needed in order to obtain a better understanding of the distribution of Cryptosporidium spp. in various waters, the human infection potential of waterborne Cryptosporidium oocysts, and the contributions of humans, farm animals, companion animals, wildlife, and other factors, such as sanitation, wastewater discharge, agriculture, recreation, and weather, to Cryptosporidium oocyst contamination of water in certain settings. Such information would be useful for scientific management of watersheds and for source water protection.

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REFERENCES


