

Infectious *Cryptosporidium parvum* Oocysts in Final Reclaimed Effluent

Angela L. Gennaccaro,* Molly R. McLaughlin,† Walter Quintero-Betancourt,
Debra E. Huffman, and Joan B. Rose‡

Department of Marine Science, University of South Florida,
St. Petersburg, Florida 33701

Received 6 December 2002/Accepted 2 June 2003

Water samples collected throughout several reclamation facilities were analyzed for the presence of infectious *Cryptosporidium parvum* by the focus detection method–most-probable-number cell culture technique. Results revealed the presence of infectious *C. parvum* oocysts in 40% of the final disinfected effluent samples. Sampled effluent contained on average seven infectious oocysts per 100 liters. Thus, reclaimed water is not pathogen free but contains infectious *C. parvum*.

Reclaimed water (treated wastewater) is being utilized in the United States and throughout the world as an alternative non-potable water source. In the United States, 18 states currently have standards and another 18 have guidelines for reclaimed water (8). These standards, for the most part, are based on total suspended solids and fecal coliforms. A variety of microbial pathogens are present in wastewater and can be detected in reclaimed water. Therefore, advanced treatment, including filtration and disinfection, is required to produce reclaimed water that does not have a negative impact on public health. In regulatory language, this means that pathogens are to be less than the limit of detection of the assay (8).

Cryptosporidium parvum, a coccidian protozoan parasite, is a potential contaminant of reclaimed water. *C. parvum* oocysts have been found to be persistent in the environment and resistant to chlorination. Because of this, physical removal by chemical pretreatment and filtration is the primary means of reducing the level of oocysts in environmental water (6). A possible risk to human health exists if filtration fails to function efficiently. This risk is greater still with reclaimed water, as to date no monitoring for *C. parvum* oocysts has been required and little information is available on the filtration efficiency in these facilities. Recently, in the state of Florida, monitoring for protozoan parasites, including *Cryptosporidium*, once every 2 years for larger facilities and once every 5 years for smaller facilities has been mandated. Sampling is recommended at a single point following disinfection (2).

In one study, *C. parvum* oocysts were detected in untreated wastewater (67% of the samples were positive) and in reclaimed water (25% of final effluent samples were positive) (5). However, only the presence of the oocysts was evaluated using fluorescence microscopy. Robertson et al. evaluated waste-

water samples for viable *C. parvum* by using vital stains; 35% of the influent samples and 46% of the effluent samples contained viable oocysts (4). In the last few years, the focus detection method–most-probable-number (FDM-MPN) cell culture technique has been developed to test the oocyst infectivity because the previously employed methods did not accurately reflect the infectious nature of the oocysts (7). The objective of the present study was to demonstrate the presence of infectious *C. parvum* oocysts in final reclaimed effluent from six reclamation facilities in the United States by using the FDM-MPN cell culture technique.

Samples were collected from influent, secondary effluent, postfiltration, and final disinfected effluent waters. Six reclamation facilities in the United States, utilizing a variety of filtration systems (shallow- or deep-bed sand and anthracite filters or fabric disk filters) and disinfection methods (chlorine gas or UV radiation), were monitored. Three facilities were monitored five times over a 1-year time period. Three additional facilities were monitored over a 5-month time period. One to 400 liters of sample, depending on the site, was filtered through Envirochek HV filters (Pall Gelman Laboratories, Ann Arbor, Mich.). After filtration, elution, and centrifugation using the guidelines provided by EPA method 1623 (9), oocysts were concentrated into a pellet by immunomagnetic separation (Dynabeads Anti-Crypto Kit; Dynal Biotech, Inc., Lake Success, N.Y.).

Half of the concentrated pellet was analyzed by the FDM-MPN cell culture technique (7). (The other half of the concentrated pellet was processed by microscopic screening of the sample after staining with monoclonal antibodies [anti-*Cryptosporidium*; Waterborne Inc., New Orleans, La.] tagged with fluorescein isothiocyanate, using an indirect fluorescent-antibody assay [IFA] to determine total counts of oocysts.) The concentrate was first bleach treated at room temperature for 8 min with a 10.5% solution of 4% sodium hypochlorite in phosphate-buffered saline (PBS; pH 7.2) to eliminate bacterial contamination as well as to trigger excystation of the oocysts. The sample was then washed once with PBS and centrifuged, after which the pellet was suspended in 1 ml of prewarmed growth medium (RPMI 1640 [Fisher Scientific, Pittsburgh, Pa.] plus

* Corresponding author. Mailing address: Department of Marine Science, University of South Florida, 140 7th Ave. South, St. Petersburg, FL 33701. Phone: (727) 553-3415. Fax: (727) 553-1189. E-mail: angelag@marine.usf.edu.

† Present address: United States Geological Survey, Center for Coastal and Regional Marine Studies, St. Petersburg, FL 33701.

‡ Present address: Department of Fisheries and Wildlife, Michigan State University, East Lansing, MI 48824.

TABLE 1. Occurrence of *Cryptosporidium parvum* oocysts in various phases of wastewater treatment^a

Sample	No. of samples	% Positive for <i>C. parvum</i>		Oocyst level				Arithmetic mean \pm SD	
		Total oocysts	Infectious oocysts	Minimum		Maximum		Total oocysts	Infectious oocysts
				Total oocysts	Infectious oocysts	Total oocysts	Infectious oocysts		
Influent	18							6,910 \pm 7,731	
Secondary effluent	18							112 \pm 153	
Postfiltration	17							37 \pm 73	
Final disinfected effluent	15							28 \pm 52	

^a All concentrations are determined per 100 liters of sample. Percent positives were calculated from values above the limit of detection. Total oocyst counts represent actual counts; infectious oocyst counts are MPN results.

additives) and placed directly on human ileocecal adenocarcinoma HCT-8 cell monolayers in eight-well chamber slides. The slides were incubated at 37°C in a 5% CO₂ incubator. After 90 min, additional growth medium was added to each well, and the slides were incubated for another 40 to 48 h.

After incubation, the slides were removed, washed with PBS, and fixed in 100% methanol. The monolayers were rehydrated for 30 min in blocking buffer (PBS with 2% goat serum [Atlanta Biologicals, Norcross, Ga.] and 10% of a 0.002% solution of Tween 20) and stained with rat anti-*C. parvum* sporozoite antibody (Waterborne Inc.) followed by fluorescein isothiocyanate-labeled anti-rat immunoglobulin G (Sigma Aldrich, Inc., St. Louis, Mo.). The slides were evaluated under an Olympus BH-2 epifluorescence microscope at 200 \times and 400 \times magnification (excitation, 340 to 380 nm; 420-nm barrier or suppression filter) for the presence of infectious foci.

The number of positive wells for each sample was entered into the MPN program, downloaded from the EPA website, to determine the number of infectious oocysts per milliliter (8). When no infectious foci were observed, the MPN program determined the detection limit for the assay. The number of infectious oocysts per 100 liters was determined from the initial volume collected and the concentrate volume analyzed after immunomagnetic separation.

C. parvum oocysts were found in all sites monitored throughout the treatment process (Table 1). As well, and more importantly, infectious *C. parvum* oocysts were found in all sampling sites (Table 1). Average concentrations of 6,910 oocysts/100 liters by IFA and 993 infectious oocysts/100 liters by MPN were found in the influent (raw wastewater). Therefore, roughly 14% of all oocysts observed were infectious in nature. At the conclusion of treatment, average concentrations of 28 oocysts/100 liters by IFA and 7 infectious oocysts/100 liters by FDM-MPN were detected. Roughly 25% of the oocysts detected were infectious in nature. Average recovery efficiencies of 5.5% from influent samples for four trials (standard deviation, ± 1.3), 15.3% from secondary effluent samples for four trials (standard deviation, ± 2.9), and 15% from postfiltered and final disinfected effluent samples for 12 trials (standard deviation, ± 11.2) have been observed in our laboratory using gamma-irradiated oocysts labeled with Texas Red. Thus, it is important to note that higher concentrations of infectious oocysts may be present in all samples, but especially final disinfected reclaimed effluent samples, since recovery efficiencies are not 100%.

During the past decade, there has been an increase in aware-

ness of the risk of illness resulting from *C. parvum* because of outbreaks such as that in Milwaukee, Wisconsin, in 1993 (3). By 1995, surveillance for cryptosporidiosis in the human population had begun in the United States. Between 1995 and 1998, the mean incidence per 100,000 ranged from 0.9 to 3 (1). The relationship to water transmission is not known. In 1999, Florida began to require periodic sampling for *Cryptosporidium* and *Giardia* in reclaimed water systems (10). In this paper, we report initial findings of infectious *C. parvum* oocysts in final reclaimed effluent as determined by the FDM-MPN method. Additional monitoring to produce a more statistically significant database and research to determine the best treatment processes are suggested as the next steps. Eventually, standards for monitoring of reclaimed water for *Cryptosporidium* should be considered. The FDM-MPN method will be a useful tool for future monitoring requirements to determine the presence of infectious oocysts and the associated health risk.

This work was funded by the Water Environment Research Foundation (project number 00-PUM-2T).

REFERENCES

- Dietz, V. J., and J. M. Roberts. 2000. National surveillance for infection with *Cryptosporidium parvum*, 1995–1998: what have we learned? Public Health Rep. 115:358–363.
- Florida Department of Environmental Protection. 1999. Reuse of reclaimed water and land application, p. 1–123. In Florida Administrative Code. Florida Department of Environmental Protection, Tallahassee.
- MacKenzie, W. R., N. J. Hoxie, M. E. Proctor, M. S. Gradus, K. A. Blair, D. E. Peterson, J. J. Kazmierczak, D. G. Addiss, K. R. Fox, J. B. Rose, and J. P. Davis. 1994. A massive outbreak in Milwaukee of *Cryptosporidium* infection transmitted through the public water supply. N. Engl. J. Med. 331:161–167.
- Robertson, L. J., C. A. Paton, A. T. Campbell, P. G. Smith, M. H. Jackson, R. A. Gilmour, S. E. Black, D. A. Stevenson, and H. V. Smith. 2000. *Giardia* cysts and *Cryptosporidium* oocysts at sewage treatment works in Scotland, UK. Water Res. 34:2310–2322.
- Rose, J. B., L. J. Dickson, S. R. Farrah, and R. P. Carnahan. 1996. Removal of pathogenic and indicator microorganisms by a full-scale water reclamation facility. Water Res. 30:2785–2797.
- Simmons, O. D., III, M. D. Sobsey, C. D. Heaney, F. W. Schaefer III, and D. S. Francy. 2001. Concentration and detection of *Cryptosporidium* oocysts in surface water samples by method 1622 using ultrafiltration and capsule filtration. Appl. Environ. Microbiol. 67:1123–1127.
- Stifko, T. R., D. E. Huffman, and J. B. Rose. 1999. A most-probable-number assay for enumeration of infectious *Cryptosporidium parvum* oocysts. Appl. Environ. Microbiol. 65:3936–3941.
- U.S. Environmental Protection Agency. 1992. Guidelines for water reuse. EPA/625/R-92/004. Office of Water, U.S. Environmental Protection Agency, Washington, D.C.
- U.S. Environmental Protection Agency. 1999. Method 1623: *Cryptosporidium* and *Giardia* in water by filtration/IMS/FA. EPA/821/R-99/006. Office of Water, U.S. Environmental Protection Agency, Washington, D.C.
- York, D. W., and L. Walker-Coleman. 2000. Pathogen standards for reclaimed water. Water Environ. Tech. 12:58–61.