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Abstract

Water samples were collected from four rivers in Washington State and two rivers in California and examined for the presence of Cryptosporidium oocysts. Oocyst-sized particles were concentrated from 20-liter samples of water by membrane filtration, centrifugation, and differential sedimentation. The particle concentrate was then deposited on a 25-mm-diameter membrane filter for oocyst identification by indirect immunofluorescence assay. The identification procedure had a limit of detection of about five oocysts per liter. Cryptosporidium oocysts were found in each of 11 river water samples examined. Concentrations ranged from 2 to 112 oocysts per liter. The finding of Cryptosporidium oocysts in all samples examined from six western rivers is noteworthy in light of recent reports indicating that Cryptosporidium sp. is a significant agent of human and animal disease. This finding suggests that waterborne oocysts of this parasite are more important than was previously recognized. More detailed studies are needed to define geographical and temporal distribution, to assess the viability of waterborne oocysts, and to determine the importance of water as a means of transmission.

Keywords

identification, water, river, oocysts, cryptosporidium

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Identification of *Cryptosporidium* Oocysts in River Water

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Water samples were collected from four rivers in Washington State and two rivers in California and examined for the presence of *Cryptosporidium* oocysts. Oocyst-sized particles were concentrated from 20-liter samples of water by membrane filtration, centrifugation, and differential sedimentation. The particle concentrate was then deposited on a 25-mm-diameter membrane filter for oocyst identification by indirect immunofluorescence assay. The identification procedure had a limit of detection of about five oocysts per liter. *Cryptosporidium* oocysts were found in each of 11 river water samples examined. Concentrations ranged from 2 to 112 oocysts per liter. The finding of *Cryptosporidium* oocysts in all samples examined from six western rivers is noteworthy in light of recent reports indicating that *Cryptosporidium* sp. is a significant agent of human and animal disease. This finding suggests that waterborne oocysts of this parasite are more important than was previously recognized. More detailed studies are needed to define geographical and temporal distribution, to assess the viability of waterborne oocysts, and to determine the importance of water as a means of transmission.

Recent reports have suggested an association between cryptosporidiosis and water supplies in the United States, the USSR, and the Caribbean (9, 18, 22). The presence of *Cryptosporidium* oocysts in water supply sources, indicating the potential for waterborne transmission, would be a matter of concern for public health and to purveyors and regulators of public water supplies.

Cryptosporidium sp. is a protozoan parasite of animals and humans and an agent of acute enterocolitis (21). Reports indicate that it causes diarrhea in children and adults worldwide (16, 20; D. P. Casemore and F. B. Jackson, Letter, Lancet ii:679, 1983). Prevalence of human cryptosporidiosis reported after examination of stool specimens from over 15,000 individuals in separate studies in Europe, the United States, and Canada ranges from 1 to 5% (14, 19, 23, 35). The disease is normally self-limiting in immunocompetent individuals, but it is potentially life threatening in immunocompromised individuals such as patients having had organ transplants, or cancer chemotherapy, or patients with acquired immune deficiency syndrome (8). No effective chemotherapeutic agents have yet been found (5). No data have been reported on the dose of *Cryptosporidium* oocysts that is infectious to humans. However, as few as 10 oocysts, or 0.02 μl of infected diarrheal stool, have been shown to infect 20- to 30-day-old nonhuman primate (*Macaca nemestrina*) infants (R. A. Miller, M. A. Bronsdon, and W. R. Morton, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, B148, p. 49).

Cryptosporidium sp. has numerous animal hosts, including nearly 40 species of domestic and wild animals (1, 30). Evidence indicates that the genus has few species, only two of which infect mammals, the most common being *C. parvum*. Evidently, *C. parvum* has broad cross infectivity among many host species (31, 33). Cryptosporidiosis in humans has been considered a zoonosis, i.e., a disease acquired by humans from animals (2, 25, 32). However, this pathway is not the only means of transmission, as indicated by clusters of cases among children attending day care centers (6). The *Cryptosporidium* life cycle includes a complex sexual and asexual reproductive phase in the intestine of host animals and the formation of environmentally hardy, infective oocysts that are excreted in the feces (7). The oocysts have been found to be resistant to chemical disinfectants (4, 10, 24).

Cryptosporidium oocysts are spherical (in *C. parvum*, about 3 to 5 μm in diameter) and are shed in numbers of up to 10^5 to 10^7 oocysts per g in calf feces. Various staining procedures for the identification of *Cryptosporidium* oocysts in fecal smears have been reported elsewhere (3, 11, 15, 29). However, locating and identifying small numbers of oocysts against a background of numerous extraneous particles isolated from a river or lake water sample present problems for which conventional staining procedures applied to glass-slide-mounted material are not well suited.

In the work described here, we modified procedures developed previously for *Giardia lamblia* monitoring (17, 27). We applied membrane filtration, centrifugation, and density gradient sedimentation procedures to concentrate particles, including *Cryptosporidium* oocysts, from river water samples. An indirect immunofluorescence assay (IFA) procedure, developed for use in identifying *Cryptosporidium* oocysts in fecal smears (29), was used.

MATERIALS AND METHODS

River water sampling. River water samples (approximately 20 liters each) were collected from six sites in Washington State and California (Fig. 1). Four samples were collected near Seattle, Wash.: from the Skykomish River at Monroe; the Snoqualmie River at High Bridge; 8 mi (ca. 13 km) north of Carnation; the Snohomish River at Snohomish; and the Skagit River, 0.25 mi (ca. 0.40 km) west of the Interstate 5 crossing north of Mt. Vernon. Samples were collected in California from the American River at Sacramento, about 0.25 mi east of the Guy West Bridge, and from the Sacramento River at Sacramento, 0.25 mi downstream from its confluence with the American River.

Sampling locations in each river were about 5 to 15 ft (ca. 1 to 5 m) from the bank, where the water was approximately 1 m deep. Water was taken from mid-depth. Care was taken to avoid material floating at the surface and not to disturb bottom sediments.

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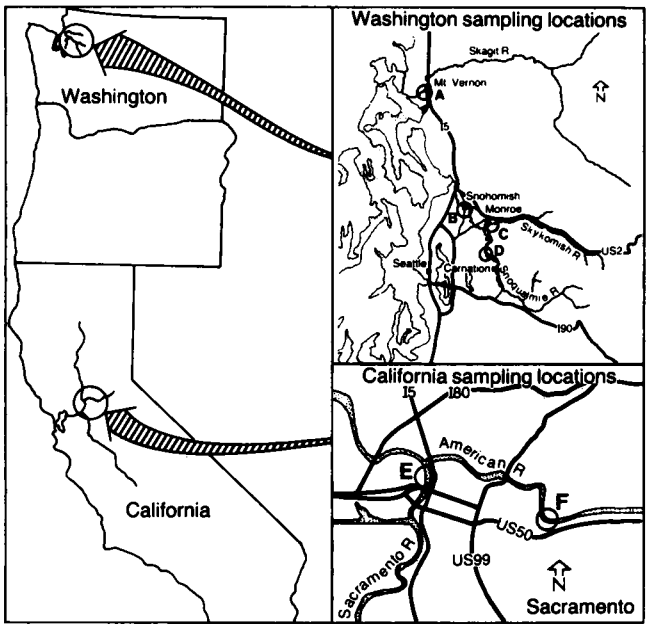

FIG. 1. River water sampling locations in Washington State and California. (A) Skagit River at Mt. Vernon, (B) Snohomish River at Snohomish, (C) Skykomish River at Monroe, (D) Snoqualmie River near Duval, (E) Sacramento River at Sacramento, and (F) American River at Sacramento.

Washington water samples were delivered to the laboratory within 4 h of collection; California water samples were shipped unrefrigerated and arrived at the laboratory within 24 h of collection. Samples were refrigerated without preservatives and processed within 48 h of collection. In addition to the 11 river water samples, 14 other samples were processed, including 5 seeded positive controls, 6 distilled water samples, and 3 tap water samples. The river water samples and accompanying controls were processed in three batches during July and August 1985.

Water sample processing. Processing for oocyst identification and counting was conducted with batches of eight or nine samples consisting of three to six river water samples plus negative controls and seeded positive controls. Positive controls were prepared by seeding 20 liters of distilled water with purified bovine-derived *Cryptosporidium* oocysts to produce concentrations ranging from 10^3 to 10^4 oocysts per liter. Distilled water samples were used for negative controls.

Water samples were processed by the following procedure. All filtrations were performed with polycarbonate membranes (Nuclepore Corp., Pleasanton, Calif.). Samples were mixed thoroughly and then prefiltered (filter diameter, 293 mm; pore size, 5 μm) under a vacuum regulated to 20 in. of Hg (ca. 6.8×10^4 Pa). Filtrate was collected and refiltered (filter diameter, 293 mm; pore size, 1 μm). The filter apparatus was wiped dry and rinsed three times with distilled water before reuse. Particles retained on the 1-μm-pore-size filters were further concentrated as follows. (i) Each filter membrane was inverted in a plastic pan (automotive oil change pan; 12 by 3 in. [ca. 30 by 8 cm]) containing 200 ml of distilled water and vibrated for 3 min at the medium setting on a Toothmaster Investment Vibrator (Whaledent International, Div. of IPCO Corp.). (ii) The liquid (300 ml including rinse water) containing particles recovered by vibration was centrifuged at 650 × *g* for 15 min and then decanted to 10% of the original volume. (iii) Particles in the size and density range of oocysts were further concentrated by layering the samples on 40% potassium citrate (specific gravity, 1.195) in 15-ml conical centrifuge tubes and then centrifuging at 650 × *g* for 1 min. Particles retained at and immediately below the interface were harvested by withdrawing 3 to 5 ml by Pasteur pipette. (iv) The recovered particles were deposited on a filter (diameter, 25 mm; pore size, 1 μm) in an in-line filter holder and rinsed three times with 10 ml of distilled water.

IFA procedure. The IFA procedure was applied to particles on the filter surface while the particles were in the in-line filter holder. Rabbit antiserum against *Cryptosporidium* oocysts (purified from dairy calf feces) was prepared as described elsewhere (29). With the exit port of the in-line filter holder stoppered, 0.6 ml of the rabbit antiserum (diluted 1:40 with 0.0175 M phosphate buffered saline [PBS; pH 7.4] containing 0.1% bovine serum albumin [essentially fatty acid free; Sigma Chemical Co., St. Louis, Mo.]) by using a tuberculin syringe to fill the inlet chamber of the filter holder. The inlet port was stoppered, and the filter was incubated at 37°C for 40 min. The serum was then expelled, the filter was rinsed with three successive 10-ml volumes of PBS, and the final PBS volume was expelled. Each operation was done with a syringe. Next, 0.6 ml of fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin G (anti-heavy and anti-light chains; Cooper Biochemical, Inc., Malvern, Pa.), diluted 1:40 with PBS containing 0.1% bovine serum albumin and combined with rhodamine-albumin (Difco Laboratories, Detroit, Mich.) diluted 1:80 with PBS, was introduced into the inlet chamber; the inlet port was stoppered; and the filter was incubated at 37°C for 40 min. The serum was then expelled, the filter was rinsed three times, and PBS was expelled as before. Finally, the filter was removed from the filter holder and mounted under 25-mm-square cover slips with Elvanol permanent resin mounting medium (13).

Oocyst identification and counting. Filters were examined by epifluorescence microscopy at ×250 or ×400 magnification (model standard 14, filter set, catalog no. 48 77 09; Carl Zeiss, Inc., New York). Oocysts were identified by the following criteria: size, shape, surface features, and stain color, distribution, and density. The normal diameter of oocysts is from 3 to 5 μm. A small portion, about 1%, are larger, i.e., 6 to 7 μm. Oocysts are approximately spherical although often slightly irregular (Fig. 2). Some appear dented or flattened, and some, having excysted, appear split open, with a pie-slice-shaped piece missing. Surface wrinkles or lines appear on most oocysts.

Portions of each IFA-stained filter were selected systematically and examined, and the oocysts were counted. The area of filter examined was recorded to allow estimation of the total oocyst concentration by assuming uniform oocyst distribution on the filter. The area of the portion of each filter selected for examination was inversely proportional to the number of oocysts identified. For the positive controls, with the slide orientation controlled by the microscope stage, an equatorial strip with a width equal to the optical-field diameter was examined. This strip contained about 2.5% of the filter area at ×400 or about 3.5% at ×250. Half of each unseeded control filter was examined. For filters with river water samples, the proportion of IFA-stained filter area examined ranged from 7 to 50%. Counting of oocysts recovered from positive controls seeded with accurately predetermined numbers of oocysts permitted estimation of the concentration of oocysts in each river water sample. Because the number of samples that could be processed in this pilot

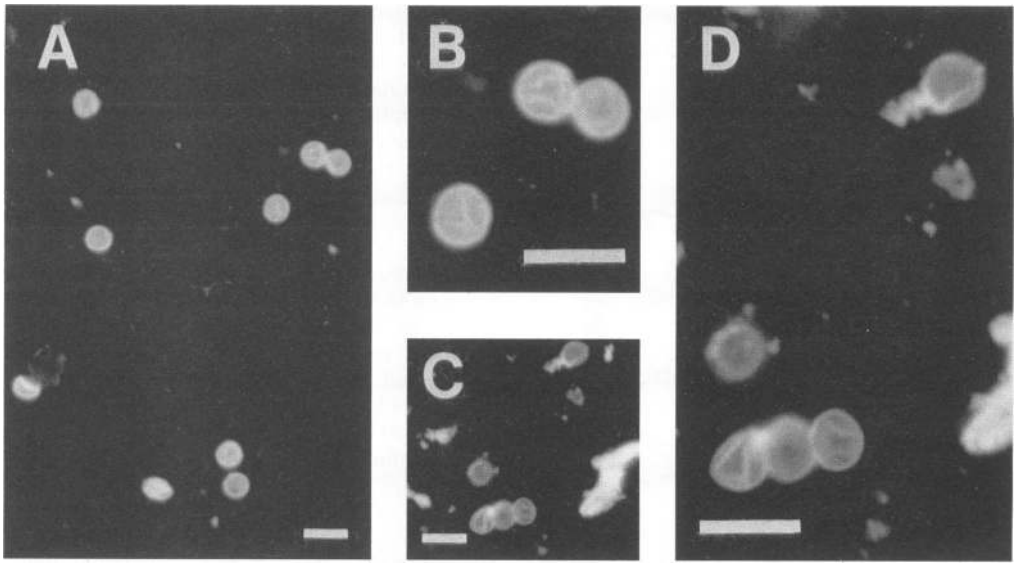

FIG. 2. *Cryptosporidium* oocysts purified from calf feces, seeded to water samples, visualized by IFA, and photographed on the surface of polycarbonate filters (pore size, 1.0 μm; Nuclepore). (A) Typical appearance of oocysts recovered from distilled water (magnification, ×700). (B) Detail of panel A (magnification, ×1,925). (C) Oocysts recovered from water, shown with debris and exhibiting characteristic bright exteriors, transparent interiors, and surface lines (magnification, ×700). (D) Detail of panel C (magnification, ×1,925). Bars are 10 μm.

study was small, the quantitative estimates reported were not usable for statistical analysis.

Oocyst purification. *Cryptosporidium* oocysts were obtained from fresh feces of naturally infected dairy calves (Holstein), primate infants (*M. nemestrina*), and humans. Samples were screened for oocyst presence and number by the acid-fast dimethyl sulfoxide procedure of Bronsdon (3). Oocysts were isolated from fresh feces by being washed through graded screens with distilled water and rewashed three times by centrifuging and resuspending the pellet in distilled water. The material was then concentrated by centrifuging on a gradient of Sheather sugar solution (12) and purified on a Percoll (Sigma)-saline step gradient (prepared with equal volumes at specific gravities of 1.01, 1.05, 1.09, and 1.13) by centrifuging at 650 × *g* for 20 min. Purified oocysts were washed and stored at 4°C in distilled water at 10^5 oocysts per ml with 2.5% Formalin as a preservative. Stability of the stock oocyst suspension was checked periodically by microscopic and numerical evaluations.

RESULTS

Oocyst recovery from river water samples and controls. At the time of sampling, water temperatures of the rivers ranged from 16°C (Skykomish River) to 23°C (Sacramento River). Turbidity values ranged from 0.4 (Skykomish River), 0.7 (Snoqualmie River), 1.1 (Snohomish River), and 1.8 (American River) turbidity units to 7.5 (Skagit River) and 7.9 (Sacramento River) turbidity units. At the time of sampling, flow and turbidity in each of the rivers were relatively low, as is characteristic of these rivers at midsummer. Weather conditions were clear and warm, although light rain, sufficient to produce some runoff, had occurred during the two days before the 29 July and 15 August 1985 samplings of the Washington rivers.

Cryptosporidium oocysts were found in all of the river water samples examined. Oocysts were round, were 3 to 5 μm in diameter, and had characteristic surface wrinkles or lines. Stained oocysts were a bright apple green, a color characteristic of fluorescein, and were sharply defined and luminous around the periphery and similarly bright on surface wrinkles. Interior areas, however, were more lightly stained and had a transparent quality. In addition to oocysts, significant numbers of aquatic organisms and debris particles were present, some of which showed nonspecific fluorescence. Such particles were readily distinguishable from *Cryptosporidium* oocysts by shape, size, color, and stain appearance.

Estimated oocyst concentrations in the river water samples ranged from 2 to 112/liter ($\bar{x}$ = 25.1, s = 30.6, n = 11) (Table 1). *Cryptosporidium* oocysts meeting the identification criteria were also found in some of the unseeded samples. The concentrations found in negative controls ranged from 0 to 16/liter ($\bar{x}$ = 3.9, s = 6.3, n = 6). The concentrations found in tap water ranged from 0 to 20/liter ($\bar{x}$ = 9.3, s = 10.1, n = 3). The positive controls, which were seeded at a concentration of 2×10^3 or 2×10^4 oocysts per liter, showed recoveries of about 70 to 3,880 per liter. The

TABLE 1. *Cryptosporidium* oocysts in river water samples

Date (1985)	Sample no.	Water source (river)	Vol (liters)	% Filter area examined	No. of oocysts	Estimated recovery (%)	Estimated no. of oocysts/liter
7/29	1	Skagit	11	10	2	5	35
	2	Skykomish	18	11	2	5	20
	3	Snoqualmie	17	11	2	5	21
	4	Snohomish	16	7	6	5	112
	5	American	20	9	2	5	23
	6	Sacramento	7	10	1	5	28
8/5	2	Skagit	20	50	5	22	2
	3	Sacramento	13	50	3	22	2
8/15	2	Skykomish	20	50	5	7	7
	3	Snoqualmie	18	50	7	7	11
	4	Snohomish	8	50	4	7	15

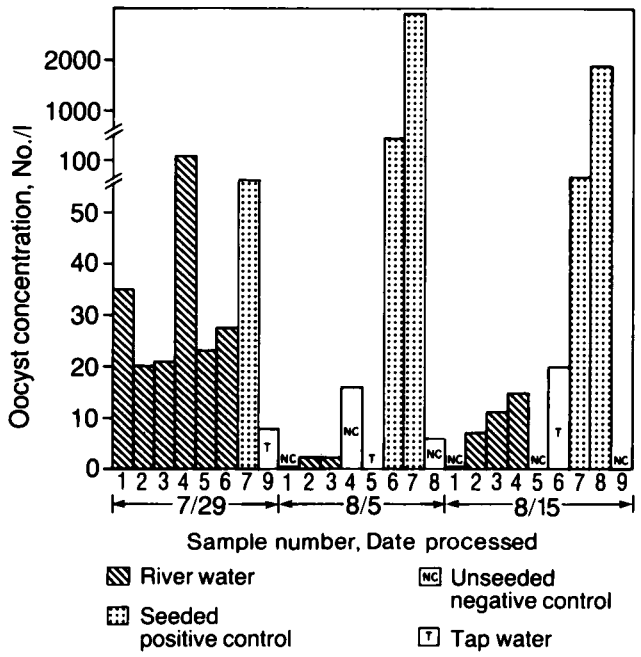

FIG. 3. Oocyst concentrations in 11 river water samples and unseeded (negative) and seeded (positive) controls shown in the exact sequence of processing. (Note scale discontinuity above concentration of 50/liter.)

percentage of oocyst recovery ranged from approximately 5 to 20%. The oocyst concentrations estimated for the 25 samples are shown in Fig. 3 in the order in which the samples were processed.

DISCUSSION

This study demonstrates that, during July and August 1985, *Cryptosporidium* oocysts were present in six rivers in Washington and California. Oocyst presence in three of the Washington rivers, the Snoqualmie, Skykomish, and Snohomish, was expected because of our previous investigations (29) of *Cryptosporidium* infections in dairy cattle living immediately upstream of the water sampling locations. Oocyst presence in the other three rivers was not unexpected. The Skagit River sampling location is also downstream of large dairy farming areas, although no information is available on the prevalence of infection in those areas. Sampling locations on the Sacramento and American rivers in California are in the midst of a large urban area and downstream of major agricultural areas that include livestock activity. In addition, the reported occurrence of cryptosporidial infections in household pets (34) and the appreciable prevalence in the human population (Editorial, Lancet, i:492–493, 1984) may contribute to oocyst presence in rivers in urban areas through storm water runoff and wastewater discharge.

The rivers sampled are not unique or unusual in their water quality. Together, these rivers exhibit a range of watershed conditions and activities that are representative of many, if not most, of the rivers in the United States. Thus, the presence of *Cryptosporidium* oocysts in each of the river water samples suggests the potential for broad geographical distribution and continuous presence of *Cryptosporidium* oocysts in surface waters.

An indication of the significance of oocyst concentrations found in this study is provided by comparing *Cryptosporidium* sp. with *G. lamblia*. *G. lamblia* is similar to *Cryptosporidium* sp. in that it infects a wide variety of animal hosts and is shed as environmentally resistant cysts that have been found in water in many areas of the United States and abroad. Giardiasis is commonly transmitted by water and, in recent years, has been among the most frequently reported waterborne illnesses in the United States (Water-Related Disease Outbreaks, Annual Summary, 1984, Centers for Disease Control, Atlanta). Data on the concentration of *G. lamblia* cysts in water have not been reported. However, concentrations have been estimated from cyst-shedding rates of riparian animals and stream flow, and the concentrations range from one cyst per 10^5 to 10^6 gal (1 gal = 3.785 liters) during high stream flow to 1 to 250 cysts per gal during low flow (17). The latter rate is similar to the range of *Cryptosporidium* oocyst concentrations found in this study, i.e., 2 to 112/liter. Also, it appears that *Cryptosporidium* sp. and *G. lamblia* have similar infectious doses for humans, i.e., between 1 and 10 oocysts or cysts (26; Miller et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1986). If waterborne *Cryptosporidium* sp. and *G. lamblia* are of comparable infectivity, it follows that the potential for waterborne transmission of cryptosporidiosis should be comparable to that of giardiasis.

The procedures used for the isolation and identification of oocysts were effective but were not without problems. The procedures are based on procedures described previously for *G. lamblia* (17, 27), but membrane filters are used for particle collection and IFA oocyst identification. Oocyst recovery rates, ranging from about 5 to 20% as determined from positive controls, have permitted detection of oocysts in 20-liter samples. This sample volume is small compared with that needed for procedures applied to *G. lamblia*, i.e., from 200 to 4,000 liters (17, 28). A sample volume of transportable size is clearly of advantage in that it permits samples to be processed in the laboratory. Field filtration is required when sample volumes exceed 20 to 40 liters.

The limit of detection of this procedure can be estimated at 0.5 oocysts per liter, or about 1.9 oocysts per gal, given the average oocyst recovery rate found in this study (10%) and a 20-liter sample volume. Whether this limit is sufficiently low may be judged relative to a commonly assumed level of human water consumption, i.e., 2 liters/day. Proportionate reduction of the detection limit would result from a consistent improvement in the recovery rate or from an increase in sample size. We expect that experience gained by processing increasing numbers of samples will lead to both more consistent and higher recovery rates.

Occasional false-positives (e.g., oocysts present in samples of distilled water) were encountered. We believe these false-positives were due to oocyst carry-over between samples or to inadequate cleaning of common processing components. Despite these occurrences, a statistical comparison by the Wilcoxon-Mann-Whitney test of the sample results reported here shows that the difference between oocyst concentrations found in river water ($\bar{x} = 25.1$, $s = 30.6$, $n = 11$) and those found in controls ($\bar{x} = 3.9$, $s = 6.3$, $n = 6$) was significant ($P < 0.005$ for a one-sided test; $P < 0.01$ for a two-sided test). Nevertheless, care must be taken to clean common processing components effectively between tests and to include sufficient negative controls.

The time required for thorough microscopic examination of a single filter (diameter, 25 mm) at ×250 at a rate of aboout 15 to 20 s per field is about 6 h. The time increases with

increasing numbers of particles on the filter. The oocyst size, 3 to 5 μm, does not permit the use of a lower magnification. In preliminary tests, we found that the use of 13-mm-diameter filters is practical as long as sample turbidities are low and particle separations are effective. Under these conditions, examination of an entire 13-mm-diameter filter can be accomplished in approximately half the time required for examining half of a 25-mm-diameter filter.

Analysis of river water samples from the western United States shows that *Cryptosporidium* sp. should be considered a waterborne organism. The presence of *Cryptosporidium* oocysts in water could have significant consequences for public health. Further work is needed to define the geographical and temporal distribution of *Cryptosporidium* sp. in water and to assess the viability of waterborne oocysts.

With membrane filtration and IFA procedures described in this pilot study, *Cryptosporidium* oocysts can be located and identified in 20-liter samples of river water, with a limit of detection of about 0.5 oocysts per liter. Further work is needed to improve the limit of detection, to increase the percentage of recovery, to reduce the variability in recovery rate, and to reduce the time required for sample processing and microscopic examination.

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