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Evaluation of Immunofluorescence Techniques for Detection of *Cryptosporidium* Oocysts and *Giardia* Cysts from Environmental Samples

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***Cryptosporidium* and *Giardia* species are enteric protozoa which cause waterborne disease. The detection of these organisms in water relies on the detection of the oocyst and cyst forms or stages. Monoclonal and polyclonal antibodies were compared for their abilities to react with *Giardia* cysts and *Cryptosporidium* oocysts after storage in water, 3.7% formaldehyde, and 2.5% potassium dichromate, upon exposure to bleach, and in environmental samples. Three monoclonal antibodies to *Cryptosporidium parvum* were evaluated. Each test resulted in an equivalent detection of the oocysts after storage, after exposure to bleach, and in environmental samples. Oocyst levels declined slightly after 20 to 22 weeks of storage in water, and oocyst fluorescence and morphology were dull and atypical. Oocyst counts decreased after exposure to 2,500 mg of sodium hypochlorite per liter, and fluorescence and phase-contrast counts were similar. Sediment due to algae and clays found in environmental samples interfered with the detection of oocysts on membrane filters. Two monoclonal antibodies and a polyclonal antibody directed against *Giardia lamblia* cysts were evaluated. From the same seeded preparations, significantly greater counts were obtained with the polyclonal antibody. Of the two monoclonal antibodies, one resulted in significantly lower cyst counts. In preliminary studies, the differences between antibodies were not apparent when used on the environmental wastewater samples. After 20 to 22 weeks in water, cyst levels declined significantly by 67%. Cysts were not detected with monoclonal antibodies after exposure to approximately 5,000 mg of sodium hypochlorite per liter.**

The enteric protozoa *Giardia* and *Cryptosporidium* species are well-documented causes of waterborne disease. From 1971 to 1985, *Giardia* species have been responsible for 92 outbreaks of disease associated with both animal and human fecal contamination of water supplies in the United States (2). *Cryptosporidium* species, newly recognized in 1980 as agents of diarrhea, have been documented in a number of outbreaks of waterborne disease in both the United States and the United Kingdom (4, 5, 8, 17). Zoonotic transmission has been demonstrated for *Cryptosporidium parvum*; therefore, many animals may also serve as sources of *Cryptosporidium* contamination of water supplies with the potential for human infection (3).

Methods to study the occurrences of *Cryptosporidium* and *Giardia* species in water by detection of oocysts and cysts, respectively, have been developed. Large volumes of water are filtered, and the concentrate is examined by microscopic techniques. Developments in methods for *Giardia* and *Cryptosporidium* detection over the last few years have included the use of yarn-wound filters for sample collection (9), various density gradients for sample clarification (15), and membrane filtration coupled with immunofluorescence techniques for cyst and oocyst detection and enumeration (10–16). Limitations of these methods include (i) poor recoveries influenced by water quality, (ii) the possibility of inadequate antigen recognition by the various antibodies, and (iii) the inability to assess identification of species or viability. Thus, interpretation of the environmental data must be made with these limitations of the methods in mind.

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The purpose of this work was to evaluate membrane filtration techniques and antibodies used for detection of enteric protozoan *Giardia* cysts and *Cryptosporidium* oocysts in environmental water concentrates. The effects of storage media, time, disinfectants, and interfering debris on the detection of oocysts and cysts with the antibodies were examined.

MATERIALS AND METHODS

Oocyst and cyst preparations. *C. parvum* oocysts were provided by Charles Sterling (University of Arizona, Tucson) and Bruce Anderson (University of Idaho, Moscow). Samples were collected from the feces of infected calves during scouring. Unpurified oocysts (shipped on ice) were received from Idaho within 24 h. The fecal preparation (50 to 100 ml) was sieved through gauze and stored at 4°C. Samples of oocysts were also received after concentration and purification with a sucrose density gradient (7). Purified samples were stored in 2.5% potassium dichromate ($K_2Cr_2O_7$) at 4°C. *Cryptosporidium baileyi* oocysts were received courtesy of William Current (Eli Lilly & Co., Greenfield, Ind.). The oocysts were recovered from chicken embryos and stored at 4°C in 2% $K_2Cr_2O_7$ or deionized (DI) water with antibiotics (penicillin, 100 U/ml; streptomycin, 100 μg/ml; and amphotericin B [Fungizone], 0.1 μg/ml). An abomasal *Cryptosporidium* isolate was also provided by Bruce Anderson (1) and stored in a fecal preparation at 4°C.

A fecal specimen from a patient infected with *Giardia lamblia* was obtained from the Arizona Health Sciences Center (Tucson) and stored in 3.7% formaldehyde at 4°C. In addition, *G. lamblia* and *Giardia muris* cysts were obtained from Swabby Gerbco, Inc. (Phoenix, Ariz.). These cysts were produced in gerbils and collected within 4 days of

excretion. Fecal samples received from infected gerbils were immediately stored in DI water, 2.5% $K_2Cr_2O_7$, or 3.7% formaldehyde at 4°C.

Antibody preparations. Monoclonal antibodies (MAb) specific for the *Cryptosporidium* oocyst were obtained from Charles Sterling (University of Arizona), Meridian Diagnostics (Cincinnati, Ohio) (6, 19), and Northumbria Biologicals (Northumberland, United Kingdom). MAb against the *Giardia* cyst were obtained from Meridian Diagnostics (21) and John Riggs (California State Health Laboratory, Berkeley) (12). A polyclonal antibody (PAb) to *Giardia* cysts was received courtesy of Judith Sauch (Environmental Protection Agency, Cincinnati, Ohio) (16). A MAb against *Giardia* trophozoites was obtained from Chemicon (El Segundo, Calif.).

Goat anti-mouse immunoglobulin M (heavy chain) [IgM(μ)] labeled with fluorescein isothiocyanate (FITC), goat anti-mouse IgG-FITC, and goat anti-rabbit IgG-FITC were obtained from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, Md.). Goat anti-mouse IgG/IgM-FITC was purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Antibodies were stored at −20°C in 1:10 dilutions in 0.5-ml volumes, thawed, and diluted as working solutions. Meridian antibodies were stored at 4°C as recommended by the manufacturer. Antibody solutions were not used after the dated shelf life of the product. The titers of each antibody were determined to obtain optimal and consistent fluorescence of cysts and oocysts as well as low background fluorescence initially on glass slides and then on membrane filters. The titers of secondary antibodies used in indirect methods were also determined to provide optimum detection of cysts and oocysts indicated by fluorescence of 100% of the cysts and oocysts detected under light microscopy.

Sample preparation. The following procedure was used consistently throughout the study for the enumeration of cysts and oocysts. Cellulose nitrate membrane filters (diameter, 13 mm) were housed in stainless steel filter holders (Millipore Corp., New Bedford, Mass.). Filters with a pore size of 5.0 μm were used for *Giardia* cysts, while 1.2-μm-pore-size filters were used for the enumeration of *Cryptosporidium* oocysts. These have previously been shown to retain 100% of the cysts and oocysts (14).

Samples containing cysts or oocysts were diluted (when necessary) with 1% Tween 80 (Sigma Chemical Co., St. Louis, Mo.) in DI water, and 0.1- to 1-ml volumes were passed through the filter holder housing the filter (using a 1-ml syringe). Equal volumes were filtered from a single preparation of cysts or oocysts and were consistent for all comparative tests on replicate membrane filters. Filters were rinsed twice with 5.0 ml of 1% Tween 80, using 5-ml syringes. The bottoms of the housings were plugged with Parafilm, and the appropriate antibodies were applied directly onto the filters (approximately 0.1 to 0.2 ml). After 30 min of incubation at room temperature, the filters were rinsed as described above. For indirect assays, the secondary antibody was applied and incubated in the same manner as described above and was then rinsed a second time. Filters were then removed from the housings and placed on glass slides in 50% glycerol–phosphate-buffered saline solution (pH 8.0), and cover slips were applied. The entire filter was examined at a magnification of ×200 by using a BHTU epifluorescence microscope equipped with a 100-W high-pressure mercury lamp (Olympus, New Hyde Park, N.Y.); cysts and oocysts were enumerated. Counts per filter, per volume filtered, or per milliliter of the original preparation were reported.

Comparative reactivity of the antibodies and stability of the fluorescence counts. The reactivity of the specific antibodies was examined for cysts from *G. muris* and *G. lamblia* and oocysts from *C. parvum*, *C. baileyi*, and the abomasal *Cryptosporidium* isolate. The ability of the antibodies to detect cysts and oocysts after storage in various solutions over time was also determined for *G. lamblia* and *C. parvum*, respectively. Unpurified cysts (used directly from gerbil feces) and oocysts were used within 6 to 10 days of collection; samples were suspended in DI water, 2.5% $K_2Cr_2O_7$, or 3.7% formaldehyde at approximately 10^4 organisms per ml and stored at 4°C as determined by hemacytometer and membrane filter counts. Cyst and oocyst concentrations were measured in all three solutions after 1 to 5 months of storage. Various antibodies were used on these samples.

The abilities of the various antibodies to detect cysts and oocysts before and after exposure to bleach (NaOCl) were determined. Concentrations of unpurified cysts (5×10^3/ml) and oocysts (1×10^4/ml) in 5-ml volumes were enumerated, mixed with an equal volume of DI water containing various dilutions of bleach to obtain final approximate concentrations of 0, 5, 50, 500, 2,500, 5,000, 10,000, 15,000, and 20,000 mg of NaOCl per liter, and incubated for 20 min at room temperature in 15-ml polypropylene tubes. After exposure, 0.1 to 1.0 ml of sodium thiosulfate (10% solution) was added to each tube to neutralize any residual NaOCl. The sample was centrifuged, and the pellets were suspended in 5 ml of 1% Tween 80. The samples were filtered and stained as described above, and the cysts and oocysts were enumerated. Any changes in morphology or intensity of fluorescence as compared with that of the control were noted.

Detection of cysts and oocysts in environmental samples. Environmental samples collected from domestic wastewater before disinfection (40 liters) were recovered from 10-in. (1 in. = 2.54 cm), yarn-wound polypropylene cartridge filters (MicroWynd II; AMF-CUNO, Meriden, Conn.) as described by Musial et al. (11) and Rose et al. (15). Filters were eluted by backflushing and then cut apart and washed in 0.1% Tween 80. A lower concentration was used to decrease foaming. The eluents were centrifuged (1,200 × *g* for 10 min), and the supernatants were discarded. Pellets were suspended in 1% Tween 80 and 1% sodium dodecyl sulfate and homogenized (setting, 30) in a VirTis homogenizer (The VirTis Co., Inc., Gardiner, N.Y.) for 3 min. After the addition of a few drops of antifoam (Sigma), the cysts and oocysts were washed by centrifugation (1,200 × *g* for 10 min) and suspended either in the detergent solution for the sucrose (1.24 g/ml) density gradient clarifications or in DI water for clarifications with potassium citrate (1.24 g/ml). After sonication (25 kHz, Branson ultrasonic cleaner; Branson Sonic Power Co., Shelton, Conn.), 10 ml of each sample was layered onto 30 ml of the density gradient solution. The gradients were centrifuged at 1,200 × *g* for 10 min for *Cryptosporidium* oocysts and 800 × *g* for 3 min for *Giardia* cysts. The entire supernatant from each sample was recovered, diluted 1:3 with 1.0% Tween 80, and centrifuged (1,200 × *g* for 10 min). The pellets were suspended in 1 to 5 ml of 1.0% Tween 80. The samples were filtered and stained with the antibodies as described above.

Evaluation of the counting procedures by using membrane filters. The precision of determining oocyst and cyst concentrations by using membrane filters and fluorescent antibody staining was evaluated. Four replicate 1-ml volumes were

TABLE 1. Antibodies tested against *Cryptosporidium* and *Giardia* species with fluorescence microscopy

Antibody designation	Antibody description				
	Protozoan/stage specificity	Immunoglobulin class	Type of fluorescence procedure	Type of antibody	Source (reference)
C1B3	*C. parvum* oocyst	IgG	Direct	MAb	Charles Sterling[a] (19)
OW3	*C. parvum* oocyst	IgM	Indirect	MAb	Charles Sterling (6); Meridian Diagnostics[b]
N	*C. parvum* oocyst	IgG	Direct	MAb	Northumbria Biochemicals[c]
JR	*G. lamblia* cyst	IgG	Direct	MAb	John Riggs[d] (12)
M	*G. lamblia* cyst	IgG	Indirect	MAb	Meridian[b] (21)
JS	*G. lamblia* cyst	IgG	Indirect	PAb	Judith Sauch[e] (16)
CH	*G. lamblia* trophozoite	IgG	Indirect	MAb	Chemicon[f]

[a] University of Arizona, Tucson.
[b] Cincinnati, Ohio.
[c] Northumberland, United Kingdom.
[d] California State Health Laboratory, Berkeley.
[e] Environmental Protection Agency, Cincinnati, Ohio.
[f] El Segundo, Calif.

filtered, stained, and counted from three samples of *Giardia* cysts and *Cryptosporidium* oocysts (approximately 20 to 80 cysts or oocysts per ml). The entire filters were scanned (at ×200), mean oocyst and cyst counts were determined, and standard deviations, standard errors, and coefficients of variation were calculated. The individuals preparing the filters were different from those individuals counting, and no more than two replicate filters were scanned by the same individual.

The distribution of *Cryptosporidium* oocysts on the membrane filter was determined by counting three replicate filters in which the number of oocysts in each field was recorded for the entire filter. The mean number of oocysts per field, standard deviations, and coefficients of dispersion were calculated.

Interference by sediments with oocyst enumeration on membrane filters was also evaluated. Known concentrations of oocysts (300 to 700 oocysts per ml) were seeded into environmental concentrates (previously ascertained to be free of oocysts) at final turbidities of 0.1, 0.2, 0.4, 0.8, 10, 50, 100, and 150 nephelometric turbidity units due to algae or clay naturally present in the samples. The samples were subsequently filtered (0.1 ml) and stained with fluorescent antibodies, and oocysts were enumerated. Mean oocyst counts, standard errors, and coefficients of variation were determined.

Statistical evaluation of the data. All cyst and oocyst counts were transformed to $\log_{10}$ values before statistical manipulation. Data were analyzed, using CoStat statistical software (CoHort Software, Berkeley, Calif.). Analysis of variance (ANOVA) was used to evaluate the differences between antibodies and storage solutions. A two-way ANOVA, completely randomized, was used to evaluate cyst counts for two antibodies in three storage solutions. A one-way ANOVA was used to compare cyst counts with three different antibodies. The *t* test was used to compare oocyst counts with two antibodies. Correlation coefficients (CoStat Software) were determined for cyst and oocyst densities versus time in formaldehyde, DI water, and $K_2Cr_2O_7$. Coefficients of variation, coefficients of dispersion, and standard errors of the means were determined for cyst and oocyst counts on membrane filters.

TABLE 2. Fluorescent-antibody reactivity against *Cryptosporidium* oocysts

Oocyst prepn[a]	Reactivity with *Cryptosporidium* MAb:		
	C1B3	OW3	N
C. parvum	+	+	+
C. baileyi	+	−	+
Abomasal *Cryptosporidium* isolate	ND[b]	−	+

[a] Preparations tested from water, 3.7% formaldehyde, and 2.5% $K_2Cr_2O_7$.
[b] ND, Not determined.

RESULTS

Seven antibodies directed against either *Giardia* or *Cryptosporidium* species were evaluated on membrane filters by using fluorescence microscopy (descriptions and abbreviations are listed in Table 1). The concentrations of oocysts and cysts were determined by using a hemacytometer and light microscopy, and the preparations were diluted to yield approximately 60 to 100 oocysts or cysts on each filter. Oocysts of *C. parvum* (calf isolate), *C. baileyi* (chicken isolate), and the abomasal *Cryptosporidium* isolate (from cattle) were tested with three MAb (N, C1B3, and OW3) (Tables 2 and 3). The N MAb reacted with all three types of oocysts, and the C1B3 MAb reacted with at least the calf and chicken oocysts. Because of a limited supply of antibody, the C1B3 MAb was not tested against the abomasal *Cryptosporidium* isolate. The OW3 MAb was found to react with only *C. parvum* oocysts. The specificities of the antibodies were maintained even after storage of oocysts in either water, 3.7% formaldehyde, or 2.5% $K_2Cr_2O_7$. All three MAb appeared to elicit a fluorescence specifically around the outside walls of the oocysts, and characteristic folds in oocyst walls were observed in 40 to 70% of the oocysts.

The specificities of MAb and PAb were determined, using preparations of *G. lamblia* and *G. muris* cysts (Table 3). The

TABLE 3. Fluorescent-antibody reactivity against *G. lamblia* and *G. muris* cysts on membrane filters

Cyst prepn	Cyst count with *Giardia* antibodies[a]:			
	JR	M	CH	JS
G. lamblia	109	86	0	121
G. muris	0	2	0	36

[a] Averages of two counts from a single preparation of cysts stored in water.

TABLE 4. Oocyst counts on membrane filters with two MAb for *C. parvum*

Sample	Oocyst storage conditions[a]	Trial	Oocyst count with *Cryptosporidium* MAb:			
			C1B3[b]	OW3[c]	C1B3[b]	OW3[d]
1	2.5% potassium dichromate, 4°C, 2 weeks	1	238	104	228	214
		2	210	90	200	250
		3	103	111	173	100
		4	240	200	213	220
		5	80	30	112	70
$\bar{x}$			174 ± 77[e]	109 ± 58	185 ± 46	171 ± 80
2	Deionized water, 4°C, 3 days	1	63			75
		2	62			60
		3	72			66
		4	96			84
$\bar{x}$			73 ± 16	ND[f]	ND	71 ± 10

[a] All oocysts were purified.
[b] C1B3 antibody directly labeled with FITC.
[c] Secondary antibody, anti-mouse IgG/IgM-FITC conjugate (Boehringer Mannheim Biochemicals, Indianapolis, Ind.).
[d] Secondary antibody, anti-mouse IgM(μ)-FITC conjugate (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.).
[e] Standard deviation.
[f] ND, Not determined.

MAb derived from trophozoites (CH) did not react with *Giardia* cysts from either species. The JS MAb reacted with both *G. lamblia* and *G. muris* cysts, while the M MAb detected *G. muris* cysts at approximately 1/10 the cyst number determined by using the PAb. The JR MAb did not react with *G. muris* cysts.

After the initial evaluation of the antibodies, further investigations were restricted to MAb C1B3, OW3, N, JR, and M and PAb JS, using *C. parvum* oocysts and *G. lamblia* cysts.

The C1B3 and OW3 MAb were compared for *Cryptosporidium* oocyst counts, using purified oocysts stored in $K_2Cr_2O_7$ and DI water. The fluorescence observed with the OW3 (and an anti-mouse IgG/IgM-FITC conjugate) was diminished or dull, and the oocyst wall appeared to be unevenly labeled with fluorescence. Increasing the titer of the primary (OW3) or secondary (anti-mouse IgG/IgM-FITC) antibody did not enhance the level of fluorescence. Oocyst counts were generally lower (37 to 63%), although this is not statistically significant when compared with levels obtained with the C1B3 antibody from the same preparation. The use of a more specific secondary antibody with the OW3 [an anti-mouse IgM(μ)-FITC] resulted in bright fluorescence and comparable oocyst counts of 185 and 171 (in $K_2Cr_2O_7$) and 73 and 71 (in DI water), using the C1B3 and OW3 antibodies, respectively (Table 4). All further evaluations with the OW3 MAb utilized the anti-mouse IgM(μ)-FITC as the secondary antibody.

Fresh, unpurified *G. lamblia* cysts and *C. parvum* oocysts were pelleted and suspended in 2.5% $K_2Cr_2O_7$, 3.7% formaldehyde, or DI water. At 0, 1, 2, 3, 4, 20, 21, and 22 weeks, cysts and oocysts were enumerated after storage at 4°C. Duplicate filters from the three *Giardia* cyst preparations were stained with the JR and M MAb and the JS PAb, while *Cryptosporidium* oocyst preparations were stained with the OW3 and N MAb. ANOVA and a *t* test were used to test for significant differences between cyst counts and oocyst counts determined with the *Giardia* and *Cryptosporidium* antibodies, respectively. The results indicated that no statistically significant difference was observed between the *Cryptosporidium* oocyst counts obtained with the two MAb (Table 5). Significantly lower (by 79 and 62%) *G. lamblia* cyst counts ($P \leq 0.01$) were obtained when using the JR MAb, in contrast to the M MAb and JS PAb (Table 5). No significant difference was observed between the counts determined with the M MAb and the JS PAb.

G. lamblia cyst counts after staining with two antibodies (M MAb and JS PAb) obtained from three storage media (DI water, 3.7% formaldehyde, and 2.5% $K_2Cr_2O_7$) were ana-

TABLE 5. Comparison of *C. parvum* oocyst and *G. lamblia* cyst counts[a] in antibody-stained preparations

Antibody	Mean cyst or oocyst count	Log_{10} mean cyst or oocyst count	Statistical test	Degrees of freedom
JR	6	0.778[b]		
M	16	1.210	ANOVA	33
JS	28	1.447		
OW3	48	1.68	*t* test	24
N	55	1.74		

[a] Counts were made from a variety of seeded samples stored in either 3.7% formaldehyde, 2.5% $K_2Cr_2O_7$, or DI water at 4°C.
[b] Significant at $P < 0.01$.

TABLE 6. Two-way ANOVA comparison of *G. lamblia* cyst counts obtained from three storage media and stained with two antibodies

Main effect	Mean cyst count	Log_{10} mean cyst count
Antibody		
M	31	1.493[a]
JS	44	1.642
Storage condition[b]		
Deionized water	31	1.485
Potassium dichromate (2.5%)	40	1.607
Formaldehyde (3.7%)	41	1.611

[a] Significant at $P < 0.05$, 78 degrees of freedom.
[b] Stored for 22 weeks.

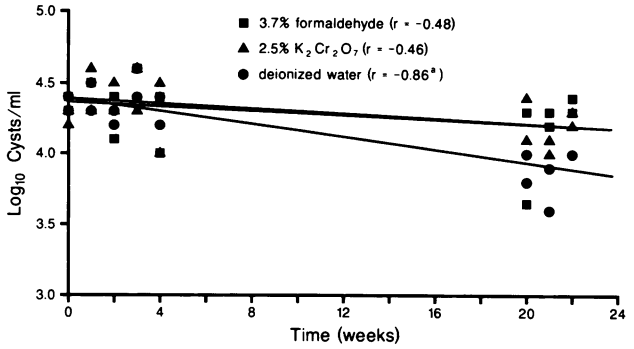

FIG. 1. Stability of stored *Giardia* cysts in DI water, formaldehyde, or potassium dichromate at 4°C. a, Significant at the 99% confidence limit.

lyzed by two-way ANOVA (Table 6). Significantly greater cyst counts (by 29%) were obtained when the cysts were stained with the JS PAb versus the M MAb ($P \leq 0.05$). No differences in cyst counts were obtained among the three storage media. Furthermore, no significant interaction was found between antibody type and storage medium.

Concentrations of oocysts and cysts (log_{10} values) were plotted against time for each storage medium, and lines of best fit were determined. The Pearson correlation coefficients were derived for time versus oocyst or cyst level. *Giardia* cyst levels averaged 2.3×10^4 during the first 4 weeks and dropped by weeks 20 to 22 to 1.6×10^4, 1.5×10^4, and 7.5×10^3 in formaldehyde, $K_2Cr_2O_7$ and DI water, respectively (Fig. 1). No significant association ($P \leq 0.01$) was observed between time and cyst counts in the formaldehyde ($r = -0.48$) or $K_2Cr_2O_7$ ($r = -0.46$); however, a significant relationship ($P < 0.01$) between decreasing cyst levels and increasing time of storage in water ($r = -0.86$) was found. After storage in water, cyst fluorescence was diminished by 50% compared with the fluorescence of cysts in the other storage media, and cyst shape was not consistently oval but more oblong. *Cryptosporidium* oocyst levels averaged 1.1×10^4 during weeks 0 to 4 and appeared to increase slightly with time (2.1×10^4 by weeks 20 to 22), with a decrease in clumping. Counts remained stable in formaldehyde (1.3×10^4) and $K_2Cr_2O_7$ (2.1×10^4). By weeks 20 to 22, oocyst levels decreased to 7.3×10^3 when stored in DI water, fluorescence was dull, and the oocysts appeared compressed (~2 μm in diameter). Correlation coefficients

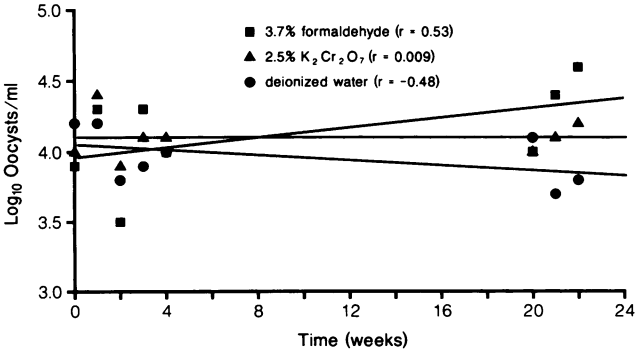

FIG. 2. Stability of stored *Cryptosporidium* oocysts in DI water, formaldehyde, or potassium dichromate at 4°C.

TABLE 7. *Giardia* cyst and *Cryptosporidium* oocyst counts from environmental sewage concentrates stained with various antibodies

Antibody	No. of samples[a]	% Positive for cysts or oocysts	Maximum count for single filter	Avg count/ filter[b]
Giardia species				
Set 1				
JR	18	68	3	1.4
M	18	85	11	2.1
Set 2				
JR	8	100	13	6.5
M	8	100	19	6.4
JS	8	100	5	3.1
Cryptosporidium species				
Set 3				
C1B3	5	40	14	3.2
OW3	5	60	16	4.0
Set 4				
OW3	9	44	2	0.7
N	9	44	3	0.5

[a] Equal and replicate volumes from each sample were filtered for each comparative test, and the entire filter was examined.
[b] Averages of two filters per antibody for all samples in each set.

for counts versus time were not significant for formaldehyde, $K_2Cr_2O_7$, and DI water (Fig. 2).

Twenty-six and fourteen environmental samples collected from sewage and stored in either 3.7% formaldehyde or 2.5% $K_2Cr_2O_7$ were evaluated for the presence of naturally occurring *Giardia* cysts or *Cryptosporidium* oocysts with various antibodies. Four sets of samples were received and in each test, replicate and equal volumes from each sample were filtered and separately stained in duplicate with the MAb or PAb. *Giardia* cysts were found in 68 to 100% of the samples in two sets of samples, at counts of up to 19 cysts per filter. *Cryptosporidium* oocysts were detected in 40 to 60% of the samples, at counts as high as 16 oocysts per filter. No significant differences in oocyst or cyst counts were observed in wastewater samples with the various antibodies

TABLE 8. Physical stability and reaction of *Giardia* cysts and *Cryptosporidium* oocysts with antibodies after exposure to bleach

Approx final NaOCl concn (mg/liter)[a]	Count[b] of:					
	Giardia cysts with antibody:			*Cryptosporidium* oocysts with antibody:		
	JR	M	JS	C1B3[c]	OW3[d]	N[d]
0	58	84	59	386	213	217
5	52	76	87			
50	81	87	61			
500	23	45	58	ND[e]	340	276
2,500				ND	109	74
5,000	1	1	20	290	ND	ND
10,000	0	0	10	212	ND	ND
15,000	0	0	13			
20,000	0	0	20	21	3	3

[a] Approximately 5×10^3 cysts or 1×10^4 oocysts were mixed with 5 ml of bleach diluted to various concentrations of sodium hypochlorite, incubated for 20 min at room temperature, and then dechlorinated with sodium thiosulfate.
[b] Averages of two trials.
[c] Tested with different oocyst suspension than were OW3 and N.
[d] Counts made on glass slides (10 μl).
[e] ND, Not determined.

TABLE 9. Counting reproducibility of *Giardia* cysts and *Cryptosporidium* oocysts on membrane filters by using fluorescence microscopy

Replicate filter	No./filter					
	Giardia cysts			*Cryptosporidium* oocysts		
	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3
1	31	31	67	81	54	48
2	32	26	76	62	47	50
3	38	24	61	80	49	47
4	34	32	70	72	43	42
$\bar{x}$[a]	33.75	28.75	68.50	73.75	48.25	46.75
SD	2.68	3.34	5.41	7.63	3.96	2.95
$\bar{x}$SD	3.81			4.85		
CV[b]	7.94	11.62	7.89	10.30	8.21	6.31
$\bar{x}$CV	9.22			8.27		
$\bar{x}$SE	1.9			2.4		

[a] Mean.
[b] CV, Coefficient of variation.

(Table 7), although the maximum number of *Giardia* cysts detected in a single subsample was consistently observed when using the M MAb.

The reaction of the antibodies to bleach-treated oocysts and cysts was tested (Table 8). Approximately 5×10^3 *Giardia* cysts and 1×10^4 *Cryptosporidium* oocysts were exposed to various concentrations of sodium hypochlorite (NaOCl) achieved by diluting bleach. No residuals were measured. *Giardia* cyst counts remained stable, as determined with all three antibodies at exposures of up to 50 mg of NaOCl per liter. However, at exposures of 500 mg of NaOCl per liter, cyst counts decreased between 35 and 57% when stained with the JR and M MAb. A few cysts and no cysts were detected after exposure to levels of 5,000 and >10,000 mg of NaOCl per liter, respectively, when stained with both of the MAb against *Giardia* species. In contrast, the *Giardia* cyst counts achieved by staining with the JS PAb remained stable after exposures of 500 mg of NaOCl per liter and decreased 66 to 83% at exposures of ≥5,000 mg of NaOCl per liter.

Cryptosporidium oocyst counts, determined by staining with the OW3 and N MAb, dropped by 49 to 66% after exposures to 2,500 mg of NaOCl per liter. Oocyst levels determined with the C1B3 MAb decreased by 34 to 45% after exposure to 5,000 to 10,000 mg of NaOCl per liter. Exposure to high concentrations of NaOCl (20,000 mg/liter) resulted in a 95 to 99% decrease in oocyst counts after staining with any of the three MAb. *Cryptosporidium* oocysts were stained with the OW3 and N MAb on glass slides. Counts were determined under phase-contrast microscopy and epifluorescence. Oocyst counts were comparable before and after exposure to bleach, in that all oocysts observed under phase-contrast microscopy were fluorescing.

The precision of determining cyst and oocyst concentrations by using membrane filters with fluorescent antibodies was examined. Four replicate volumes were filtered from three preparations of purified oocysts and cysts and stained with C1B3 and JR MAb, respectively (Table 9). The relative variation from the mean (coefficient of variation) ranged from 7.89 to 11.62 for cyst counts and averaged 9.22 for *Giardia* cyst counts at densities between 24 and 70 cysts per filter. The coefficient of variation averaged 8.27 for *Cryptosporidium* oocyst counts between 42 and 81 cysts per filter. The standard error for the total variation was 1.9 and 2.4 for *Giardia* cyst and *Cryptosporidium* oocyst counts, respectively.

TABLE 10. Determination of *Cryptosporidium* oocyst distribution on membrane filters by fluorescence microscopy

Trial	Mean no. of oocysts/field	SD	Coefficient of dispersion[a]
1	0.3977	0.4269	1.07
2	0.3614	0.3498	0.9679
3	0.4477	0.4802	1.07

[a] Values near 1 indicate a Poisson distribution.

Oocyst distribution on the membrane filter was determined by using a purified oocyst suspension and recording the numbers of oocysts per field on an entire filter (440 fields) at a magnification of ×400. The coefficients of dispersion for three trials were 1.07, 0.97, and 1.07 (Table 10), which indicated a Poisson distribution of oocysts (in which the coefficient of dispersion equals 1.0).

The use of membrane filters for the accurate and precise counting of oocysts in the presence of environmental sediments was evaluated. Oocysts were added to three surface water samples (determined free of oocysts) containing algae or clay at turbidities ranging from 0.1 to 150 nephelometric turbidity units. Mean oocyst counts ranged from 2 to 76 per filter, and the standard error averaged 7.2 (range, 3.5 to 13). The coefficient of variation averaged 22 (range 12 to 36). Algae and clay concentrates at turbidities of 50 nephelometric turbidity units were shown to interfere with the detection of oocysts (Fig. 3). Counts were depressed 20 to 95%. No oocysts were detected on membrane filters in 0.1 ml of samples with turbidities of 150 nephelometric turbidity units due to clays.

DISCUSSION

A number of antibodies have been developed and used for the detection of *Cryptosporidium* or *Giardia* species by fluorescence microscopy in both clinical and environmental samples (6, 10–16, 19–21). The detection of enteric protozoa in water samples is plagued by problems which are not apparent in fecal samples. Only antibodies developed against the cyst and oocyst stages are applicable to environmental samples, as the other stages would not be found in water. The numbers of cysts and oocysts are generally low in water

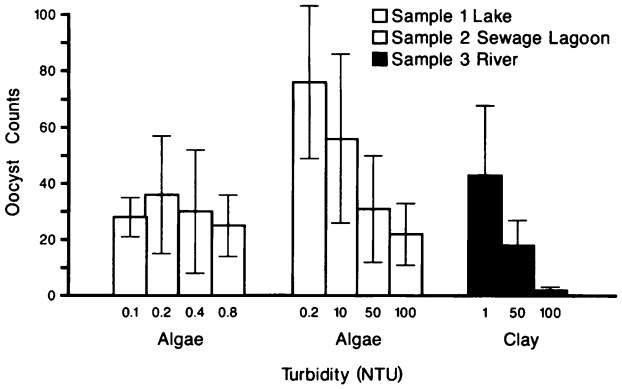

FIG. 3. Interference by sediments with the detection of *Cryptosporidium* oocysts on membrane filters under fluorescent microscopy. NTU, Nephelometric turbidity units.

compared with those in fecal samples and, therefore, sensitivity becomes an issue. Algae, diatoms, pollen, etc., are very common in most waters and may interfere with the detection of the target organism. Animals as well as humans may be contributing cysts or oocysts found in water, and identification of species is difficult to ascertain. Viability and potential infectivity are uncertain.

It is possible that the JR and OW3 MAb may be used to examine water samples more specifically for *G. lamblia* and *C. parvum*, respectively (12, 20, 22). Such specificity would be beneficial since other species, such as *G. muris* (the mouse species) and *C. baileyi* (the chicken isolate), have not been shown to cause infection in humans. The OW3 MAb did not react with *C. baileyi* oocysts, which confirms previous reports (19), or with oocysts of an abomasal *Cryptosporidium* isolate which has been found in cattle but has not been described in humans (1). Darlington and Blagburn (M. V. Darlington and B. L. Blagburn, Abstr. Annu. Meet. Southeastern Soc. Parasitol., abstr. no. 34, 1988) have reported, however, that the OW3 MAb will react with oocysts from *C. meleagridis*, a turkey isolate. The other two MAb against *Cryptosporidium* species (C1B3 and N) were less species specific, reacting with the oocysts of *C. baileyi*. The C1B3 MAb has also been reported to react with yeasts (19) which (because of the similar size and shape of yeasts) may result in false-positives for *Cryptosporidium* species if no other confirmatory test is used.

The JR MAb did not react with *G. muris* cysts, and it has been reported that it does not react with cysts from muskrats (12, 22). The M MAb demonstrated a lower reactivity for *G. muris* cysts when compared with counts obtained by staining with the JS PAb. Both of the MAb against *Giardia* species may somewhat limit the detection of *Giardia* cysts originating from mice and perhaps other animals in water samples. *Giardia* species are inadequately defined, and until species and their host specificities are better described, it is unlikely that any single antibody will identify cysts in water which only pose a health risk to humans.

No differences were observed in either seeded preparations or environmental samples for *Cryptosporidium* oocyst levels with the C1B3, OW3, or N MAb, except in two trials. The C1B3 MAb gave higher counts than the OW3 MAb when oocysts stored for 2 weeks in water were used. However, because of the limited supply of C1B3 MAb, the difference could not be validated. Madore et al. (10) have reported oocyst levels as high as 5.8×10^3/liter in wastewater from Arizona, using the C1B3 MAb, in contrast to levels reported in Texas and Colorado (4.0 oocysts per liter), using the OW3 MAb (13). This could result from either a decreased efficiency in the detection of oocysts in water when using the OW3 MAb or the detection of yeasts when using the C1B3 MAb or a true difference in the occurrences of *Cryptosporidium* species due to differences in the infections in the populations producing the sewage.

In seeded trials with *G. lamblia* cysts derived from gerbils, the JR MAb resulted in significantly lower counts, while the JS PAb resulted in the highest counts. The direct fluorescent procedure used with JR MAb may allow only limited detection of cysts because of the high background fluorescence; or perhaps, with the greater specificity of the MAb, more cysts failed to bind to the antibody, having lost that particular epitope. In wastewater concentrates stained with the various antibodies, differences in cyst detection were not readily apparent, and more comparative information for not only sewage but surface waters (which may have greater fecal contributions from animals) will be needed.

Water samples once concentrated are generally stored in 3.7% formaldehyde or 2.5% $K_2Cr_2O_7$. Potassium dichromate has been previously used for *Cryptosporidium* species and maintains the viability of oocysts (3), and formaldehyde has been used for *Giardia* species. It was of interest to examine the stability of the reaction of the antibody with the organisms in water and in these preservatives. *Giardia* cyst levels declined slightly after 22 weeks when stored in potassium dichromate or formaldehyde and demonstrated a more significant decline in water. In addition, after 20 weeks of storage in water, the cysts had lost their characteristic shape and demonstrated a nonuniform fluorescence around the cyst wall. The decline in cyst levels may have been due to disruption of cyst integrity or failure of the antibodies to react.

A similar trend was seen with stored *Cryptosporidium* oocysts. Counts appeared to increase slightly in formaldehyde, probably because of the dispersion of oocyst clumps commonly seen in these unpurified preparations, which produced a more homogeneous suspension. The physical shape of the oocyst and the level of fluorescence were best when stored in formaldehyde, whereas in the water, the fluorescence was dull and the oocysts appeared to be compressed and folded.

Exposure to a disinfectant may also decrease the immunofluorescence detection of cysts and oocysts. Decreases in oocyst counts were observed only after exposure to >2,500 to 20,000 mg of sodium hypochlorite per liter and were probably due to physical disintegration of the oocyst wall, as fluorescing oocyst counts were equal to counts detected by phase-contrast microscopy on the same fields. Decreases in *Giardia* cyst counts were observed after exposures to 500 mg of sodium hypochlorite per liter. The MAb were not able to bind to *Giardia* cysts, which were detectable by using the PAb, probably as a result of oxidation of the epitope. It appears that the *Giardia* cyst wall is more susceptible to damage by chlorine than is the *Cryptosporidium* oocyst wall. The antibody binding site on the oocyst or cyst may be stable at chlorine concentrations used in drinking water (1 to 3 mg of NaOCl per liter). We have demonstrated that after exposure to 50 mg of NaOCl per liter, both MAb and PAb would bind to the cysts and oocysts, permitting detection by immunofluorescence with all the antibodies tested. The effects of other disinfectants, such as ozone, on the abilities of cysts and oocysts to bind to antibodies need to be examined.

Cyst and oocyst quantitation is important in determining the potential exposure and risk. The use of membrane filters for enumerating cysts and oocysts from environmental water samples has several advantages. Large volumes of the sample may be processed so that an adequate percentage of the sample may be examined. Routinely, we filter and examine a minimum sample volume equivalent to the volumes of 40, 100, and 400 liters which were collected from wastewaters, surface waters, and drinking waters, respectively, for each protozoan. Spaulding et al. (18) demonstrated a relative variation of 4.5 to 12.2 for *Giardia* cyst counts on filters, using a clearing procedure and light microscopy. Our results are very similar for *Cryptosporidium* oocysts and *Giardia* cysts, employing immunofluorescence in clean preparations. However, environmental samples may contain various debris and other organisms and have fairly high turbidities even after processing on density gradients. This results in a decrease in the volume which can be passed through the membrane filters, greater error in counting, and interference with the detection of the organisms. New ap-

proaches for separating cysts and oocysts from the extraneous debris will be needed to overcome these problems.

We have developed a set of criteria for identifying *Giardia* cysts or *Cryptosporidium* oocysts when examining environmental samples. The criteria include (i) the degree of fluorescence, at least 50% of that seen in controls of fresh oocysts or cysts seeded into environmental samples; (ii) a distinct fluorescence around the wall; (iii) a general spherical shape of 4 to 6 μm for *Cryptosporidium* species and an oval shape of 8 to 18 μm by 5 to 15 μm for *Giardia* species; and (iv) a folding in the oocyst wall (seen in 60% of fresh oocysts). For cysts and oocysts stored in water for more than 20 weeks, only 55 and 20%, respectively, would have been identified as *Giardia* and *Cryptosporidium* species by using that set of criteria, underestimating the levels of cysts and oocysts by approximately 50 to 80%. Sauch (16) has developed a test for *Giardia* cyst detection in water samples in which the membrane filters are cleared and immunofluorescence as well as phase-contrast microscopy can be used. After initial detection with the fluorescent antibody, cysts may be confirmed by visualizing internal morphology under phase microscopy. This procedure may also be applicable to *Cryptosporidium* species. Determinations of immunofluorescence, size, and shape may be used as presumptive screens for *Giardia* and *Cryptosporidium* species, followed by some other type of confirmatory test, if needed.

It is obvious that the underestimation of *Cryptosporidium* oocysts and *Giardia* cysts in environmental waters is a significant problem. This may be partly the result of the length of time and conditions under which the oocysts or cysts have been exposed to water, the type of antibodies used (both primary and secondary), and the amount of debris in the samples. Another concern is that there is no information available on the association between detection and viability. Despite these limitations, immunofluorescence has greatly enhanced the ability to detect *Cryptosporidium* oocysts and *Giardia* cysts in water at sensitivities of 0.25 cysts or oocysts per 100 liters; the methods have been used during investigations of disease outbreaks, demonstrating that levels of 63 oocysts per 100 liters are associated with significant waterborne disease (8, 13, 14). As the methods are further developed and utilized, the health significances of the occurrences of protozoa in the environment will be elucidated.

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