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Decrease in *Cryptosporidium parvum* Oocyst Infectivity In Vitro by Using the Membrane Filter Dissolution Method for Recovering Oocysts from Water Samples

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Exposure of *Cryptosporidium parvum* oocysts to solutions used for cellulose acetate membrane (CAM) dissolution filtration reduced their infectivity in HCT-8 cells. Ethanol (95% [vol/vol] and 70% [vol/vol]) alone and short exposure times to acetone decreased infectivity. These findings contrast with similar experiments using excystation assays and infectivity in mice.

Successful detection of *Cryptosporidium parvum* oocysts in water samples depends on efficient filtration methods to recover oocysts. Method 1623 from the U.S. Environmental Protection Agency involves filtering a 10-liter water sample using the Gelman Envirochek membrane capsule filter that traps oocysts. The filter is then shaken to recover the oocysts in an elution buffer (15, 25). Other filtration methods, such as the use of polycarbonate membranes or polypropylene cartridge filters, have been described (13, 17–19). The purpose of these methods is to recover oocysts from raw or finished water samples. Suitable filtration methods should not render oocysts noninfective through chemical or mechanical treatments so infective oocysts in samples can be determined.

An alternative filtration method involves capture of oocysts on a cellulose acetate membrane (CAM) that is dissolved in acetone following filtration and is subsequently centrifuged, rinsed in ethanol, and eluted in a buffer for final recovery (1). This method has an average rate of recovery up to 70.5%, making it more reliable than other methods (1, 2). When modified into a Millipore Glass Microanalysis system, the method resulted in higher oocyst recoveries, particularly when 1 liter of the elution buffer per 25 liters of low-turbidity water was used (10).

The viability of *C. parvum* oocysts can be determined by vital dye staining, exposing oocysts to excystation solutions, and testing their infectivity by infecting mice. In recent years various cell culture methods have been developed whereby *C. parvum* oocysts or sporozoites are applied to cells grown in vitro (5, 6, 12, 20–22, 24). As an alternative to the other assays, we used the HCT-8 cell line to study the effects of the various components of the CAM dissolution procedure on *C. parvum* infectivity.

Oocysts of *C. parvum* (GCH1 isolate) were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infections Diseases, National Institutes of Health, through McKessonHBOC BioServices, Rockville, Md. For all experiments, oocysts were between 2 and 7 months old. The oocysts were stored in 2.5% (wt/vol) potassium dichromate at 4°C throughout the experimentation period.

Oocyst suspensions were centrifuged for 3 min at 11,750 × *g* in sterile 1.5-ml Eppendorf tubes, and pellets were resuspended in phosphate-buffered saline (PBS), pH 7.2. Approximately 10^6 control and experimental oocysts (determined by hemocytometer counts of stock solutions) were aliquoted into tubes.

To simulate the CAM dissolution method, a procedure similar to that of Aldom and Chagla (1) was used. A 47-mm-diameter CAM with an average pore size of 8 μm (Millipore Corp., Bedford. Mass.) was dissolved in 32 ml of acetone. One milliliter of this solution was added to the experimental oocysts, and the tube was vortexed for 15 s. In one experiment the oocysts were held in the solution for 30 min. In subsequent experiments they were incubated for 15, 2, and 1 min, respectively. At the end of each of these exposure times, oocysts were centrifuged for 4 min at 11,750 × *g* at 22°C. Subsequently the pellet was washed, in succession, with 1 ml of acetone, 95% (vol/vol) ethanol, 70% (vol/vol) ethanol, and sterile PBS elution buffer containing 0.1% (vol/vol) Tween 80 (SIGMA-Aldrich Canada, Oakville, Ontario, Canada), 0.1% (wt/vol) sodium dodecyl sulfate, and 0.001% (vol/vol) Sigma antifoam (SIGMA-Aldrich Canada). In each wash, 1 ml of solution was added and the pellet was resuspended and centrifuged for 4 min at 11,750 × *g*. At this step, control oocysts were also suspended in the elution buffer. All tubes were centrifuged for 4 min at 11,750 × *g* and the supernatant was removed. Oocysts were resuspended in 90 μl of elution buffer, and 10 μl of 10% (vol/vol) sodium hypochlorite (Javex bleach solution; 5.25% [wt/vol] sodium hypochlorite) was added. The tubes were placed on ice for 8 min and then centrifuged, and the supernatant was removed. The oocysts were washed in 500 μl of elution buffer and resuspended in 1 ml of growth medium (see below). A 10-fold serial dilution series of oocysts was prepared in growth medium. Oocysts were enumerated using a hemocytometer.

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TABLE 1. Experiments designed to test the effects of the CAM dissolution protocol on *C. parvum* infectivity[a]

Expt	Description
2-min CAM	Exposure of oocysts to dissolved CAM in acetone for 2 min, 4-min centrifugation, rinse in 1 ml of acetone, centrifugation; rinse in 1 ml of 95% ethanol, centrifugation; rinse in 1 ml of 70% ethanol, centrifugation; rinse in 1 ml of elution buffer, centrifugation ; wash in bleach
1-min CAM	Exposure of oocysts to dissolved CAM in acetone for 1 min; 4-min centrifugation; rinse in 1 ml of acetone, centrifugation; rinse in 1 ml of 95% ethanol, centrifugation; rinse in 1 ml of 70% ethanol, centrifugation; rinse in 1 ml of elution buffer, centrifugation; wash in bleach
CAM-acetone	Exposure of oocysts to dissolved CAM in acetone for 2 min; 4-min centrifugation; rinse in 1 ml of acetone, centrifugation; rinse in 1 ml of elution buffer, centrifugation; wash in bleach
CAM only	Exposure of oocysts to dissolved CAM in acetone for 2 min; 4-min centrifugation; rinse in 1 ml of elution buffer, centrifugation; wash in bleach
Acetone only	Oocysts washed in 1 ml of acetone; 4-min centrifugation; rinse in 1 ml of elution buffer, centrifugation; wash in bleach
Ethanol only	Oocysts washed in 1 ml of 95% ethanol; 4-min centrifugation; oocysts washed in 1 ml of 70% ethanol, centrifugation; rinse in 1 ml of elution buffer, centrifugation; wash in bleach
Elution buffer only	Oocysts washed in 1 ml of elution buffer; 4-min centrifugation; wash in bleach; control oocysts washed in PBS before bleach treatment

[a] Following bleach treatment, the oocysts were rinsed in elution buffer, centrifuged for 4 min at 11,750 × *g*, and resuspended in 1 ml of growth medium. For each experiment, complementary control samples were used at the same time and from the same oocyst lot.

The full set of CAM-acetone, acetone, 95% ethanol, and 70% ethanol treatments were individually tested at 30- and 15-min exposure times at three replicates per dilution. Subsequent exposures to CAM-acetone for 2 and 1 min were tested at six replicates per dilution. The individual effects of CAM-acetone mixtures, acetone, ethanol, and the elution buffer were also tested. A summary of the seven experiments is shown in Table 1.

Human ileocecal (HCT-8) cells (American Type Culture Collection [Manassas, Va.] no. CCL-244) were maintained in 75-mm^2 culture flasks at 37°C and 5% CO_2 atmosphere. Cells were grown in a maintenance medium consisting of RPMI 1640 supplemented with 5% fetal bovine serum, L-glutamine, HEPES (ICN Biomedicals Inc., Aurora, Ohio), Opti-MEM (Life Technologies, GIBCO BRL, Burlington, Ontario, Canada), penicillin, streptomycin, and amphotericin (SIGMA-Aldrich Canada) and passaged every 3 days by trypsinization with 1× Trypsin-EDTA (ICN Biomedicals Inc.). During infectivity experiments with cells growing on chamber slides (see below), a growth medium was used in which the concentration of fetal bovine serum in the maintenance medium was increased from 5 to 10% (vol/vol).

Trypsinized cells from 95 to 100% confluent 75-mm^2 culture flasks were washed and suspended in 5 ml of maintenance medium. Cells were diluted 1:20 in maintenance medium, and 800 μl was added to each chamber in an eight-well chamber slide (Falcon culture slides; Becton Dickinson, Franklin Lakes, N.J.). Slides were incubated at 37°C under a 5% CO_2 atmosphere. After 48 h, medium was removed and replaced with 700 μl of growth medium. Oocyst dilutions (300 μl) were added to each chamber, and slides were returned to the incubator. After 24 h, the growth medium was removed, the chambers were rinsed with PBS, and 700 μl of fresh growth medium was added to each chamber. The slides were further incubated for 24 h. The growth medium was removed from each chamber, the chambers were rinsed with PBS, and the cell monolayers in each chamber were fixed in methanol for 20 min. The methanol was removed, chamber walls were removed from the slides, and the slides were air dried for 30 min.

Fixed, dried cell monolayers on each slide were covered in antibody dilution-blocking buffer (PBS, pH 7.4; 1% bovine serum albumin; 10% normal goat serum; 0.02% sodium azide; Waterborne Inc., New Orleans, La.) for 30 min. The buffer was removed and replaced with a fluorescein-labeled rat immunoglobulin G to sporozoites of *C. parvum* (Sporo-Glo; Waterborne Inc.) (1:20 dilution in dilution-blocking buffer from 20× stock antibody solution). The slides were placed in a lightproof box and incubated at 22°C for 1 h. The antibody solution was removed, and the slides were rinsed four times with PBS. Coverslips were placed on the slides with a 2% DABCO mounting medium [2% 1,4-diazabicyclo(2,2,2) octane (SIGMA-Aldrich), in 90% glycerol–10% PBS], and each chamber was viewed by epifluorescence at a magnification of ×100 using a Nikon Eclipse E600 microscope. Foci of infection appeared as bright green clusters on monolayers at an excitation wavelength of 460 to 500 nm and an emission wavelength of 510 to 560 nm. Each well was recorded as positive or negative, and the numbers of foci per well were determined. For each set of experiments and controls (six replicates of each), the most probable number (MPN) of infectious oocysts was calculated using the Most Probable Number Calculator version 4.02 (available from the U.S. Environmental Protection Agency, Risk Reduction Engineering Laboratory, Cincinnati, Ohio). The extent of infectivity was determined by dividing the MPN per milliliter by the microscopic oocyst count per milliliter of stock as described by Slifko et al. (23).

For comparison, oocysts treated with the solutions described above were examined by vital dye staining using a method modified from that of Campbell et al. (4). Oocysts were treated as described above. After resuspension in elution buffer, oocysts were centrifuged for 4 min at 11,750 × *g* at 22°C and the supernatant was removed. Oocysts were resuspended in 200 μl of Hanks balanced salt solution (HBSS) (14). Ten microliters each of 4′,6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, Oreg.) (stock solution, 2 mg/ml in methanol) and propidium iodide (PI) (Molecular Probes) (1 mg/ml in 0.1 M PBS) were added to 100 μl of oocyst suspension and incubated for 2 h at 37°C in the dark. By epifluorescence microscopy, DAPI-positive oocysts stained blue at an excitation wavelength of 330 to 380 nm and an emission wavelength of 420 nm. Oocysts that were PI-positive stained bright red when viewed at an excitation wavelength of 460 to 500 nm and an emission

TABLE 2. Effects of various treatments on *C. parvum* oocyst infectivity[a]

Expt	Oocyst infectivity [negative log (MPN, ml/oocysts per ml of stock)]		
	Experimental	Control	Difference
2-min CAM			
Trial 1	6.42	2.69	3.73
Trial 2	6.92	2.80	4.12
1-min CAM			
Trial 1	6.74	2.45	4.29
Trial 2	7.03	2.37	4.66
CAM-acetone			
Trial 1	6.51	2.49	4.02
Trial 2	6.08	3.72	2.36
CAM only			
Trial 1	4.94	2.79	2.15
Trial 2	4.38	3.76	0.62
Acetone only			
Trial 1	4.35	3.53	0.82
Trial 2	4.51	3.78	0.73
Ethanol only			
Trial 1	5.05	1.59	3.46
Trial 2	5.76	2.74	3.02
Elution buffer only			
Trial 1	2.45	2.31	0.14
Trial 2	2.32	1.98	0.34

[a] Control oocysts were processed at the same time as experimental samples and were not exposed to treatment components as described in Table 1.

wavelength of 510 to 560 nm. DAPI-negative and PI-negative oocysts exhibited no fluorescence. In replicates of three 10-μl aliquots, 100 oocysts were evaluated for uptake of dyes and scored as viable or nonviable. Only DAPI-positive and DAPI-negative, PI-negative oocysts were deemed viable.

For the membrane dissolution protocol, oocysts remained in the CAM-acetone solution for 2 min plus an additional 15 min during centrifugation. No foci of infection were observed in experiments with longer exposure times (15 and 30 min) to CAM-acetone. Individual foci were detected after 2 min of exposure to the solution plus 4 min of centrifugation before rinsing in acetone. At 2- and 1-min exposure times to the full protocol simulation, a decrease of oocyst infectivity was detected (Table 2). This result was observed in the total number of foci counted at any dilution and MPN values, in both cases relative to control values. Chamber slide wells containing high numbers of oocysts (10^6 to 10^5) had few or no foci of infection, the highest number being two foci observed in one well inoculated with 10^6 oocysts. The wells were observed to contain brownish, apparently dead oocysts. In control wells, hundreds of foci of infection were observed at the same dilutions. The extensive overlap of these foci made it difficult to determine the precise numbers of foci per well.

Lowered rates of infectivity were also observed from the CAM-acetone treatments. In one well at the 10^6-range oocyst inoculation, four foci of infection were observed. However, no foci were observed at lower dilutions. Infectivity was retained in CAM-only experiments (Table 2) at a higher number than for the oocysts exposed to full protocols. There was reduced infectivity in the acetone-only and ethanol-only experiments, although the ethanol exposure reduced infectivity more than the acetone or CAM-only exposures. This pattern indicates that prolonged exposure to ethanol or acetone may reduce infectivity. Excystation (98%) of *C. parvum* oocysts exposed to 90% ethanol for 30 min at 22 and 37°C has been demonstrated (3). Threshold values for prolonged exposure of oocysts to either ethanol or acetone were not determined in this study. However, the short exposure times to these solutions in the CAM dissolution protocol were shown to decrease infectivity considerably.

The numbers of viable oocysts detected using DAPI and PI staining showed a pattern similar to that observed in the infectivity experiments. The 2- and 1-min protocols resulted in the lowest numbers of viable oocysts relative to control samples (Table 3). The CAM-acetone and ethanol treatments also caused reduced viability. However, the CAM-only, acetone-only, and elution buffer treatments resulted in higher counts of DAPI-positive and DAPI-PI-negative oocysts. This pattern did not correspond to the reduced infectivity detected in cell cultures. However, the numbers of PI-positive oocysts were, with the exception of the CAM-acetone treatments, somewhat higher than in treated samples.

The effect of pretreating oocysts with bleach on the experimental treatments is unknown. We used a dilute sodium hypochlorite solution (1% solution of the original 5.25% stock) following experimental treatments. In a separate experiment, we performed the 2-min CAM experiment (Table 1) on oocysts in which the bleach treatment was omitted. The results were

TABLE 3. Effect of various treatments on *C. parvum* oocyst viability as observed by vital dye staining[a]

Treatment	% Viable oocysts[b]		% Nonviable oocysts[c]	
	Experimental	Untreated control	Experimental	Untreated control
2-min CAM	26.67 ± 15.14	43.33 ± 5.51	74.00 ± 14.00	56.67 ± 5.51
1-min CAM	36.33 ± 7.37	48.67 ± 15.57	63.67 ± 7.37	51.33 ± 15.57
CAM-acetone	35.00 ± 1.73	37.33 ± 3.06	65.00 ± 1.73	62.67 ± 3.06
CAM only	39.67 ± 8.08	26.00 ± 2.00	60.33 ± 8.08	74.00 ± 2.00
Acetone only	39.67 ± 10.02	38.33 ± 8.08	60.33 ± 10.02	61.67 ± 8.08
Ethanol only	35.33 ± 6.66	37.33 ± 6.81	64.67 ± 6.66	62.67 ± 6.81
Elution buffer only	30.00 ± 4.58	28.33 ± 5.13	70.33 ± 4.16	71.67 ± 5.13

[a] Each values is the average of three counts of 100 oocysts ± standard deviation.
[b] DAPI positive and PI negative or DAPI- and PI-negative.
[c] PI positive.

similar to those observed from treated oocysts. There were small numbers of foci in the 10^6 inoculum wells, but no foci were observed at any other dilutions; control wells had infective foci similar to those of the experiments in which bleach treatment was included (unpublished observations).

Previous studies have shown that *C. parvum* oocysts recovered from the membrane filter dissolution method retain their excystation rates and their infectivity in BALB/c mice following processing by the CAM dissolution protocol (11). In contrast, our experiments using infectivity assays in HCT-8 cells have shown reduced infectivity after oocysts are exposed to the various solutions used in the protocol. Our results are similar to those of McCuin et al. (16), who detected reduced viability using vital dye staining. The stages of exposure to the CAM dissolved in acetone, acetone washing, and ethanol washing appear to have an additive effect in decreasing infectivity as the foci of infection were lowest under the full protocol conditions. These findings indicate that the full CAM filter dissolution protocol can considerably reduce infectivity of *C. parvum* oocysts. This could potentially lead to a significant underestimation of oocyst infectivity.

There are several possibilities accounting for the differing results obtained by the cell culture method and by the mouse model. These include the different *C. parvum* strains used (AUCP-1 in the mouse study, GCH1 in the present study), age of oocysts (2 weeks in the mouse infectivity study, more than 30 days in the present study), and the degree of resolution of both methods. There are few reports of comparisons of infectivity of the various known *C. parvum* isolates. Rates of infectivity in mice of bovine isolates of *C. parvum* from Colombia and Spain were shown to be similar (26), but the isolates used in the present study have not been compared with others.

Although both cell culture and mouse infectivity models are suitable indicators of *C. parvum* infectivity, the two methods have not been researched with respect to their relative suitability. For the membrane filter dissolution method, rates of infectivity could not be demonstrated using the mouse model, as all of the mice became infected with the same number of oocysts (5×10^5) (11). The effects of the membrane filter dissolution method on reducing rates of infectivity could not be observed in the mouse model, even though infectivity can occur following exposure to the protocol. At the 10^6 range of oocyst inoculum, we detected single foci of infection in three of the six replicates. However, no other foci were detected at the other dilutions. It is not precisely known how many oocysts cause various degrees of infection intensity in mice, although the mean infective dose in neonatal mice has been estimated to be 60 oocysts (7). This makes the effects of chemical treatments on oocysts more difficult to detect. Here, the use of the membrane filter dissolution method-MPN method has been advantageous in providing a higher degree of resolution to quantify rates of oocyst infectivity. Both methods can identify infectious oocysts, but the cell culture method can resolve relative rates of infectivity.

Staining oocysts with vital dyes has been shown to correlate well with in vitro excystation methods (4, 9). They have been used for studying disinfection methods for *C. parvum* (8) and have been used here to study a possible correlation with the cell culture data. Based on counts of DAPI-positive oocysts, the 2- and 1-min protocols both resulted in decreased counts of viable oocysts. Ethanol-treated oocysts also resulted in lowered viability. By contrast, three of the treatments resulted in higher viability counts than the control samples even though PI-positive counts were generally higher. These patterns, in addition to the high counts of DAPI-positive and DAPI-PI-negative oocysts for all of the experiments, suggest that the dye permeability assay provided results that overestimate infectivity of the parasites processed by the CAM dissolution protocol.

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