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Recovery of waterborne oocysts of *Cryptosporidium* from water samples by the membrane-filter dissolution method

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Abstract The cellulose-acetate membrane (CAM)-filter dissolution method implemented into a Millipore Glass Microanalysis system was used for recovery of *Cryptosporidium parvum* oocysts seeded into 25 l of drinking water in polyethylene carboy aspirator bottles. CAM-entrapped oocysts were detected by immunofluorescence microscopy. From 65 to 94 oocysts/l (mean 75 oocysts/l), 34.7% overall of the inoculated oocysts, were unrecovered as determined after the water had been drained from the bottle, rinsed with 1 l of eluting fluid (EF), and CAM-filtered. Efficiency rates of oocyst recovery ranged from 24.0% to 64.0% (mean 44.1%), without the use of EF and from 72.1% to 82.3% (mean 78.8%) when EF was used. To ensure a high recovery efficiency of *Cryptosporidium* oocysts from sampled water by the CAM-filter dissolution method, it is recommended that 1 l of EF per 25 l of water be used.

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Introduction

Human cryptosporidiosis is a zoonotic diarrheal disease (Fayer et al. 1990), with waterborne outbreaks being linked to contamination of drinking and recreational waters by oocysts (Lisle and Rose 1995). The method of the American Society for Testing and Materials (ASTM; Anonymous 1993), the Standard Methods for the Examination of Water and Wastewater (Anonymous 1994), and the Alternate Method (Hansen and Ongerth 1991) for recovery of waterborne oocysts use a yarn-wound cartridge filter or a membrane filter. Recovered oocysts are detected in the particulate matter by immunofluorescent antibody (IFA) staining. The recovery rates of *Cryptosporidium parvum* oocysts from 20 l of water seeded with oocysts has ranged from 9% to 50% (Nieminski et al. 1995). The efficiency of recovery is lowered by oocyst losses during processing of the filter device with the entrapped oocysts (LeChevallier et al. 1995). However, a recent membrane-filter dissolution method (Aldom and Chagla 1995) has increased the recovery of oocysts up to 70.5%. This is because dissolving of the entire 1.2-μm-pore cellulose-acetate membrane (CAM) allows direct enumeration of membrane-entrapped oocysts.

The estimated concentration of oocysts in surface waters as based on 20-l samples collected in standard polyethylene containers and processed by the polycarbonate membrane-filtering method was within the limits of 2–112 (Ongerth and Stibbs 1987) and 0.2–65 oocysts/l (Hansen and Ongerth 1991). A volume range of 20–25 l of water is convenient for sampling and allows transportation and, consequently, processing of the samples in the laboratory.

The purpose of the present study was to evaluate a standard method for the recovery of waterborne *Cryptosporidium* oocysts that uses 25-l plastic containers for water sampling. To recover the oocysts from water samples, we used the membrane-filter dissolution method, which is known to have a superior efficiency of oo-

cyst recovery as compared with other membrane-filtering methods (Nieminski et al. 1995). As part of this evaluation the effect of an eluting procedure on the recovery of waterborne ocysts was also tested.

Materials and methods

Cryptosporidium parvum (AUCP-1) oocysts from experimentally infected Holstein calves were purified by cesium chloride gradient centrifugation (Kilani and Sekla 1987), numerically evaluated with a hematocytometer (Fayer and Ellis 1993), and stored at 4 °C in 5.0 ml of 2.5% $K_2Cr_2O_7$. Oocysts (4 week old) were washed five times with phosphate-buffered saline (PBS, pH 7.4) by centrifugation (700*g*, 15 min) and resuspended in 5 ml of water that had been filtered by the Corning 500-ml Filter System (Sigma Chemical Company, St. Louis, Mo. USA) through a 0.22-μm-pore CAM.

Oocyst numbers were evaluated with a hemacytometer, and 5 oocyst suspensions were prepared with 1.38×10^4, 6.88×10^3, 4.58×10^3, and 3.44×10^3 oocysts/500 μl. When added to 25 l of water, each inoculum of 500 μl, was calculated to result in concentrations of 550, 275, 183, and 138 oocysts/l, respectively. The 50% infective dose (ID_{50}) for *C. parvum* (Iowa strain) infection in immunocompetent humans has been shown to be 132 oocysts (DuPont et al. 1995). The inoculum dose was based on the assumption that an average person will ingest an ID_{50} of 132 oocysts in 1, 2, 3, or 4 cups of drinking water (1 cup = 8 fluid oz. = 240 ml). Consequently, 1 cup of water from each concentration category was expected to contain 132, 66, 44, and 33 oocysts, respectively. To minimize variations in the number of oocysts among inocula, we stored the suspension tubes at 4 °C for 1 week, shaking them before sampling, and performed all replicates using the four original suspensions.

A 25-l Nalgene polyethylene carboy aspirator bottle (Thomas Scientific, Swedesboro, N.J., USA) was brushed inside with 1.0 l of PBS (pH 7.4) eluting fluid (Aldom and Chagla 1995), washed with distilled water, drained, and dried out in an oven (27 °C) for 15 min. The PBS eluting fluid, which contains 0.1% Tween 80, 0.1% sodium dodecyl sulfate (SDS), and 0.001% antifoam agent (Sigma Chemical Company, St. Louis, Mo., USA), prevents the adhesion of a particulate matter to a surface. The bottle was filled with drinking water processed by the Filterite 10-μm-pore yarn-wound cartridge (Memtec America Corp., Baltimore, Md., USA).

The 500-μ l inoculum was added to the bottle and, after 48 h at room temperature (RT, approx. 21 °C), the water was filtered through a 25-cm-diameter, 25-μm-particle-retention funnel filter paper (VWR Scientific, Bridgeport, N.J., USA) mounted on a tripod base stand above the Glass Microanalysis system with a 25 mm-diameter, 1.2-μm-pore CAM (Millipore, Bedford, Mass., USA). The Glass Microanalysis system was attached to a vacuum/pressure pump regulated up to 76 cmHg (30 in. Hg). Surface water sampling utilized a 20-l sample, stored unrefrigerated and processed 48 h after the collection (Ongerth and Stibbs 1987). The filter paper was used because a yarn-wound cartridge of 1-μm nominal pore size contains a range of pores measuring up to 30 μm (Clancy et al. 1994). After filtration, 1 l of PBS eluting fluid was added to the bottle, which was then rolled manually for 15 min to facilitate fluid contact with all the internal surface. The fluid was filtered in the manner described above, and the container was washed as previously described to prevent oocyst carryover between samples due to inadequate cleaning of processing components (Ongerth and Stibbs 1987).

The number of oocysts in the 500-μl inoculum was directly enumerated by spiking of the inoculum with 15 ml of the PBS eluting fluid upon filtration with the Glass Microanalysis system with the 25-mm-diameter, 1.2-μm-pore CAM. A control trial was performed in which 25 l of unspiked Filterite-processed drinking water was filtered as described above. The experiments with the four oocyst concentrations, including oocyst enumeration, were performed in quadruplicate, and each quadruplicate was associated with one unspiked control trial. In another experimental design, 1 l of PBS eluting fluid was added to the carboy before it was filled with 25 l of the Filterite-processed drinking water. The water was spiked separately with 500 μl of inoculum containing 1.38×10^4, 6.88×10^3, 4.58×10^3, and 3.44×10^3 oocysts; stored for 48 h at RT; and filtered in the same manner. One trial was performed with each oocyst concentration.

After filtration, the CAMs were removed from the Glass Microanalysis system, folded separately into 1.5-ml Eppendorf tubes, dissolved in 1.0 ml of acetone for 2 min (Aldom and Chagla 1995), and processed according to the ASTM protocol (Anonymous 1993). The centrifugal force was 7,000 *g*, and the final pellet was washed twice with 0.22-μm-pore CAM-filtered water. Pellets were separately resuspended in 500 μ l of 0.22-μm-pore CAM-filtered water, and two 200-μ l samples from each resuspension were examined by immunofluorescence microscopy (Graczyk et al. 1996) using the Meri**f**luor *Cryptosporidium*/*Giardia* test kit according to the manufacturer's instruction. Fluorescent identification of the oocysts from the filtration was based on the comparison with the fluorescent features of the enumerated oocysts and followed the described criteria (Hansen and Ongerth 1991). The confirmation approach (Clancy et al. 1994) was used to rule out presumptive *Cryptosporidium* oocysts, and the scoring of fluorescence followed the protocol of Garcia et al. (1992).

Statistical analysis was performed with STATISTIX 4.1 (Analytical Software, St. Paul, Minn., USA). The recovery efficiencies (see Table 1) were computed for each oocyst suspension by comparison of the enumeration values with the numbers of oocysts recovered from the water and PBS eluting fluid or from the water containing the PBS eluting fluid. Total recovery efficiency represents the mean rate of oocyst recovery from all trials. The oocyst concentration was computed according to the equation (oocysts/l) = $100 \times [(\textit{oocyst count for 25 l}) / (25\ l \times \%\ \text{of recovery efficiency})]$ (Hansen and Ongerth 1991). Reported mean (x) values were associated with standard deviations (SD), and total mean values were associated with coefficients of variation (CV). The degree of linear association between variables was compared using Pearson's correlation coefficient (r). A nonparametric Kruskal-Wallis analysis of variance (*ANOVA*) was used for the comparison of recovery efficiency derived from all trials. Statistical significance was considered to be $P < 0.05$.

Results

The four control trials gave zero oocyst counts for *Cryptosporidium* oocysts; however, immunofluorescence examination revealed round particles within the size of 10–18 μm in diameter ($x = 15.7 \pm 1.9$ μm). Light and phase-contrast examinations of these particles revealed algae morphology.

The direct enumeration of the oocysts from 500-μl inocula seeded to the 15-ml of PBS eluting fluid revealed that the seeded oocyst numbers were lower than expected by an average of 8.0% (range 2.3–12.1%). The recovery rates obtained without PBS eluting fluid for oocysts seeded to 25 l of water varied within the limits of 64.0% and 24.0% and were significantly correlated with the decrease ($r = 0.87$, $P < 0.05$) in the numbers of seeded oocysts (Table 1). From 56.7% to 18.3% ($x = 34.7 \pm 16.2\%$) of the seeded oocysts were recovered from the PBS eluting fluid used to wash the 25-l bottle from which the water with seeded oocysts had been drained (Table 1). The percentage of oocysts recovered from PBS eluting fluid increased significantly when the number of seeded oocysts decreased ($r = 0.77$,

Table 1 Recovery efficiency of *Cryptosporidium parvum* (AUCP-1 strain) oocysts seeded to 25 l of drinking water in the polyethylene carboy aspirator bottle by dissolution of a 25-mm-diameter, 1.2-μm-pore CAM. Each experiment was performed in quadruplicate

Seeded oocysts[a]			Mean number (%) of oocysts recovered		
Number	Concentration		From 25 l of water	Eluted after draining of the water	Total
	per liter	per cup[b]			
12,863	514	123	8,236 (64.0)	2,350 (18.3)	10,575 (82.3)
6,200	248	59	3,172 (51.2)	1,800 (29.0)	4,975 (80.2)
4,680	187	45	1,744 (37.3)	1,630 (34.8)	3,375 (72.1)
3,050	122	29	732 (24.0)	1,730 (56.7)	2,550 (80.7)

[a]Based on the enumeration from 15 ml of PBS eluting fluid
[b]Equal to 8 liquid oz. (240 ml)

$P < 0.05$). The total recovery rate was within the range of 82.3–72.1% (Table 1), and the recovery efficiencies were not correlated with the number (or concentration) of seeded oocysts.

The mean recovery rate in this trial was 78.8% (CV = 5.6%). From 17.7% to 27.9% ($x = 21.2 \pm 4.6\%$) of the oocysts were lost during processing. The number of oocysts recovered from the water and the total number of recovered oocysts (Table 1) were significantly correlated with the number of seeded oocysts ($r = 0.98$, $P < 0.05$). The numbers of oocysts that were retained in the container after water drainage were within the limits of 2,350 and 1,630 (Table 1). The concentration of unrecovered oocysts due to elimination of the PBS eluting fluid from the procedure were within the limits of 94–65 ($x = 75 \pm 13$) oocysts/l (Table 1). The percentage of unrecovered oocysts significantly increased when the number of seeded oocysts decreased ($r = 0.74$, $P < 0.05$).

When 1 l of the PBS eluting fluid was added to 25 l of water with 12,100, 6,350, 4,820, and 3,015 seeded oocysts, the recovery rates were 79.4%, 75.4%, 75.1%, and 76.6%, respectively. From 20.6% to 24.9% ($x = 23.4 \pm 2.0\%$) of the oocysts were lost in this experiment. The mean recovery rate was 76.6% (CV = 3.3%).

Comparison of the recovery rates obtained when PBS eluting fluid was added to the water with the results of washing of the plastic container showed no significant difference (Kruskal-Wallis ANOVA; $F = 1.41$, $P < 0.05$). The overall mean recovery efficiency of *Cryptosporidium* oocysts from 25 l of water using 1 l of PBS eluting fluid by the CAM dissolution method was 77.7% (CV = 5.7%). Without the use of PBS eluting fluid the overall mean recovery efficiency was 44.1%.

Discussion

The recovery rates of *Cryptosporidium* oocysts from water by the polycarbonate membrane-filtering method have ranged from 5% to 50.0% (Ongerth and Stibbs 1987; Hansen and Ongerth 1991). The overall mean recovery rate of 77.7% obtained in the present study places the CAM membrane-filter dissolution method at the high end of the reported ranges and confirms the previously reported high recovery efficiency of 70.5% for this method (Aldom and Chagla 1995).

As demonstrated in the present study, the recovery efficiency of oocysts from water samples can be increased by addition of the eluting fluid. Monitoring of surface waters for *Cryptosporidium* oocysts by filtering of 20-l samples through polycarbonate membranes has shown that oocyst concentrations fall within the limits of 2–112 (Ongerth and Stibbs 1987) and 0.2–65 oocysts/l (Hansen and Ongerth 1991). It has been demonstrated that the presence of *Cryptosporidium* oocysts in surface waters is continuous as opposed to intermittent (Hansen and Ongerth 1991). Although 0.1% Tween 80 has successfully been used for backflushing fiber water filters (Musial et al. 1987), none of the eluting agent(s) was used in the studies monitoring potable waters for the oocysts (Ongerth and Stibbs 1987; Hansen and Ongerth 1991).

The concentration of *Cryptosporidium* oocysts in surface water samples is evaluated according to the equation $(oocysts/l) = 100 \times [(oocyst\ count\ for\ 25\ l)/(25\ l \times \%\ of\ recovery\ efficiency)]$ (Hansen and Onghert 1991), where *% of recovery efficiency* represents a recovery rate from oocyst-spiked water (positive control). Although the oocyst concentration in sampled water is computed using the recovery efficiency from a positive control, a method with a low recovery efficiency may not be sufficient to recover oocysts from environmental samples, particularly when their numbers are low. Whereas the estimates of oocyst concentration based on the low and high recovery efficiency methods may end up with similar values, a method with high recovery efficiency increases the chance of oocyst recovery. Thus, such a method enhances the accuracy of evaluation of oocyst concentration when oocyst numbers are low.

On the basis of the results of the present study, it is recommended that 1 l of PBS eluting fluid be added to each 25 l of water before or soon after collection so as to enhance oocyst recovery. However, it is not recommended that the PBS eluting fluid detergents be added directly to the water or to the empty container because these detergents are not readily water-soluble.

As shown in the present study, elimination of the PBS eluting fluid from the oocyst recovery procedure resulted in an average of 18 (range 23–16) oocysts/cup of water that remained unrecovered. Considering the infectivity data on *C. parvum* in healthy volunteers (DuPont et al. 1995), 2 cups of unboiled water misdiagnosed as oocyst-free due to the elimination of the eluting agent could be sufficient to establish infection. It is assumed that the level of human water consumption is 2 l/day (approx. 8 cups; Ongerth and Stibbs 1987); thus, in the present case the oocyst number would exceed the number determined as the ID_{50} (132 oocysts; DuPont et al. 1995). Because the ID_{50} was determined only for the Iowa strain of *C. parvum*, differences in ID50 for various *C. parvum* isolates cannot be excluded.

The present study showed that an average of 34.7% (range 56.7–18.3%) of oocysts were not drained with the water when the eluting agent was eliminated from the oocyst recovery process. In addition, an average of 21.2% (range 17.7–27.9%) of the oocysts were lost during processing. Thus, if the oocyst concentration is low, the water sample can be misdiagnosed as negative. Negative detection should be interpreted as no oocyst being recovered but not as the absence of oocysts in the water sample.

The previously reported 30% overall losses in oocyst recovery by the membrane dissolution method were explained by variation among the seed suspensions and difficulties related to the interpretation and classification of the fluorescently stained objects (Aldom and Chagla 1995). Although we eliminated these factors from our procedure, the overall losses of seeded oocysts nonetheless exceeded 21%.

It has been demonstrated that an average recovery efficiency of 26% is sufficient to recover *Cryptosporidium* oocysts from the surface waters (Hansen and Ongerth 1991). The recovery rates of the polycarbonate membrane filtering decrease with decreasing concentrations of seeded oocysts (Ongerth and Stibbs 1987). In contrast, the oocyst recovery rates of the CAM dissolution method applied to the different oocyst concentrations examined in the present study did not vary significantly.

The numbers of oocysts retained in the plastic container after water drainage were not related to the numbers of oocysts seeded to the container, and the percentage of oocysts recovered from PBS eluting fluid increased significantly when the number of seeded oocysts decreased. These data indicate that the plastic containers retain certain and relatively stable numbers of oocysts that adhere to the internal container surface during transport and storage. However, because the oocysts used in the present study were stored in $K_2Cr_2O_7$, which may oxidize the outer oocyst surface, the magnitude of the adherence of oocysts present in the surface waters may be different.

The polyethylene containers, particularly white ones, facilitate algae growth if they are inadequately cleaned between water samplings, which may result in a decrease in the filtration rate and an increase in the algae load on the membrane. Algae may interfere with fluorescent staining; 2 of 11 commercial laboratories have misdiagnosed *Cryptosporidium* oocysts on the basis of a positive fluorescent reaction with the algae *Oocystis minuta* (Clancy et al. 1994). In addition, inadequate cleaning facilitates oocyst carryover between samplings, which may result in misdiagnosis.

The presence of *Cryptosporidium* oocysts in potable waters has been shown to have a significant effect on public health (Moore et al. 1994). The dose of oocysts that is hazardous to public health has been determined (DuPont et al. 1995), and currently it is necessary that oocyst concentrations in the water be measured using the most reliable, efficient, and economical method. The present study demonstrates that the CAM dissolution method incorporated into the Glass Microanalysis system has a high oocyst-recovery efficiency when applied to 25 l of low-turbidity water. A complete processing time of only 1 h for CAM-entrapped material, which includes 20 min of fluorescent examination, represents an advantage as compared with the 6 h required for the same size CAM by the AM (Ongerth and Stibbs 1987). Furthermore, the CAM dissolution method can be implemented as a tool for watershed management in monitoring the public water supply and for monitoring of treated drinking water.

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