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Note

Enhanced concentration and isolation of *Cyclospora cayetanensis* oocysts from human fecal samples

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Abstract

Cyclospora cayetanensis is the causative agent of cyclosporiasis, an emerging infectious disease. We present a new method for the purification of *C. cayetanensis* oocysts from feces using a modified detachment solution and Renocal-sucrose gradient sedimentation. This method yields oocysts free from adherent fecal debris and amenable to processing using flow cytometry.
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Cyclospora cayetanensis, a coccidian parasite, is the causative agent for cyclosporiasis, a diarrheal illness transmitted via contaminated water or food (Centers for Disease Control and Prevention, 1997). Outbreaks in North America have been linked to imported produce (Herwaldt, 2000; Mansfield and Gajadhar, 2004). *C. cayetanensis* oocysts are round; 8 to 10 μm in diameter and are auto-fluorescent (Ortega et al., 1993). The infectious dose is thought to be small but remains unknown as attempts to infect laboratory animals and human volunteers have been unsuccessful (Eberhard et al., 2000; Chu et al., 2004; Alfano-Sobsey et al., 2004). Since neither animal models nor cell culture methods for *C. cayetanensis* exist, little is known about this parasite.

At present, *C. cayetanensis* oocysts for research must be recovered from human stool, however low numbers are passed in the feces making efficient recovery imperative. Methods for purifying and concentrating oocysts from feces have been proposed (Ortega et al., 1993; Kimura et al., 2004; Chu et al., 2004; Entrala et al., 2000). These methods yield only a small fraction of the intact oocysts that were present in the fecal matter, and the products are not free of fecal debris (data not presented). These oocysts, especially those to be sorted by flow cytometry must be free from fecal debris (Lindquist et al., 2001). The use of flow cytometry allows for further purification and sorting of exact cell numbers useful for molecular analysis of samples. Here we describe how use of a modified "detachment solution" (Venosa et al., 1996), followed by discontinuous Renocal-sucrose gradient sedimentation, as adapted from Everson et al., 2002, enables efficient purification of *C. cayetanensis* oocysts.

Human fecal samples from Nepal, preserved in 2.5% (w/v) potassium dichromate, were homogenized by shaking. These samples were obtained as the waste byproduct of a clinic, and no information is available about the individuals who produced them. The volume of the samples including the preservative was about 500 ml. The samples had been stored at room temperature since their collection. Most of the samples were collected within 2 years prior to use. Eleven samples were each sieved through polyester tulle (Hirschberg Schutz Co., Union, NJ), wrapped around a 14 mesh cross-stitch plastic grid (Darice Inc., Strongsville, OH) and placed into a 15 cm diameter glass funnel. Samples were washed through the filter with 200 ml sterile distilled water. The filtrate was distributed equally between two 250 ml plastic conical bottles. The volume of each bottle was increased to 250 ml with sterile distilled water. Each bottle was centrifuged at 3000 ×g for seven minutes using a swinging bucket

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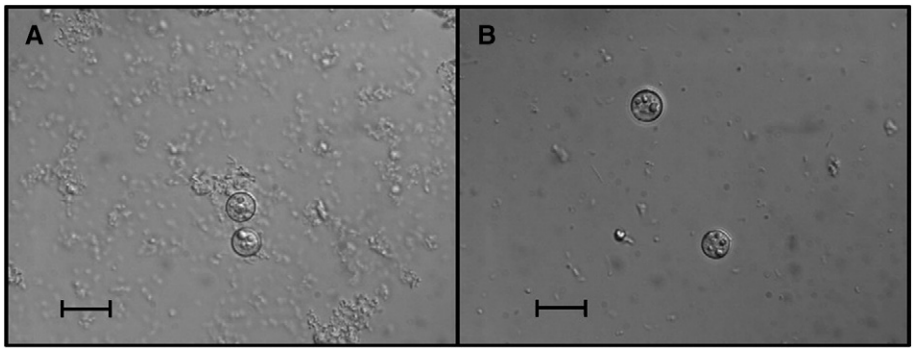

Fig. 1. Micrographs of the final product of floatation using 0.01% Tween 20 solution (A), or detachment solution (B). Debris and fecal matter are more abundant in the sample treated with 0.01% Tween 20 while the sample treated with detachment solution has less debris. Samples were combined with 10 µl of distilled water and observed with the 100× objective and oil immersion using differential interference contrast microscopy. Scale bar=10 µm.

rotor at 4 °C with no brake (PR-7000 M, International Equipment Co., Needham Heights, MA). The supernatant was aspirated off. This washing procedure was repeated two additional times to remove the potassium dichromate. After the third wash, each pellet was re-suspended to 200 ml with either a modified aqueous detachment solution (0.563 mM $H_2Na_2P_2O_7$, 42.8 mM NaCl) (Venosa et al., 1996) or aqueous 0.01% Tween 20. Both bottles were placed on their sides on an orbital shaker (Lab-Line, Barnstead International, Dubuque, IA) and agitated at 400 rpm for 30 min at 20 °C. Samples were centrifuged for 7 min at 3000 ×g, the supernatant was aspirated off and the pellets re-suspended in 35 ml sterile water. The suspension was underlain with a discontinuous density gradient using 35 ml of 5%, 10%, 15%, 20%, 25%, and 30% diatrizoate meglumine (Renocal-76, Bracco Diagnostics, Princeton, NJ) in 0.25 M sucrose respectively. After a 1 h centrifugation at 1000 ×g, 6 sequential 35 ml fractions were collected from each gradient. Each fraction was transferred into a new 250 conical bottle using a 50 ml pipette and aspirating only the air/liquid interface. The fractions were diluted to 250 ml with 0.1 M PBS (pH=7.2) and centrifuged at 3000 ×g for 7 min. The supernatant in each bottle was aspirated down to 5 ml including the pellet. The volume in each bottle was increased to 50 ml with 0.1 M PBS and the pellet re-suspended by vortexing. Following centrifugation at 3000 ×g for 7 min, the supernatants were aspirated, leaving 1 ml including the pellets, which were re-suspended by vortexing. Ten µl of each fraction was combined with 10 µl sterile water on a slide, and examined using bright field microscopy at 400× magnification to determine the concentration of oocysts retained in that fraction of the Renocal-sucrose gradient (data not shown). After this examination, the 6 fractions were recombined, centrifuged for 7 min at 3000 ×g, aspirated to 1 ml, and vortexed briefly. Ten µl samples of the final products were counted using a 0.100 mm deep Brightline Hemocytometer to determine the number of oocysts recovered per treatment. The quality of the preparations was determined based on how free the preparations were of fecal debris, whether fecal material was adhering to the oocysts, and the number of intact oocysts in each preparation. Oocyst preparations were filtered onto 13 mm diameter, 0.4 µm porosity polycarbonate track etched membranes (Whatman, Florham Park, NJ), and stained by the Kinyoun's acid fast technique (Becton Dickinson, Cockeysville, MD) to determine if purification changed their staining characteristics. Statistical analysis was performed using the SPSS statistical package (SPSS, Chicago, IL).

Examination of gradient fractions by bright field microscopy showed variability in the maximum oocyst yielding fraction from sample to sample. Combining all fractions increased the quantity of oocysts without loss of quality (data not shown). The detachment solution–Renocal-sucrose gradient produced markedly cleaner preparations (see Fig. 1A and B). These preparations,

Table 1
Comparison of *C. cayetanensis* oocyst recoveries

Sample ID	Oocyst[a] count with detachment solution treatment	Oocyst[a] count with Tween 20 solution treatment	Percentage intact oocysts with detachment solution treatment	Percentage intact oocysts with Tween 20 solution treatment
Fecal sample 1	117±13	55±10	92%±2%	90%±4%
Fecal sample 2	353±24	95±5	90%±2%	88%±5%
Fecal sample 3	543±10	62±4	88%±2%	90%±3%
Fecal sample 4	218±23	64±8	92%±2%	94%±2%
Fecal sample 5	88±9	5±1	92%±4%	90%±9%
Fecal sample 6	241±34	85±10	92%±2%	93%±3%
Fecal sample 7	218±23	34±8	87%±2%	89%±3%
Fecal sample 8	168±12	25±6	87%±0%	93%±5%
Fecal sample 9	45±9	27±5	87%±3%	92%±3%
Fecal sample 10	444±34	409±19	94%±1%	93%±1%
Fecal sample 11	60±11	6±2	95%±5%	97%±5%

Oocyst counts were done using a 0.100 mm deep Brightline Hemacytometer with 6 counts per sample.
[a] $p<0.001$ paired *T*-test.

when examined under the microscope, were found to contain sparse fecal debris, and also, the oocysts were free from what fecal debris remained. Samples purified using a Tween 20 solution–Renocal-sucrose gradient treatment contained plentiful fecal debris. The oocysts, while clearly visible under bright field microscopy, tended to clump and were often associated with fecal debris making isolation using flow cytometry difficult (data not shown). Table 1 shows that the detachment solution significantly increased oocyst recoveries, as determined by a paired *T*-test ($p<0.001$), by as much as 18 fold over the Tween 20 treatment. We believe the addition of the detachment solution to the fecal pellet frees the oocysts from the fecal matrix allowing them to be more readily retained in the Renocal-sucrose gradient. There was no difference in the number of fragmented oocysts between these 2 preparations; in both treatments on average less than 10% of the oocysts recovered were not intact. Oocysts from both preparations were variably acid fast, which is consistent with what has been reported by other authors (Eberhard et al., 1997; Visvesvara et al., 1997).

In summary, purification of oocysts from stool using the detachment solution and Renocal-sucrose gradient technique yielded *C. cayetanensis* oocysts that were abundant, intact, largely free from fecal debris, and ready to research using flow cytometric methods. These and similar methods will aid in the study of the environmental distribution of *C. cayetanensis* and other aspects of its biology.

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