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Comparative Evaluation of Several Techniques for Purification of *Cryptosporidium parvum* Oocysts from Rat Feces

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Three methods of isolating *Cryptosporidium parvum* oocysts from rat feces were evaluated. Oocyts were initially isolated by sucrose density gradient centrifugation. They were further purified by passage through a glass bead column or a Percoll gradient or by dialysis. Although oocysts recovered from the glass bead column and by dialysis were relatively free of fecal debris, only oocysts recovered from the Percoll gradient were free of bacteria. Recovered oocysts retained their antigenicity and infectivity. The ability to effectively recover oocysts from rat feces suggests that the laboratory rat may be a convenient substitute for ruminants in the propagation and maintenance of *C. parvum* oocysts for in vitro and in vivo use.

Cryptosporidium parvum, a common opportunistic protozoan, causes severe, protracted, and potentially life threatening diarrhea in immunocompromised patients (4, 6, 11). In immunocompetent individuals infection by *C. parvum* leads to self-limiting diarrhea (4, 12). Recently, this protozoan has caused several waterborne outbreaks of diarrhea (5, 8, 10). *C. parvum* has a wide host range, and infected animals can be sources of contamination for food and water supplies (3, 10).

C. parvum oocysts used in research are usually obtained from specific-pathogen-free calves that are reinfected on a maintenance schedule. Unfortunately, many research laboratories are unable to house large domestic ruminants for this purpose. Further, because they are frequently infected by this organism, calves selected for inoculation with an experimental isolate might already harbor *C. parvum*. Their subsequent inoculation would lead to contamination of the experimental isolate. Thus, the ability to continuously maintain a single *C. parvum* isolate without contamination in a small laboratory animal would be advantageous.

We developed and evaluated three methods for isolating *C. parvum* oocysts from the feces of infected rats. In these procedures, oocysts are first isolated from a discontinuous sucrose gradient, then purified further by being passed through glass beads or Percoll or by dialysis. Percoll gradients and glass bead columns effectively separate *C. parvum* oocysts from calf feces (2, 7, 9, 18). However, these methods may fail to separate oocysts from the feces of nondiarrheic rats, which contain less water and more fiber than feces from calves with diarrhea. Further purification through dialysis, however, is less likely to rupture the oocysts than the other two techniques. Identification of an effective method for isolating viable oocysts from rodent feces is necessary before rodents can replace large ruminants as hosts for the maintenance and propagation of *C. parvum* isolates for research purposes.

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MATERIALS AND METHODS

Collection of fecal oocyst suspension. A *C. parvum* isolate of bovine origin maintained by continuous passage in immunosuppressed rats was used to infect the rats that served as the source of oocysts in this study. Female Sprague-Dawley rats (Harlem Sprague-Dawley, Inc., Indianapolis, Ind.) weighing 200 to 250 g received 0.25 mg of dexamethasone per kg of body weight (Roxane Laboratories, Columbus, Ohio) daily in their drinking water for 38 days (16). After 10 days of immunosuppression, the rats were inoculated orally with 1.5×10^6 oocysts on days 10 and 11. Beginning at the onset of oocyst shedding (the eighth day after oocyst inoculation), the feces were collected daily in 15 ml of 2.5% potassium dichromate, mixed, and stored as a 25% suspension at 4°C. This fecal suspension was passed through 11 stainless steel mesh sieves with decreasing pore sizes ranging from 425 to 20 μm. The oocysts were counted in a hemocytometer under bright-field microscopy, and the oocyst suspension was stored at 4°C.

Oocyst purification on discontinuous sucrose gradient. The sucrose gradient was prepared as described previously (8). Briefly, Sheather's sucrose solution (500 g of sucrose, 9 ml of phenol, 0.5 ml of Tween 80, in 320 ml of deionized water) was diluted 1:2 and 1:4 with sterile phosphate-buffered saline (PBS) (0.1 M, pH 7.2). The sucrose gradient was prepared by placing 10 ml of the 1:2 solution into 50 ml of polypropylene centrifuge tubes (Falcon; Becton Dickinson, Franklin Lakes, N.J.) and then slowly adding 10 ml of the 1:4 solution. Thirty milliliters of the fecal oocyst suspension (1.5×10^6/ml) was layered onto the 1:4 gradient. The sucrose gradient was centrifuged at $1,700 \times g$ for 30 min at 4°C. Following centrifugation, the potassium dichromate layer and half of the 1:4 gradient were collected with a pipette and then discarded. The remainder of the 1:4 gradient and 0.5 ml of the 1:2 gradient above the interface were recovered. This aspirate was dispersed into 80 ml of PBS, and the oocysts were counted.

Oocyst purification on glass bead column following sucrose gradient centrifugation. Twelve centimeters of 3-mm-diameter glass beads (Fisher Scientific, Fair Lawn, N.J.) was placed in a 22-by-300-mm glass chromatography column. Before use, the column was washed with nitric acid and PBS several times. The column was filled with sterile PBS between uses. For each run, the effluent end of the column was fitted with a new 10-μm polycarbonate filter (Millipore, Bedford, Mass.). The oocysts (80 ml) from the sucrose gradient were added to the column and then eluted with 520 ml of sterile PBS. The 600-ml oocyst eluent was centrifuged at $1,500 \times g$ for 30 min, and then the pellet was resuspended in 80 ml of sterile PBS and the oocysts were counted. A sample of the suspension was streaked onto brain heart infusion agar, incubated at 37°C, and checked for bacterial growth after 48 and 72 h.

Oocyst purification by dialysis following sucrose gradient centrifugation. The oocyst suspension (80 ml) obtained from the sucrose gradient was placed in dialysis tubing and dialyzed against 1,000 ml of PBS overnight at 4°C with constant stirring. The dialysate was recovered and the tubing was thoroughly washed with PBS to release any adhered oocysts. Approximately 220 ml of PBS was added to the oocysts in the dialysate, and the solution was centrifuged at $1,500 \times g$ for 30 min. The pellets were resuspended to 80 ml in sterile PBS. The suspension was assessed for purity and sterility on brain heart infusion agar as described above, and the oocysts were counted.

Oocyst purification by Percoll gradient following sucrose gradient centrifugation. The oocysts (80 ml) from the sucrose gradient were pelleted by centrifugation and then brought to 2 ml with sterile PBS. The Percoll gradient was prepared by mixing 6 ml of Percoll (Sigma, St. Louis, Mo.) with 0.66 ml of 10×

TABLE 1. Percentage of oocysts lost upon purification

Method	% Oocysts lost	
	Mean[a] (±SD)	Range
Glass bead	12.9 ± 4.1	6.7–16.7
Dialysis	17.1 ± 2.9	12.5–20.0
Percoll gradient	28.8 ± 6.8	18.8–35.3

[a] Each value is a mean of five separate runs.

Alsevier's solution and 6 ml of 1× Alsevier's solution. The resuspension was divided, and each half (1 ml) of the pellet suspension was layered onto a Percoll gradient and then centrifuged at 16,000 × *g* for 20 min at 4°C. The band just above the pellet, previously determined to contain whole oocysts, was collected and pelleted at 1,500 × *g* for 30 min. Following centrifugation, the two pellets were washed with PBS, and each of the two final pellets was brought to 40 ml with sterile PBS and combined as an 80-ml oocyst suspension. The oocysts were counted and the suspension was assessed for bacterial contamination as described earlier.

Immunogenicity. The oocysts obtained by the Percoll gradient method were further used to separate oocyst walls and sporozoites. Rabbits were immunized by the intramuscular route three different times with 0.5 ml of each of these fractions. The initial immunization was with complete Freund's adjuvant, and the subsequent two immunizations were with incomplete Freund's adjuvant. Serum was harvested for antibody at 3 and 5 weeks. Antibody titers and specificity were determined by an indirect fluorescence assay. Briefly, 1×10^5 oocysts and 4×10^5 sporozoites were incubated with twofold serial dilutions of rabbit antiserum specific for sporozoites or oocyst wall fractions for 60 min at 37°C and washed three times with PBS (0.1 M, pH 7.2). Suspensions of antibody-coated oocysts and antibody-coated sporozoites were incubated with 1:400 of fluorescein-labelled goat anti-rabbit immunoglobulin G for 2 h at 37°C, washed three times, and observed for green fluorescence with a fluorescence microscope at a wavelength of 480 nm.

Viability assay. Oocyst viability was determined with fluorescein diacetate (FDA; Eastman Kodak, Rochester, N.Y.) and propidium iodide (PI; Sigma) as described previously (1). Briefly, FDA solution was prepared by mixing 0.04 ml of FDA stock solution (10 mg of FDA in 1 ml of acetone stored at −20°C) with 10 ml of PBS at pH 7. *C. parvum* oocysts (10^5/ml) were incubated with 0.1 ml of the diluted FDA solution and 0.15 ml of PI solution (1 mg of PI in 50 ml of PBS) at room temperature for 5 min. Total oocysts were counted under bright-field microscopy, then observed at excitation wavelengths of 455 to 490 nm (FDA) and 545 to 546 nm (PI). The number of FDA-positive oocysts in a total of 100 oocysts yielded the percentage of viable oocysts.

Statistics. The data were evaluated in an analysis of variance that compared the percentage of oocysts lost with each method. The significance of the difference was expressed at a P value of 0.05.

RESULTS AND DISCUSSION

The three techniques were evaluated on the basis of the percentage of oocysts recovered and the presence of bacterial contamination in the final fraction of oocysts. The percentages of oocysts lost after purification by the glass bead method or dialysis were not significantly different ($P > 0.5$). The percentage of oocysts lost during the Percoll gradient procedure was significantly larger than that during the other two methods (Table 1) ($P < 0.05$). However, in a viability assay performed on one of the replicate runs, more viable oocysts were recovered with the Percoll gradient method (97%) than with the other two methods (60%). In addition, only the oocyst suspension prepared by the Percoll gradient method was free of bacterial contamination.

On the basis of these results, Percoll gradient purification yields a pure fraction of oocysts. However, relatively fewer oocysts are collected by this method. In addition, large volumes cannot be efficiently processed by this method, since it is time-consuming and labor-intensive. The oocysts obtained from the Percoll gradient were free of bacterial contaminants as determined by aerobic cultures of gradient aliquots as well as microscopic examination of wet mounts and Gram stains of the aliquots for microbes. Therefore, the oocysts are suitable for immunologic studies requiring minimal interference from extraneous particulate antigenic material. Polyclonal antibody produced against the oocyst walls and excysted sporozoites had titers of 1:1,280 and 1:12,000, respectively, without cross-reaction between them at a dilution of 1:2.

The bacterial contamination associated with the glass bead and dialysis procedures can be eliminated with 1.75% hypochlorite as previously described (13, 17). Therefore, addition of this treatment to those methods would enable collection of concentrated oocysts from larger volumes. The resulting oocysts are suitable for infectivity studies required when drugs are screened for therapeutic activity against *C. parvum* (14).

Oocysts obtained by all three techniques were infectious when added to HCT-8 (human colon tumor) tissue culture monolayer and administered to immunosuppressed rats and mice. No difference in infection of immunosuppressed rats has been observed with oocysts obtained from rat feces and those obtained from calves (14, 15). As previously indicated, the oocysts also retained sufficient antigenicity to elicit polyclonal antisera when inoculated into rabbits. The data suggest that all three methods described effectively purify oocysts from rat feces. Therefore, the laboratory rat can replace ruminants in the propagation and maintenance of *C. parvum* oocysts for in vitro and in vivo use.

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