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Separation of Cryptosporidium Oocysts from Fecal Debris by Density Gradient Centrifugation and Glass Bead Columns

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A method was developed to obtain purified cryptosporidium oocysts from fecal samples. Oocysts were initially collected by centrifugation through a sucrose density gradient and further purified by passage through glass bead columns. The purified oocysts were antigenically active and sufficiently pure for immunological studies.

In vitro immunological studies of cryptosporidiosis, a coccidial zoonotic infection, are dependent on purified preparations of oocysts. Flotation in Sheather sugar solution or in 33% zinc sulfate and sedimentation with Formalin-ether or Formalin-ethyl acetate are effective concentration techniques for the clinical diagnosis of cryptosporidiosis (1). Since oocysts are very small (4 to 8 μm [8]) and are mixed with fecal debris, the concentrates must be stained with periodic acid-Schiff, acridine orange, Giemsa, iodine, or acid-fast stain, the last being the most effective (2). Preparations of fecal oocysts obtained by flotation techniques are heavily contaminated with fecal material. The usefulness of such concentrates for immunological studies that use fluorescent-antibody staining techniques is limited by nonspecific binding caused by the presence of fecal bacteria, yeasts, and vegetable matter. Furthermore, experience in our laboratory with fluorescein-labeled antibody has shown that treatment with Formalin-ether alters the antigenicity of oocysts.

Discontinuous (4) and continuous (6) sucrose gradients and glass bead columns have been used to purify coccidial oocysts other than cryptosporidium from feces. Wagenbach (9) separated *Eimeria tenella* sporozoites from oocysts by eluting Ringer solution through 200-μm glass beads, and Ryley (5) separated *E. tenella* oocysts from feces by passing a fecal suspension through glass beads, followed by a 2-liter rinse with 5% Tween 80. The sporozoites passed through with the Ringer solution, and the oocysts, which attached to the beads with the Ringer solution, passed through the column with the 5% Tween 80.

We have developed a modified method for the preparation of purified cryptosporidium oocysts from feces that uses sucrose density centrifugation and glass bead columns.

Sucrose density centrifugation. An initial sucrose solution (454 g in 355 ml of distilled water) was diluted with distilled water to yield 10, 20, 30, and 50% solutions for use in density centrifugation. The four sucrose solutions were autoclaved and used under sterile conditions thereafter. Refrigeration at 4°C prevented mixing of the layers. Starting with the 50% solution, 10 ml of each successive dilution was transferred dropwise with a Pasteur pipette into a 50-ml screw-cap centrifuge tube (Corning Glass Works).

Fecal specimens from infected calves were stored at 4°C in 2% potassium dichromate, which maintains the viability of organisms for as long as 6 months, as determined by infectivity studies (7). Samples were rinsed five times by centrifuging 10 ml of the specimen with 40 ml of saline at 650 × *g* for 5 min at 10°C and decanting the supernatant after each rinse. The final ratio of stool to saline was 1:2. A 5-ml sample of the washed suspension was layered on top of the sucrose gradient and centrifuged in a clinical centrifuge (Damon/IEC) at 650 × *g* for 20 min at 10°C. The 10, 20, and 30% layers were transferred by aspiration to three 50-ml centrifuge tubes containing 40 ml of saline or distilled water. The tubes were centrifuged at 650 × *g* for 10 min at 10°C, and the supernatants were removed with a pipette and discarded. The pellets were washed with saline to remove excess sucrose, and one part per hundred of an antibiotic-antimycotic mixture (GIBCO Laboratories) containing penicillin (10,000 U/ml), streptomycin (10,000 μg/ml), and amphotericin B (Fungizone; E. R. Squibb & Sons) (25 μg/ml) was added. Each layer was inspected for oocysts with the high-power objective of a light microscope. Oocysts were quantitated by averaging the number of oocysts in 30 high-power fields evenly distributed among the top, middle, and bottom rows of the cover slip.

Glass bead columns. Wheaton glass columns (no. 265133) (22 by 200 mm) equipped with a sintered glass disk and stopcock were filled with 6 cm of 3-mm glass beads. The glass bead column was cleansed with concentrated nitric acid and then rinsed with saline. Sufficient saline was left in the column to cover the beads. The 10, 20, or 30% layer from the sucrose density centrifugation was then layered on top of the beads and eluted through the column with 600 ml of saline. The eluent was collected in 50-ml aliquots. Saline was used for the glass bead elutions instead of 5% Tween 80 (5) because the latter is difficult to remove from oocysts. The fritted glass retaining disk clogged easily and had to be cleansed either with concentrated nitric acid or by pumping soapy water through the disk with a scrub brush after each 600-ml run.

Each 50-ml aliquot was centrifuged in a clinical centrifuge at 350 × *g* for 10 min at 10°C, with acceleration and deceleration of centrifugation performed over 2 min to avoid disruption of the pellet. The supernatant was removed with a pipette, leaving concentrated oocysts. Aliquots 5 through 8 (200 to 400 ml) and aliquots 9 through 12 (400 to 600 ml) were combined and reconcentrated. All samples were examined microscopically for cryptosporidium oocysts.

Serum from several subjects with previously documented

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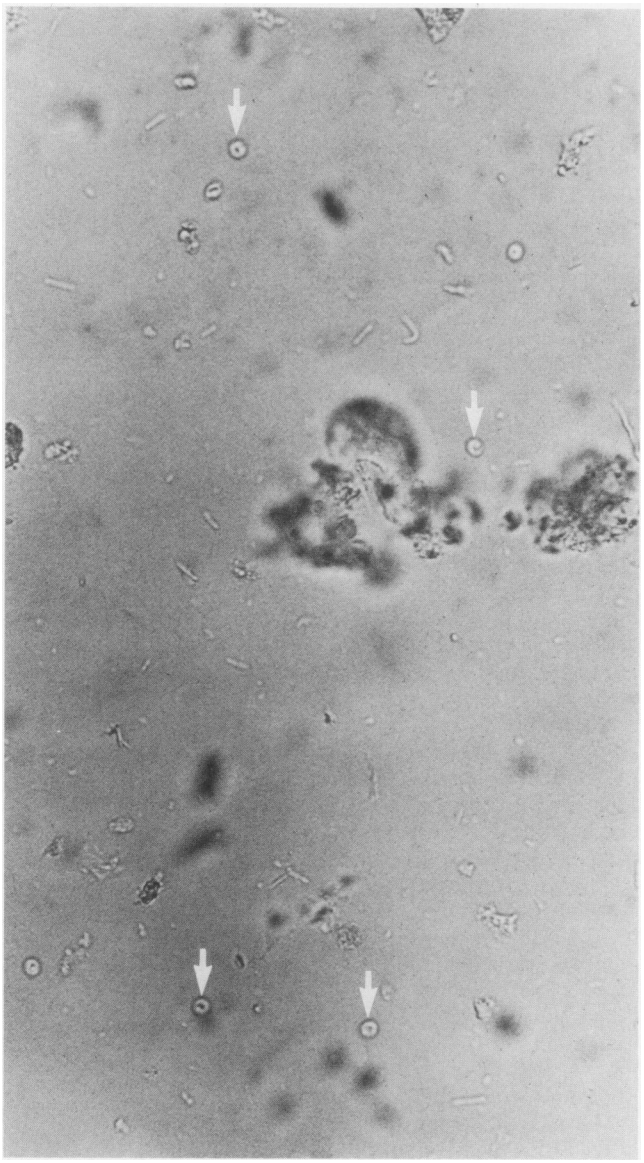

FIG. 1. Cryptosporidium oocysts (arrows) after sucrose density centrifugation. Oocysts were mixed with other fecal matter. Unstained; ×400.

cryptosporidium infections was used as a source of antibody in the indirect immunofluorescence reaction to determine whether the purified oocysts retained their antigenicity. Meloy fluorescein-conjugated goat anti-human immunoglobulins G, M, and A with high fluorescein-to-total-protein ratios were used for the immunofluorescence assay. The antibody-coated oocysts were viewed under a Leitz fluorescence microscope.

Results and discussion. The majority of cryptosporidium oocysts were found in the 30% layer of the sucrose density gradient (Fig. 1). Unlike the Sheather sugar flotation technique (3), phenol was not added to the sucrose density column to avoid changes in oocyst antigenicity. The sediment contained heavy debris but no oocysts. The 10% layer contained about one-sixth and the 20% layer contained about one-third as many oocysts as the 30% layer.

The glass beads and sintered glass disk trapped much of the fecal debris but allowed the oocysts to pass through the column. An increasing ratio of oocysts to debris was observed in each successive aliquot. Combinations of aliquots 5 through 8 and aliquots 9 through 12 were relatively clean, each containing ratios of oocysts to debris of about 1:1 to 2:1 (Fig. 2).

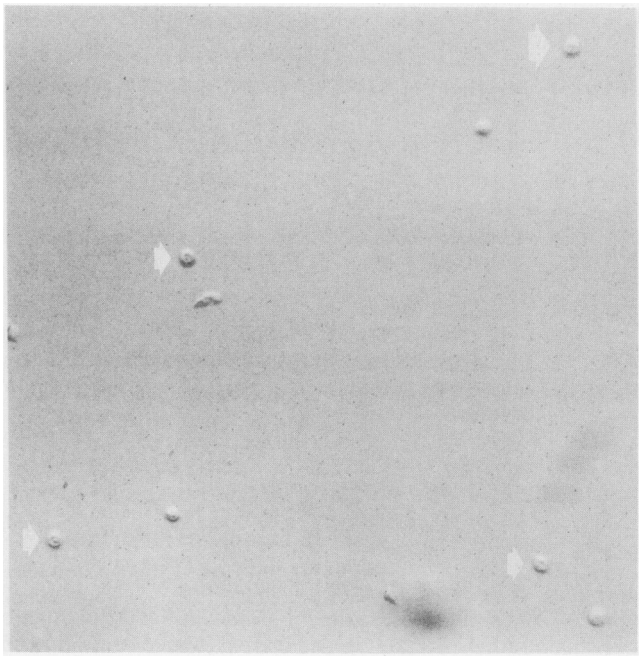

FIG. 2. Cryptosporidium oocysts (arrows) after cleansing through glass bead columns. Unstained; ×300.

Serum from patients with known cryptosporidium infections determined by fecal analysis yielded antibody titers of up to 1:640, as compared with titers not exceeding 1:20 in serum from uninfected healthy subjects.

A major advantage of the sucrose density gradient-glass bead method is that the reagents used should not alter the antigenicity of the oocysts. Although the size of the oocysts (4 to 8 μm) makes complete purification of oocysts from feces difficult, the method described provides preparations of sufficient purity for immunological studies, such as immunofluorescent-antibody labeling of cryptosporidium oocysts, without excessive interference from extraneous antigenic material.

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