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Separation of *Cryptosporidium* Species Oocysts from Feces by Using a Percoll Discontinuous Density Gradient

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***Cryptosporidium* oocysts were concentrated by an ether–phosphate-buffered saline sedimentation technique and then separated by density gradient centrifugation. This two-step method yielded highly concentrated oocysts largely free of bacteria and fecal debris.**

Laboratory work with *Cryptosporidium* oocysts has been hindered by the lack of availability of large numbers of oocysts free of fecal contaminants. Previous purification techniques have dealt primarily with the concentration of oocysts for diagnostic purposes only (1, 4). In our experience, such flotation or sedimentation techniques have proven to be unsatisfactory in providing the concentrated or purified oocysts required for in vitro cultivation studies and for the production of hyperimmune sera for immunodiagnostic tests.

This report describes a new method of purifying *Cryptosporidium* oocysts by a two-step procedure in which oocysts are first concentrated by sedimentation and then separated on a discontinuous density gradient.

Oocysts were concentrated from the feces of naturally infected calves by a modified Formalin-ether sedimentation technique in which phosphate-buffered saline (PBS) (pH 7.2) was used instead of Formalin. Approximately 15 ml of fecal homogenate (20% [vol/vol] in PBS) was centrifuged in a 20-ml centrifuge tube for 10 min at 500 × *g*. The supernatant was discarded, and the sediment was resuspended in 15 ml of PBS. A 3- to 5-ml layer of ether was added and thoroughly mixed with the suspension, and the mixture was then centrifuged for 10 min at 500 × *g*. Four layers, a solvent, a plug of debris, PBS, and sediment, usually resulted. The layer of debris at the interface between the two liquids was loosened by passing a swab stick gently around the circumference of the tube. The top three layers were then discarded, allowing only the last few drops to return to the sediment on the bottom. In the sediment, oocysts accounted for up to 75% of the deposit, with the rest consisting of assorted fecal debris and microorganisms. The morphological integrity of the oocysts was verified by examination of Giemsa- or acid-fast-stained smears or both.

Density gradients were prepared with Percoll (Pharmacia South Seas Australia). The stock solution (starting density, 1.133 g/ml) was diluted in accordance with the specifications of the manufacturer to yield solutions with densities of 1.04 and 1.08 g/ml. Gradients with sharp interfaces were achieved by layering 3 ml of 1.04-g/ml Percoll on an equal volume of 1.08-g/ml Percoll in 10-ml conical glass centrifuge tubes by using a syringe fitted with an 18-gauge needle. PBS-ether sediment (0.5 ml) was then layered on top of the Percoll in each of the gradient tubes, and the tubes were centrifuged at 250 × *g* for 10 min at room temperature.

After centrifugation, distinct bands at the upper and lower interfaces and a pellet at the bottom were formed. The bands were collected, suspended in 10 volumes of PBS, and centrifuged at 500 × *g* for 10 min. The resulting pellets were examined by Giemsa and acid-fast staining and for the exclusion of trypan blue. The original pellet consisted of fecal debris only, the pellet obtained from the upper band contained bacteria and damaged oocysts, and the pellet obtained from the lower band contained purified oocysts at a concentration of approximately 10^5/ml (Fig. 1).

These purified oocysts were shown to be infectious by inoculation of two 1-day-old gnotobiotic piglets (2, 3). Both piglets shed oocysts after 3 days and had typical gut histology on necropsy.

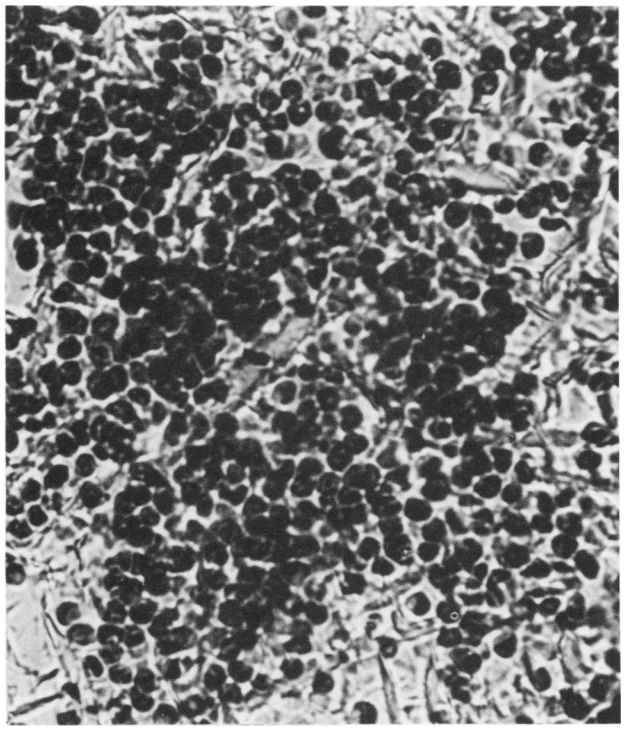

FIG. 1. *Cryptosporidium* oocysts after treatment with PBS-ether and separation on a Percoll density gradient. Giemsa stain. ×1,000.

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The superiority of PBS-ether sedimentation over Sheather sugar flotation as an initial concentration step is due largely to the ability of ether to extract lipids, therefore essentially dispersing the oocysts in the aqueous phase. It is thus imperative to ensure that adequate mixing of the two phases takes place. The present results showed that the combination of PBS-ether sedimentation and Percoll discontinuous density gradient centrifugation provided a simple and efficient method of obtaining highly concentrated and clean oocysts from feces or from intestinal contents of animals, as required for laboratory studies.

The Percoll purification technique has greatly facilitated the propagation of *Cryptosporidium* oocysts in cell cultures and chicken embryos because the method provides concentrated and largely bacterium-free inocula and because the Percoll solutions have low osmolality and cell toxicity. This technique has also been useful in providing a source of antigen for preparing high-titer antisera against *Cryptosporidium* oocysts in rabbits. Further investigations should allow the use of culture-derived antigen in the development of new immunodiagnostic procedures and other tests required for the study of the epidemiology of cryptosporidiosis.

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