

Flow Cytometric Detection of *Cryptosporidium* Oocysts in Human Stool Samples

LUIS M. VALDEZ,^{1,2} HAN DANG,¹ PABLO C. OKHUYSEN,^{1,2} AND CYNTHIA L. CHAPPELL^{1*}

Center for Infectious Diseases, School of Public Health,¹ and Division of Infectious Diseases, School of Medicine,²
The University of Texas Houston Health Science Center, Houston, Texas

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***Cryptosporidium parvum* is an important pathogen that causes diarrhea in virtually all human populations. Improved diagnostic methods are needed to understand the risk factors, modes of transmission, and impact of cryptosporidiosis. In the present study, we fluorescently labeled and counted *C. parvum* oocysts by flow cytometry (FC) and developed a simple and efficient method of processing human stool samples for FC analysis. Formed stool (suspended in phosphate-buffered saline) from an asymptomatic, healthy individual was seeded with known concentrations of oocysts, and oocysts were labeled with a cell wall-specific monoclonal antibody and detected by FC. The method described herein resulted in a mean oocyst recovery rate of 45% ± 16% (median, 42%), which consistently yielded a fourfold increase in sensitivity compared to direct fluorescent-antibody assay of seeded stool samples. However, in many instances, FC detected as few as 10³ oocysts per ml. Thus, FC provides a reproducible and sensitive method for *C. parvum* oocyst detection.**

Cryptosporidium parvum is a coccidian parasite that causes disease in virtually all human populations (3). The disease, which is characterized by diarrhea, abdominal pain, and nausea, is self-limited in immunocompetent persons. However, in malnourished children and in immunodeficient persons, such as those with advanced AIDS, *Cryptosporidium* infection may cause severe, prolonged diarrhea and is an important cause of morbidity and mortality in these patients (5, 12). DuPont et al. have shown recently that a small inoculum (50% infective dose, 132 oocysts) is sufficient to cause infection in seronegative, healthy volunteers (4). This low 50% infective dose helps to explain the potential for person-to-person transmission and the occurrence of waterborne transmission, as was evidenced during the 1993 Milwaukee outbreak (10).

Studies have also shown how some individuals may have biopsy-proven cryptosporidial infection in the absence of diarrhea and with negative stool examinations by the currently available diagnostic methods (9, 13). The existence of undetected asymptomatic infection, coupled with the fact that state-of-the-art water treatment plants cannot completely eliminate the risk of infection (6), underscores the importance of having more sensitive diagnostic methods to understand better the risk factors, modes of transmission of the disease, and impact of the disease in high-risk individuals and the general population.

Several methods have been used for the detection of oocysts in stool samples, and the currently available diagnostic methods have been compared (2, 8, 11, 17). These studies show that direct immunofluorescence assay (DFA) and enzyme immunoassay methods provide greater sensitivity and specificity than conventional staining methods. However, the detection of *Cryptosporidium* spp. in seeded specimens by DFA or enzyme immunoassay requires approximately 10,000 oocysts per g of watery stool or more oocysts in the case of formed stool specimens (17). Therefore, light infections or stool-to-stool variation in oocyst concentration may result in false-negative

findings. The sensitivity of currently available methods is influenced by the stool processing method used (i.e., suspension, centrifugation, filtration).

Cryptosporidium oocysts, as discrete particles, can be detected by flow cytometry (FC) if labeled with an appropriate fluorescent tag. Arrowood et al. (1) recently described the use of this method, which employed a monoclonal antibody for evaluation of experimental cryptosporidial parasite loads in infected mice with severe combined immunodeficiency (SCID). The FC method was found to be approximately 10 to 15 times more sensitive than conventional DFA. Flow cytometry is also under investigation as a sensitive method of detecting oocysts from environmental water samples (15, 16).

In the present study, we confirmed that oocysts can be fluorescently labeled and counted by FC and we modified and extended the method of Arrowood et al. (1) to human stool samples. We developed a simple and efficient method of processing human feces for flow cytometric analysis and also compared the sensitivity of this method of oocyst detection with that of DFA by using seeded human stool samples.

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MATERIALS AND METHODS

***C. parvum* oocysts.** The *C. parvum* isolates used for this study were the Iowa isolate (originally obtained by Harley Moon, University of Iowa, Ames) and the UCP isolate (Joseph Crabb, ImmuCell Corporation, Portland, Maine), both of which were originally collected from and passaged in calves. The Iowa isolate was provided by Charles Sterling (University of Arizona, Tucson) and stored at 4°C in 2.5% potassium dichromate (K₂Cr₂O₇). The UCP isolate was stored at 4°C in 0.15 M phosphate-buffered saline (PBS; pH 7.2). Oocyst concentrations were adjusted as desired after counting in triplicate with a hemacytometer.

Preparation, seeding, and processing of human stool samples. Human stool samples from healthy volunteers were used (4). All stool samples were diluted (1:4) in formalin and, as part of our routine laboratory protocol, stored at 4°C, although there is no data supporting the notion that such a temperature is needed for better preservation of stool samples. For the negative control and as a medium for seeded samples, we selected a stool sample from an asymptomatic adult who showed no serological evidence of previous exposure to *C. parvum* by enzyme-linked immunosorbent assay or active infection by DFA. Known concentrations of *C. parvum* oocysts (UCP isolate) in 10 µl were resuspended in 90 µl of PBS, and twofold serial dilutions were prepared. Each oocyst dilution (100 µl) was added to 900 µl of the negative human stool sample to obtain serial

* Corresponding author. Mailing address: Center for Infectious Diseases, University of Texas School of Public Health, 1200 Herman Pressler W 732, Houston, TX 77030. Phone: (713) 500-9372. Fax: (713) 500-9364. E-mail: cchappell@utspsh.sph.uth.tmc.edu.

concentrations of seeded stool samples (ranging from 5.3×10^2 to 1.4×10^5 oocysts/ml). All of these samples were processed and assayed as described below.

Processing of samples. Feces (stored in formalin) were vortexed for 30 s and allowed to settle for an additional 30 s. A 100- μ l aliquot of the suspension was removed and added to 900 μ l of PBS. The sample was vortexed and then centrifuged at $1,880 \times g$ (3,000 rpm) for 30 min, and the supernatant containing formalin was removed. The pellet containing oocysts was then resuspended in 1 ml of PBS and vortexed for 30 s. From this suspension, 180 μ l was removed and mixed with 20 μ l of a 0.5-mg/ml solution of an oocyst-specific monoclonal antibody conjugated with fluorescein isothiocyanate (FITC) (OW50-FITC; Meridian Diagnostics, Cincinnati, Ohio). The mixture was incubated in the dark at 37°C for 1 h and then centrifuged for 15 min at $1,880 \times g$. The supernatant was discarded, and the pellet was resuspended in 1 ml of PBS. The samples were then vortexed for 30 s, transferred to a polystyrene tube (Falcon 2054; Becton-Dickinson), and stored at 4°C in the dark until analyzed. All samples were analyzed on the same day as they were processed.

Oocyst detection assays. The optical characteristics of purified oocysts were evaluated on a flow cytometer (FACSTRAK; Becton Dickinson Immunocytometry Systems, San Jose, Calif.) by using LYSYS II acquisition software (Becton-Dickinson Immunocytometry Systems). Operating conditions included log scales on all detectors (forward scatter, side scatter, and fluorescence detectors), a forward scatter threshold voltage setting of 300 V, a forward scatter photodiode setting of E-01, a side scatter voltage setting of 319 V, and an FL1 detector setting of 550 V (1, 14).

Samples were evaluated by using a sampling interval of 102 s. The acquisition gate was set based on the fluorescence and forward scatter characteristics of a sample containing pure oocysts in PBS incubated with oocyst cell wall-specific monoclonal antibody. The negative control consisted of a negative stool sample processed as described above. To calculate the sample volume analyzed, tubes were weighed before and after flow cytometric analysis and a standard curve was used to relate the sample weight and volume analyzed. A 102-s sampling interval typically analyzed an approximately 100- μ l test volume. All samples were assayed in triplicate, and the mean number of oocysts and standard deviation were calculated. After each sample run, the system was flushed with deionized water to prevent cross-sample contamination. After acquisition, a second region was defined for all gated events based on the forward and side scatter characteristics of the pure oocysts (positive control). Only events meeting both criteria were counted as oocysts. All comparisons were made against the negative control, and a statistically significant difference between the mean of triplicate observations and the negative control was considered a positive result.

The quantification of oocysts was done by DFA as previously described (4, 7), by using the same FITC-labeled monoclonal antibody employed for FC (Merifluor Cryptosporidium/Giardia Direct Immunofluorescent Detection Procedure; Meridian Diagnostics). Briefly, stool samples preserved in formalin were vortexed for 30 s. From each sample, 5 μ l was transferred to each of three wells. Slides were allowed to dry for 30 to 45 min. After drying, detection reagents were added and slides were incubated for 30 min in a humidifying chamber at room temperature. Slides were then gently washed in the buffer solution provided in the kit and lightly tapped to remove excess buffer. Oocysts in 10×40 fields were counted by immunofluorescence microscopy, and if the organisms were not seen, the whole area of the slide was scanned with a $20\times$ objective and the presence of oocysts was confirmed with a $40\times$ objective. The oocyst count was the mean count of the three wells.

Statistical analysis. Flow cytometric data was compared by using the Student *t* test (two tailed) and the chi-square test (with the Yates correction) to compare proportions, by using Instat software (Graphpad Software, San Diego, Calif.). A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Stool processing and assay optimization. A simplified processing method was developed that included two centrifugations and a 1:10 dilution step. Removal or disaggregation of large particles was done to prevent clogging of the flow cytometer. Initially, we tested different methods of removing large debris by using filters of different compositions and pore sizes and by varying the centrifugation times and speeds (data not shown). Although filtration decreased the amount of debris, it also trapped a significant number of oocysts, resulting in 3% recovery compared with processing of pure oocysts in the same way. Extensive flushing of the filters was ineffective in significantly increasing oocyst recovery. As a result of this, we decided not to include a filtration step in our stool processing procedure and used the method outlined in Fig. 1.

The final method selected involved extensive vortexing to break up the particulate and centrifugation to separate oocysts from formalin and excess antibody. Dilution and vortexing of

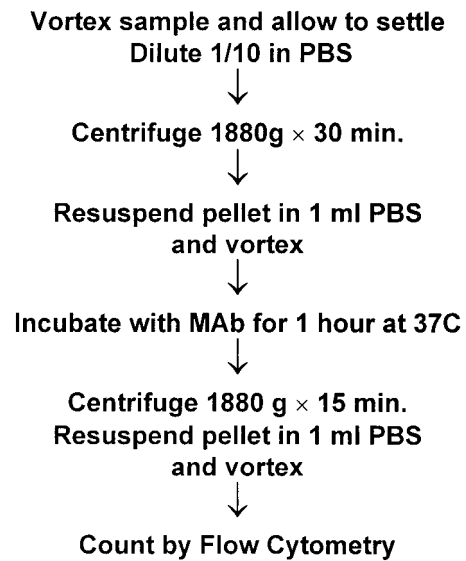


FIG. 1. Flow chart for processing of human stool samples.

samples yielded a greater oocyst recovery rate than the above filtration methods, presumably by releasing oocysts trapped in the particulate. When experiments were done with pure oocysts, each centrifugation step resulted in approximately 20% oocyst loss, presumably due to variability in counting, nonspecific attachment to the tube wall in the absence of a carrier protein, and disruption of oocysts. The optimal centrifugation speed was determined empirically; forces greater than $2,500 \times g$ decreased recovery due to oocyst disruption. When seeded stool samples with known numbers of oocysts were subjected to this process, the recovery rate was as high as 75% (mean recovery, $45\% \pm 16\%$ [standard deviation]).

For each run, pure oocysts were used to set gates for FC and as a positive control for the antibody reagent. Labeled oocysts were counted by FC, and the data were expressed as a dot plot (Fig. 2) depicting the number of particles according to forward scatter (size) and fluorescence intensity. An acquisition gate (R1) was set around the area of highest fluorescence. In samples using pure oocysts suspended in PBS, approximately 70 to 80% of the pure oocysts were consistently found within the acquisition gate. This percentage correlated with the excystation rates of the oocysts used (data not shown). When oocysts with a higher or lower excystation rate were used, the percentage of gated oocysts increased or decreased proportionally, probably reflecting changes in particle fluorescence after excystation.

To determine the antibody concentration needed for optimal labeling, pure oocysts were incubated with different concentrations of specific, FITC-conjugated monoclonal antibody (Fig. 3). As the concentration of antibody was decreased, there was a left shift and a broadening of the peak fluorescence, indicating decreased intensity in the labeled oocysts. However, the number of oocysts counted at each antibody concentration, i.e., the area under the curve, was not significantly different. An antibody concentration of 5 μ g/100 μ l of sample was considered optimal for additional experiments, since it yielded a peak that was well separated from the area of the background stool particulate.

A comparison of oocyst detection by FC and DFA was carried out. Known concentrations of *C. parvum* oocysts were

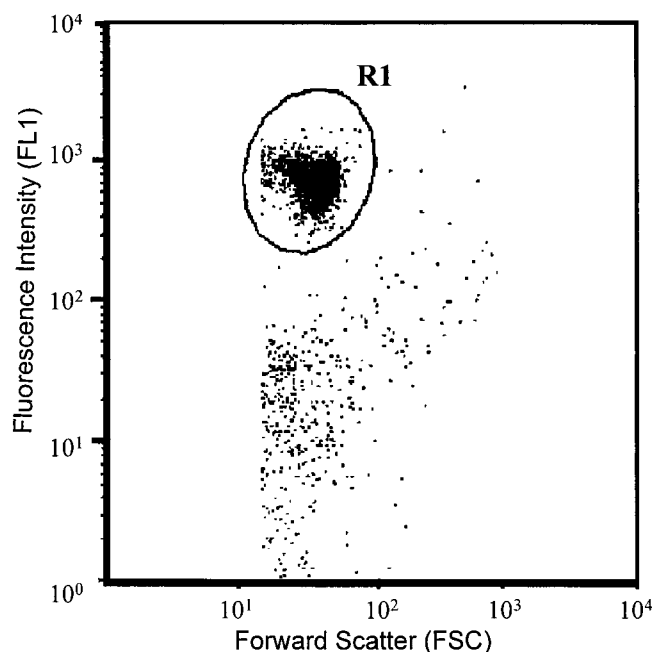


FIG. 2. Dot plot (forward scatter versus fluorescence intensity) of purified *C. parvum* oocysts labeled with monoclonal antibody OW50-FITC (Meridian Diagnostics). The acquisition gate defined by an area of high fluorescence is indicated (R1).

added to human stool samples, and aliquots were taken for assay by each method. Flow cytometric analysis consistently detected 1.4×10^4 or more oocysts per ml of stool (Fig. 4). Of the eight samples with seeded oocysts at a concentration lower than 1.4×10^4 (range, 5.3×10^2 to 8.5×10^3) oocysts per ml of stool, FC identified five samples that met the criteria for positivity (statistically significantly different from the negative control), detecting as few as 1×10^3 oocysts/ml (inset in Fig. 4). DFA was consistently positive with concentrations of 5.8×10^4

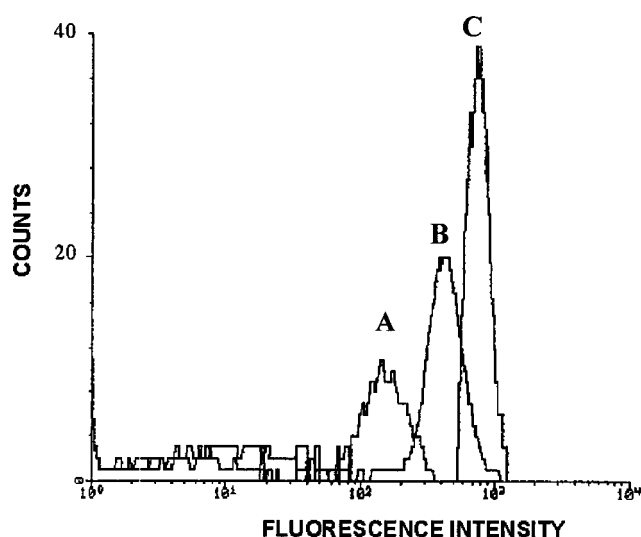


FIG. 3. Comparison of fluorescence signal intensities of purified *C. parvum* oocysts labeled with various OW50-FITC antibody concentrations. Each peak represents a different concentration: A, 1.5 $\mu\text{g}/100 \mu\text{l}$; B, 3 $\mu\text{g}/100 \mu\text{l}$; C, 5 $\mu\text{g}/100 \mu\text{l}$.

or more oocysts/ml (Fig. 4). In a comparison of the proportion of positive results obtained with each test by using seeded samples, FC was more sensitive than DFA. Of 16 seeded samples tested, 13 were positive by FC (81.3%; 95% confidence interval, 54.35 to 95.95) and only 3 were positive by DFA (18.75%; 95% confidence interval, 4 to 45.6) ($P = 0.002$).

DISCUSSION

Currently available diagnostic methods for detection of cryptosporidiosis have been compared (2, 8, 11, 17), and although some feel that the methods are equally sensitive (8), others feel that the most commonly used methods may fail to detect cryptosporidiosis in many immunocompromised and immunocompetent individuals (17). Methods capable of detecting lower oocyst concentrations will have an important impact on epidemiological investigations that assess risk factors, frequency of asymptomatic individuals, and modes of *Cryptosporidium* transmission.

In flow cytometry, it is important to eliminate large debris particles that might clog the instruments. Centrifugation proved better than filtration in decreasing debris and background fluorescence and resulted in a better oocyst yield. An inverse relationship between sample dilution and oocyst recovery was observed. This observation may reflect, in some way, what was described by Weber et al. (17), who demonstrated that it was easier to recover oocysts from watery stools than from formed stools. The centrifugation steps were kept to a minimum but were necessary to remove the formalin in preserved samples and to eliminate the excess background fluorescence from unbound antibodies.

Because flow cytometry detects labeled particles, it is very important to achieve a fluorescence intensity that discriminates from nonspecific fluorescing particles. The optimal antibody concentration was found to be 5 $\mu\text{g}/100 \mu\text{l}$ of sample. By using this monoclonal antibody concentration, we obtained a sensitive acquisition gate. Lower antibody concentrations, such as 1 to 3 $\mu\text{g}/100 \mu\text{l}$, did not significantly affect oocyst detection but resulted in readings that overlapped with the background fluorescence. Specificity was also increased by defining a second gate based on the forward scatter and side scatter characteristics of pure oocysts. An interesting relationship between the excystation rate and the oocyst labeling intensity was observed and deserves further study. The excystation rate correlated with the number of pure oocysts that were labeled intensely (within the acquisition gate). The fluorescence intensity of the remaining oocysts was decreased by 10- to 100-fold. These observations were made with oocysts with different excystation rates (80 to 98%); however, more careful experiments are required to confirm these findings. If these observations are confirmed, flow cytometry might be of value for assessing oocyst excystation. Interestingly, the fact that the OW50 cell wall protein, the target of the monoclonal antibody used in these studies, diminishes over time in preserved samples (2a) may be the basis for this finding.

By using this procedure, we have confirmed that flow cytometry provides a sensitive method for the detection of *C. parvum* oocysts and that this method is 4 to 34 times more sensitive than the DFA method when seeded human stool samples are used. In a recent report, Arrowood et al. used experimentally infected SCID mice to demonstrate that FC was 10-fold more sensitive than DFA (1). In that study, oocysts were purified prior to FC evaluation to eliminate stool debris and background fluorescence. However, only 7.3% of the seeded oocysts were recovered. In the present study, we adapted this FC method for human stool samples. A simplified stool prepara-

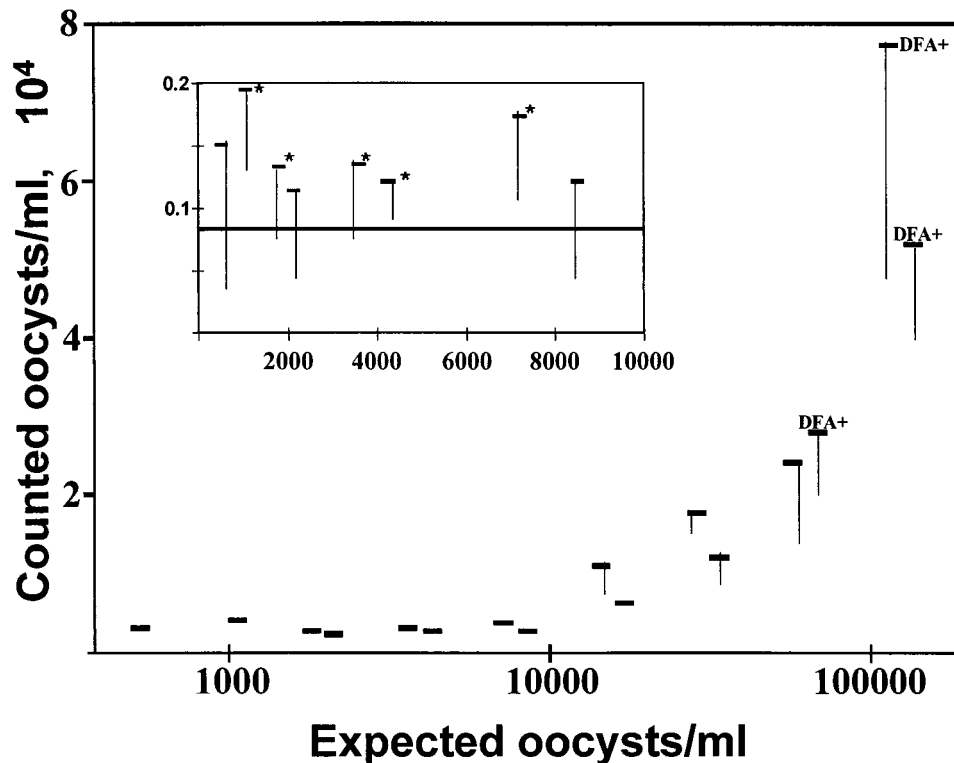


FIG. 4. Relationship between the expected number of *C. parvum* oocysts/ml of seeded human stool and the actual number counted by flow cytometry. Each data point represents the mean of triplicate assays. Error bars indicate 1 standard deviation below the mean. The inset is an expanded view of the first eight points. The horizontal line represents the mean of the negative control plus 2 standard deviations. Only three samples tested positive by DFA (DFA+). An asterisk denotes a statistically significant difference from the negative control ($P < 0.05$).

tion procedure achieved an oocyst recovery rate of $45\% \pm 16\%$ with no loss in sensitivity due to background fluorescence. The variation in recovery may be influenced by sampling error or by occasional clogging of the flow cytometer by particulate. This may be a problem especially at oocyst concentrations below $1.4 \times 10^4/\text{ml}$, which sometimes make it difficult to discriminate between oocysts and false-positive events. False positives may be minimized by defining more stringent acquisition gates and/or using a second labeled monoclonal antibody, such as phycoerythrin.

Although FC requires an expensive setup and technical expertise, the increased sample volume tested (20-fold or higher if needed) and increased sensitivity may be of clinical use in individuals with *C. parvum*-related diarrhea who may be shedding oocysts that are currently below the level of detection afforded by the DFA and enzyme-linked immunosorbent assay techniques. Prospective studies are needed to validate the clinical utility of our method. In preliminary studies, we have used FC to verify the presence of oocysts in clinical cases of DFA-confirmed cryptosporidiosis and have examined stool samples from symptomatic volunteers experimentally challenged with *C. parvum* and identified a low level of infection (unpublished data).

FC has been previously described as a method of *C. parvum* oocyst detection in a SCID mouse model and in water samples, and to our knowledge, this is the first report extending the use of FC to human stools. We conclude that with a simple stool processing method, flow cytometry is sensitive and offers greater sensitivity than DFA when seeded human stool samples are used. The application of this sensitive method may be very helpful, particularly in outbreak situations in which infec-

tion cannot always be documented in symptomatic individuals or when the diagnosis of low-grade infection and asymptomatic carriage is important.

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