A Flow Cytometric Protocol for Detection of Cryptosporidium spp.

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Abstract
Cryptosporidium parvum is transmitted through water and can cause severe diarrhea. The diagnosis is usually based upon observer-dependent microscopic detection of oocysts, with rather low sensitivity and specificity. Our objective was to optimize a flow cytometric (FC) protocol for the detection of C. parvum. A specific monoclonal antibody conjugated with R-phycoerythrin was incubated with dead oocysts to determine the optimal antibody concentration. Serial concentrations of oocysts were stained with the optimized concentration and analyzed by FC. The lower detection limit was determined, and the possibility of cross-reaction was investigated using prokaryotic and eukaryotic microorganisms. A FC protocol was optimized to detect oocysts in spiked human stools. The optimal antibody concentration was found to be 3.0 µg/ml. The lowest number detectable was $2 \times 10^3$ oocysts/ml. Staining procedure was specific, as no cross-reactions were observed. This reliable and easy FC protocol allow the specific detection of Cryptosporidium oocysts, even at very low concentrations, which is important for public health and further studies of treatment efficacy.

Key terms
Cryptosporidium parvum; detection by flow cytometry; monoclonal antibodies; stool parasitology diagnosis; water analysis

Cryptosporidium parvum is a coccidian parasite that can cause watery diarrhea and abdominal pain both in immunocompetent or immunocompromised individuals and which is of particular concern in HIV-infected patients. Among such patients, up to 15–40% suffer from parasitic diarrhea, frequently caused by C. parvum (1); this is an important cause of morbidity and mortality (2) and may even be life threatening. The microorganism is transmitted by the ingestion of contaminated food and especially by drinking water, which has been the source of several large outbreaks. In fact, oocysts of C. parvum are resistant to the chlorine concentrations usually employed for the disinfection of drinking water and can survive in aquatic environments for months (3–5), mainly due to the protective nature of the double cell wall (6). The infectious dose of C. parvum oocysts is very low, and it has been estimated that, as few as 30 oocysts, can cause disease in healthy volunteers (7). The level of viable oocysts in domestic water samples that poses a health risk is still unknown (4), but has been estimated to be as low as 10 oocysts (3). In the United Kingdom, legislation set a legal limit of 1 oocyst/10 l of water. However, this level was established as a standard for water treatment and do not reflect any possible public health risks (4). The detection of oocysts is highly dependent on the methods used to collect and analyze samples (3, 8–10). Conventional methods, which include microscopic observation of stained stool samples, are too time consuming and dependent on the technician’s skill and expertise, thus showing rather low sensitivity and specificity (11, 12). Several alternative methods have been evaluated for the detection of oocysts, for example, immunofluorescence and enzyme immunoassays, which have a higher
sensitivity and specificity than microscopy (13–15). However, the sensitivity of these methods is still low, with the detection limit ~10,000 oocysts per gram of watery stool and lower in the case of more formed stools (2). Cytometry may be considered as an alternative, and a few studies have already addressed the cytometric detection of Cryptosporidium in feces and water (2, 3, 5, 16).

The aim of this study was to develop an optimized flow cytometric (FC) protocol to detect C. parvum oocysts, using specific monoclonal antibodies. The detection limit was determined as well as the possibility of cross-reactions with other microorganisms.

**Material and Methods**

**Microorganisms**

*C. parvum* oocysts used for this study were provided from Waterborne, the Iowa isolate, calf source treated by heat (15 min, 70°C) and stored at 5% formalin at the concentration of 1 × 10⁶ (Waterborne™, New Orleans, LA). They were stored at 4°C until used. Bacteria and fungi for cross-reaction studies were obtained from American Type Culture Collection (ATCC): *Escherichia coli* (strain 35218), *Staphylococcus aureus* (strain 25923), and *Candida albicans* (strain 10231). *Giardia lamblia* were obtained from Waterborne.

**Optimization of the Stain**

*C. parvum* oocysts were diluted in sterilized phosphate-buffered saline (PBS; pH 7.4) to a final concentration of 2 × 10⁵ oocysts/ml and incubated with serial concentrations of a *Cryptosporidium* oocysts mouse monoclonal antibody (5.0 μg/ml, 3.0 μg/ml, 1.5 μg/ml, 0.3 μg/ml, and 0.03 μg/ml) conjugated with R-phycoerythrin (RPE) (Crypt-a-Glo, RPE, 20X concentrate—Waterborne) at 37°C for 45 min in the dark. The suspensions were centrifuged at 600g for 15 min, and the supernatant was discarded; the pellet was resuspended in 1 mL of sterile PBS, vortexed for 30 s, transferred to a propylene tube, and analyzed by flow cytometry. All the experiments were performed three times.

**Flow Cytometry Analysis**

The optical characteristics of purified oocysts were evaluated on a flow cytometer (FACSCalibur BD Biosciences, Sydney) standard model, with three PMTs equipped with standard filters (FL1: BP 530/30 nm; FL2: BP 585/42 nm; FL3: LP 650 nm); a 15 mW 488 nm Argon Laser and with cell Quest Pro software (version 4.0.2, BD Biosciences, Sydney). Acquisition settings were defined using a nonstained sample (autofluorescence), adjusting the PMTs voltage to the first logarithmic (log) decade. Instrument controls followed standard procedures.

**Assessment of Detection Limit and Possibility of Cross-Reactions**

Serial concentrations of *C. parvum* oocysts were prepared: 2 × 10²; 2 × 10³; 2 × 10⁴; 1 × 10⁵; and 2 × 10⁶/ml stained with the previously optimized antibody concentration and analyzed by flow cytometry.

Contamination with other microorganisms: suspensions with 10⁷ oocysts/ml of *C. parvum* were mixed with the microorganisms mentioned above at 0.5 MacFarland density: *E. coli* (1.5 × 10⁶ cells/ml), *S. aureus* (1.5 × 10⁶ cells/ml); *C. albicans* (1.5 × 10⁶ cells/ml); *G. lamblia* (2 × 10⁵ cysts/ml).

The mixtures were stained with *Cryptosporidium* antibody conjugated with RPE at the previously optimized conditions. The suspensions were then centrifuged at 600g for 15 min, and the supernatant was discarded; the pellet was resuspended in 1 ml of sterilized PBS, vortexed for 30 s, transferred to a propylene tube, and analyzed by flow cytometry.

**Detection of *C. parvum* Oocysts in Human Faeces**

Stool samples from healthy human volunteers who showed no serological evidence of previous exposure to *C. parvum* by ELISA were diluted in formalin (1:4) and stored at 4°C until used. All the samples were divided in two and in one of them *C. parvum* oocysts in 2 × 10⁵/ml were used for stool sample spiking. The Faust concentration method (17) was used: faecal suspensions were filtered through gauze, and the material on the filter was diluted to 10 ml with sterile water. This suspension was centrifuged at 500g for 2 min in conic propylene tubes, followed by supernatant decantation; sterile water was then added to achieve 10 ml of total volume; and the process was repeated until a clear supernatant was obtained. Because of the amount of debris that might interfere with fluorescence, other methods were tested for removing large debris, for example, two different brands of filter papers with 20-μm pore diameter, Whatman (Whatman PLC, Brentford, UK) and MN (Macherey-Nagel, Easton, PA). The final pellet was resuspended in 10 ml of ZnSO₄·7H₂O (703 g/l, 33%, specific gravity 1.118) for flotation; NaCl (360 g/l, specific gravity 1.21) was also assayed as a flotation solution. Velocity (200g and 500g), time of centrifugation (5 and 10 min) for supernatant clearance was tested, and waiting times of 45 min and 24 h were compared. After flotation, the upper 1 ml of samples was transferred to Eppendorf tubes and stained with specific monoclonal antibody followed the previously optimized conditions.

**Results**

As the concentration of antibody increased, there was an increase of mean intensity of fluorescence in labeled oocysts (Fig. 1). Similar staining was obtained with concentrations of 3.0 and 5.0 μg/ml of specific probe; 3.0 μg/ml was therefore considered optimal for additional experiments, yielding histograms that showed a clear separation of the oocysts from the autofluorescence background.

Figure 2 shows the intensity of fluorescence of *C. parvum* oocysts labeled with the optimal antibody concentration. Although 2 × 10⁶ oocysts/ml was the smallest concentration analyzed, the lowest concentration that could still be detected with certainty was 2 × 10⁵ oocysts/ml.

To determine the specificity of the antibody, microorganisms usually present in samples such as environmental water...
or feces were mixed with pure C. parvum oocysts and then incubated with the monoclonal antibody. Although an increase in events was detectable when high concentrations of other microorganisms were used, these showed up in the autofluorescence zone and were easily distinguishable from the labeled C. parvum oocysts, represented in the R region. These results are available as supplementary material.

Analysis of Faecal Samples

Although filtration with gauze (Faust method) decreased the amount of debris, it also trapped a significant number of oocysts, and the use of filter paper with 20-μm pore diameter yielded no real improvement in oocyst recovery. The gauze procedure provided optimal recovery (70–80%). In relation to different flotation solutions, improved separation of oocysts from debris was observed when using a ZnSO₄ · 7H₂O solution and a prolonged recovery time, namely 24 h. Centrifugation for 500g for 10 min was optimal for the flotation method, resulting in clear suspensions, allowing recovery of a good percentage of oocysts. Nonnegative samples (without oocysts contamination) showed unspecific fluorescence, false positives.

DISCUSSION

Cryptosporidiosis is often undiagnosed in microbiology laboratories, leading clinicians to establish a clinical diagnosis without laboratory confirmation. Symptomatic patients with cryptosporidiosis usually display large numbers of oocysts in their stools; decreasing as the patient becomes asymptomatic (18). C. parvum is the most common species.

Although cytometry allows both morphofunctional evaluation and quantification of individual microorganisms, it remains underutilized in microbiology in general and in clinical microbiology in particular. Our team has used cytometry with great advantage in detection and antimicrobial susceptibility evaluation of Mycobacterium tuberculosis (19) and fungi (20,21). Even though cytometry currently requires expensive equipment, this is present in many immunology laboratories and often could be shared by microbiologists. The costs of the reagents and consumables for the test described here are relatively low, around 2.5€. Our optimization steps involved the determination of the ideal concentration of the fluorescent stain and the establishment of the cytometric analytical protocol. The concentration of the probe must allow a clear separation of the two populations of events in histograms or scatter plots, that is, autofluorescence and stained oocysts. Insufficient staining will not allow a clear separation, while excessive concentration could result in unspecific staining. The limit of detection of the assay has relevant clinical implications. Using flow cytometry with specific-labeled antibodies, we found an inverse relation between sample dilution and oocyst detection, as previously described (2). Valdez et al. (2) found a threshold of detection for flow cytometry of $5 \times 10^4$ oocysts/ml. We were able to decrease this threshold to $2 \times 10^3$ oocysts/ml. Only PCR techniques showed lower detection limits (22–24); however, PCR is disadvantageous in terms of its price and inability to determine viability (25). Another advantage of the FC protocol is its capacity of discriminating C. parvum oocysts from debris or other microorganisms; no cross-reactions occurred between C. parvum antibody and the most common microorganisms present in stool or water. We were able to detect labeled C. parvum oocysts when mixed with other microorganisms.

Studies involving oocyst detection in water usually reveal low values, ranging from 0.01 to 15 oocysts/100 l in reclaimed water, and from 17 to 200 oocysts/100 l in surface water (26). Because studies differ in sample volumes analyzed and have been done at different seasons of the year, periodicity and quantity of the samples have to be defined in future studies.

Laser scanning cytometry has been shown to be an interesting

![Figure 1](image1.png)

**Figure 1.** Mean intensity of fluorescence of Cryptosporidium parvum oocysts labeled with serial concentrations of specific antibody conjugated with R-phycoerythrin (Waterborne). All the experiments were performed three times.

![Figure 2](image2.png)

**Figure 2.** Comparison of fluorescence signal intensities of Cryptosporidium parvum oocysts analyzed at FL2 showing: A: autofluorescence of $2 \times 10^6$ oocysts/ml and different concentrations of labeled oocysts with specific antibody; B: $2 \times 10^6$ oocysts/ml ( ), C: $1 \times 10^5$ oocysts/ml ( ), D: $2 \times 10^5$ oocysts/ml ( ), and E: $2 \times 10^3$ oocysts/ml (fill).
tool for water analysis (3,25), but the equipment needed is more expensive than flow cytometry equipment, and stool analysis may be more difficult.

In this study, in which human stool samples were analyzed by flow cytometry, it was essential to clear samples of large particle’s debris that might clog the apparatus. Although the use of gauze rather than paper filters resulted in faster and more efficient recovery, such filters need a vacuum system, making the method more expensive and time consuming. The use of ZnSO$_4$·7H$_2$O solution flotation for 24 h combined with increased centrifugation time resulted in recovery of good concentrations of oocysts without undesired background fluorescence, as was noted in a previous study (16).

Our flow cytometry protocol is now usable to detect and quantify Cryptosporidium in samples such as water or stools, as well as to perform a follow-up of positive patients.

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LITERATURE CITED


