Applied and Environmental Microbiology, Mar. 2004, p. 1827–1829 Vol. 70, No. 3
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.3.1827–1829.2004

Detection of *Cryptosporidium parvum* in Soil Extracts†

Mark Walker[1]* and Douglas Redelman[2]

Department of Natural Resources and Environmental Sciences[1] and Sierra Cytometry and University of Nevada Cytometry Center,[2] University of Nevada, Reno, Nevada 89557

Received 21 August 2003/Accepted 17 November 2003

Epifluorescent microscopy and flow cytometry were used in different combinations with fluorescein isothiocyanate-labeled immunoglobulins M and G3 to estimate the numbers of *Cryptosporidium parvum* oocysts in soil extracts containing 10 to 10,017 oocysts/ml. No combination had a systematic effect on accuracy or precision. Background debris may have produced overestimates at low oocyst concentrations when flow cytometry was used.

Cryptosporidium parvum oocysts are detected in soil, water, and animal waste by using fluorochrome-labeled immunoglobulins with fluorescent microscopy (1, 6) and flow cytometry (4, 7). The antibody recommended for examination of water samples is an immunoglobulin M (IgM) conjugated with fluorescein isothiocyanate (FITC) (9). Genus-specific monoclonal IgG3 antibodies are also useful. The number of binding sites on IgM antibodies potentially results in groups or clumps of oocysts, which may affect the precision and accuracy of estimates.

Nonspecific immunoglobulin binding and the presence of naturally fluorescing particles may lead to false positives when analytic methods are used (5). This study examined the precision and accuracy of microscopy and flow cytometry with IgM and IgG3 immunoglobulins applied to soil sample extracts.

Experimental design. Oocysts were added to soil extract obtained from a loamy sand (3% gravel, 78% sand, 9% silt, 10% clay [as determined by a hydrometer]), with 6% organic matter (as determined by using loss on ignition). The experiments followed a full factorial design (two analytic methods, two antibodies, and three replicates for each of 11 concentrations).

Soil extract preparation. Soil extracts were prepared by using differential sucrose gradients (12) with 5 g of soil. Replicate analyses by microscopy of extracts combined with IgM antibodies found no *C. parvum* oocysts.

Analytic equipment. A Beckman-Coulter XL/MCL flow cytometer with an argon ion blue laser (15 mW, 488 nm) was used to collect forward and orthogonal light scatter and green (520-nm-wavelength) fluorescence signals. Purified oocysts labeled with both antibodies were identified by observed fluorescence and light scatter signals. The flow rate used was 23 μl/60 s, with flow times of 120 s for ≥1,490 oocysts/ml and 300 s for ≤624 oocysts/ml.

Microscopy was carried out with a Nikon E2000 microscope equipped with a 100-W mercury vapor bulb and a 100× Plan Fluor oil immersion objective (numerical aperture, 1.3).

Source of oocysts. Oocysts were obtained per rectum from naturally infected dairy calves in Fallon, Nev., and purified using differential sucrose gradients (10). Stocks were stored at 4°C with 100 U of penicillin G sodium/ml, 100 μg of streptomycin sulfate/ml, and 0.25 μg of amphotericin B/ml. The observed morphology of the oocysts (using differential interference microscopy at 1,000× magnification) corresponded with expectations (9). We successfully used immunoglobulins and amplified DNA target sequences with forward and reverse primers provided by the National Institutes of Health AIDS Reagent Program (catalog no. 1558).

Preparation of replicates. Stock concentrations were adjusted serially with pipettes calibrated by the manufacturer with distilled water (starting concentration, 1,001,722 oocysts/ml [n = 16 replicate counts]). Serial dilutions were prepared immediately prior to each experiment. For each dilution, we applied IgG3 to 10 aliquots of 0.100 ml each to estimate concentrations and examined 100 randomly selected fields from a 0.020-ml subsample of each aliquot beneath a coverslip (22 by 22 ml) on an agar-coated slide. The stocks with ≤7,639 oocysts (Table 1) were estimated as having a mean of ~7,639 oocysts/2^n and a standard deviation of ~7,228 oocysts/2^n, with n equal to the number of serial dilution steps, each a 50% concentration of the previous dilution (7,639 and 7,228 are the mean and standard deviation, respectively, of oocysts after the seventh serial dilution).

The solutions used for the trials were prepared by adding 0.010 ml of oocyst suspension to 0.990 ml of soil extract to ensure minimal change in the concentration of background materials. All experiments were performed with a single batch that was less than 6 months old, as recommended previously (3).

Oocyst isolation from extracts and application of antibodies. IgM (Waterborne, Inc., New Orleans, La.; catalog no. AFL100) and IgG3 (ImmuCell, Portland, Maine; catalog no. LR-50) antibodies were used according to the manufacturer's instructions. For microscopy, the entire 1.000 ml of soil extract solution and 1.000 ml of distilled water were passed through a 13-mm black filter with a pore diameter of 0.2 μm (Isopore membrane; Millipore catalog no. GTBP01300) (11). Random-field microscopy was then performed (for samples containing

* Corresponding author. Mailing address: Department of Natural Resources and Environmental Sciences, MS 370, FA132, University of Nevada, Reno, NV 89557-0013. Phone: (775) 784-1938. Fax: (775) 784-4789. E-mail: mwalker@equinox.unr.edu.

† Publication 52031318 of the Nevada Agricultural Experiment Station.

TABLE 1. Results of trials with combinations of microscopy and flow cytometry and IgM and IgG3 labeling

No. of oocysts added to extract	Mean no. of oocysts recovered (SD)[a]			
	M-IgM	M-IgG	F-IgM	F-IgG
10,017 (2,213)	8,254 (898)	7,859 (940)		
4,826 (610)	4,652 (703)	4,083 (794)	3,430 (1,626)†	3,330 (1,290)†
3,200 (999)	2,196 (237)*	2,042 (147)*	2,483 (446)	1,378 (745)*
1,490 (580)	1,135 (315)	1,356 (164)	904 (225)	1,313 (274)
624 (196)	656 (111)	663 (89)	466 (241)	572 (264)
554 (248)	435 (207)	378 (47)	648 (258)	720 (354)
286 (45)	70 (56)*	164 (72)*	180 (45)	303 (187)†
76 (72)	60 (21)	60 (16)	35 (37)	118 (96)
38	30 (24)	34 (8)	67 (81)†	63 (37)
19	10 (15)	20 (18)	68 (32)*†	78 (59)*†
10	0	0	16 (22)†	95 (35)*†

[a] Results of replicate measurements ($n = 5$) are given for combinations of microscopy (M) and flow cytometry (F) with the indicated antibodies. *, Significantly different from estimated stock concentrations ($\alpha = 0.05$). †, Significantly greater variance than might be expected given the variance in the stock ($\alpha = 0.05$).

≥1,490 oocysts/ml, 50 fields were examined; for samples containing <1,490 oocysts/ml, 100 fields were examined). For flow cytometry, samples were diluted 1:3 with 0.01 mM phosphate-buffered saline–1,4-diazabicylo-[2.2.2]octane (DABCO) to prevent fluorescence quenching. The results are listed in Table 1 and displayed in Fig. 1.

Accuracy. The evaluation of the accuracy of the results took place in two steps. First, differences between means of sample counts and stock suspension counts were compared by a one-way analysis of variance. Second, if the difference was significant ($\alpha = 0.05$), Dunnett's test was used to identify which sets of results were different, with the stock considered as a control (8).

When 10 oocysts were present, microscopy produced estimates of no oocysts present and flow cytometry overestimated the number of oocysts, regardless of the type of antibody used.

Precision. We assessed the variances of the estimates (samples versus stock) with an *F* test of the null hypothesis of equivalence of variances (8). The variance was greater than expected for flow cytometry when both antibodies were used with oocyst concentrations of ≤19/ml and 4,826/ml. This suggests that at very low oocyst concentrations, flow cytometry results may not be as precise as those obtained by microscopy. It is possible that fluorescing background debris created false-positive signals.

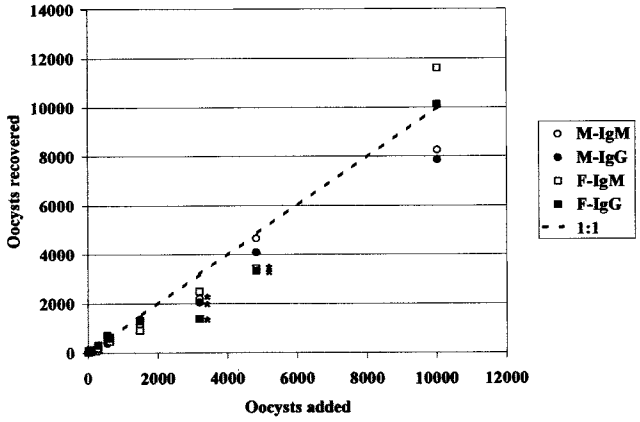

FIG. 1. Mean numbers of oocysts recovered by microscopy (M) with FITC-labeled IgM and IgG3 antibodies and by flow cytometry (F) with FITC-labeled IgM and IgG3 antibodies. Results are plotted against mean numbers of oocysts added to soil extracts, with a 1:1 correspondence indicated by a dashed line. Points marked with an asterisk are significantly different than added amounts.

Conclusions. Replicate counts of zero oocysts were determined by microscopy when 10 oocysts/ml were present. Assuming that the binomial distribution represents the likelihood of occurrence of oocysts in microscopic fields, the probability of detecting no oocysts in 100 fields at this concentration is >50%. The choice of immunoglobulins had no identifiable effect on either precision or accuracy. For flow cytometry, the analytic limit of detection under the conditions considered may be between 19 and 38 oocysts/ml, based on false-positive results at the lowest concentrations examined. Raw fecal material from infected neonatal calves may contain millions of oocysts per milliliter (2). When used with either antibody, microscopy and flow cytometry will detect oocysts present in a minimum of 0.01 μl of fecal material per g of soil, provided soil extraction methods are not excessively inefficient. The experimental conditions used for these trials involve a single soil. The performance of either method may change significantly with different types and amounts of fluorescing background debris in the soil extracts.

This research was supported in part by Hatch and USDA-NRI grants (projects NEV0533F and NEV052HL, respectively) and by the Nevada Agricultural Experiment Station.

REFERENCES

1. **Arrowood, M. J., and C. R. Sterling.** 1989. Comparison of conventional staining methods and monoclonal antibody-based methods for *Cryptosporidium* oocyst detection. J. Clin. Microbiol. **27:**1490–1495.
2. **Blewett, D. A.** 1989. Quantitative techniques in *Cryptosporidium* research. First International Workshop on Cryptosporidiosis. Morendun Research Institute, Edinburgh, Scotland.
3. **Dufour, A. P., M. Feige, H. D. Linquist, M. Messner, S. Regli, C. Rodgers, F. W. I. Schaeffer, S. Schaub, J. Sinclair, and L. J. Wymer.** 1999. Criteria for evaluation of proposed protozoan detection methods. U.S. Environmental Protection Agency, Washington, D.C.
4. **Ferrari, B. C., G. Vesey, K. A. Davis, M. Gauci, and D. Veal.** 2000. A novel two-color flow cytometric assay for the detection of *Cryptosporidium* in environmental water samples. Cytometry **41:**216–222.
5. **Ferrari, B. C., G. Vesey, C. Weir, K. L. Williams, and D. A. Veal.** 1999. Comparison of *Cryptosporidium*-specific and *Giardia*-specific monoclonal antibodies for monitoring water samples. Water Res. **33:**1611–1617.
6. **Jakubowski, W., S. Boutros, W. Faber, R. Fayer, W. Ghiorse, M. LeChevallier, J. Rose, S. Schaub, A. Singh, and M. Stewart.** 1996. Environmental methods for *Cryptosporidium.* J. Am. Water Works Assoc. **88**(9)**:**107–121.

7. **Medema, G. J., F. M. Schets, H. Ketelaars, and G. Boschman.** 1998. Improved detection and vital staining of *Cryptosporidium* and *Giardia* with flow cytometry. Water Sci. Technol. **38(12):**61–65.
8. **Montgomery, D.** 2001. Design and analysis of experiments. John Wiley and Sons, New York, N.Y.
9. **U.S. Environmental Protection Agency.** 1999. Method 1622: *Cryptosporidium* in water by filtration/IMS/FA. Office of Water, U.S. Environmental Protection Agency, Washington, D.C.
10. **Vetterling, J.** 1969. Continuous-flow differential density flotation of coccidial oocysts and a comparison with other methods. J. Parasitol. **55:**412–417.
11. **Walker, M., K. Leddy, and E. Hagar.** 2001. Effects of combined water potential and temperature stresses on *Cryptosporidium parvum* oocysts. Appl. Environ. Microbiol. **67:**5526–5529.
12. **Walker, M., C. Montemagno, J. Bryant, and W. Ghiorse.** 1998. Method detection limits of PCR and immunofluorescence assay for *Cryptosporidium parvum* in soil. Appl. Environ. Microbiol. **64:**2281–2283.