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# Fluorescence Detection of *Cryptosporidium* Oocysts in Human Fecal Specimens by Using Monoclonal Antibodies

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**With the discovery that the coccidian parasite *Cryptosporidium* sp. can cause severe symptoms in humans, implementation of many diagnostic techniques rapidly followed. The infection is self-limiting in patients with normal immune systems but chronic in the immunosuppressed patient. With the eventual development and use of therapeutic agents, it will become very important to find *Cryptosporidium* sp., even in low numbers, in fecal specimens. Production of a highly specific and sensitive antibody by use of cloning techniques has provided another diagnostic tool. Formalinized positive human fecal specimens ($n$ = 99) and negative specimens ($n$ = 198), of which 115 contained yeastlike fungi and other organisms, were tested in blind trials by use of a monoclonal antibody. Sensitivity was 100% with 3- to 4+ fluorescence on all cryptosporidial oocysts, both in light and heavy infections. The organisms were round and easily visible (4 to 6 μm), showing apple-green to yellow fluorescence against a dark background free of nonspecific fluorescence. Specificity was also 100% with all 99 positive *Cryptosporidium* sp. specimens exhibiting fluorescence and all 198 negative specimens showing no fluorescence. All positive and negative specimens were previously confirmed by the hot modified acid-fast technique. However, seven specimens previously considered negative by this acid-fast method were positive by the monoclonal antibody technique. These specimens were confirmed as positive, after extensive examination of additional smears prepared by the modified hot acid-fast method revealed rare organisms, emphasizing the increased sensitivity of the monoclonal antibody technique. Since acid-fast stains do not always consistently stain all oocysts, the increased sensitivity of the monoclonal reagent provides an excellent screening method.**

With the increased awareness that *Cryptosporidium* sp. can cause severe symptoms in humans, the development and implementation of many diagnostic techniques have been reported, including various concentration and staining methods (1–3, 6–9, 12). The majority, but not all, of the patients diagnosed with this infection were symptomatic, with various degrees of diarrhea. In many of these patients, the large number of organisms present ensures that the infection can be diagnosed by many of these techniques. However, for patients with very few oocysts in the stool, the routine methods such as the various acid-fast techniques and concentrates alone may not be sufficient to allow recovery of the organisms, even with increased expertise with the techniques. We saw a correlation between the number of organisms in the stool and the clinical presentation of the patient: the more formed the stool, the fewer the organisms. The number of organisms passed by a patient also varied from day to day and week to week.

Monoclonal antibody reagents with potential increased sensitivity were thought to offer an excellent alternative method, particularly when large numbers of patients or those with minimal symptoms are screened for *Cryptosporidium* sp. Data generated from this method would also reflect a more accurate incidence rate for those patients tested. This method with monoclonal antibodies would also eliminate the possibility of false-positives and false-negatives that are seen with routine staining methods for stool parasites.

## MATERIALS AND METHODS

**Specimens.** Human fecal specimens ($n$ = 297) were collected in 10% Formalin and submitted to the laboratory. Most of the patients (both inpatients and outpatients) were immunocompromised; 31 of them had the acquired immune deficiency syndrome. There were 99 cryptosporidial positive specimens and 198 cryptosporidial negative specimens, of which 115 contained yeastlike fungi, parasites, other organisms, or combinations thereof.

**Monoclonal antibody reagent.** This reagent was prepared and supplied by Charles Sterling and Michael Arrowood, Department of Veterinary Science, University of Arizona, Tucson (10, 11). Oocyst walls were isolated by sonicating 5 $\times 10^6$ intact oocysts, shocking freed sporozoites with distilled water, and washing in 0.025 M phosphate-buffered saline to remove debris. Spleen cells of adult RBN/Dn mice immunized on days 0, 14, and 28 were fused on day 32 with FOX/NY mouse myeloma cells by use of polyethylene glycol. Hybrid cells were grown in 24-well culture plates.

One hybridoma (oocyst wall 3) producing an immunoglobulin M monoclonal antibody, as determined by double diffusion in agar against an isotype-specific goat anti-mouse immunoglobulin, was positive for oocysts by indirect immunofluorescence assay. After cloning, this hybridoma was injected into pristane-primed mice to produce ascites tumors. The ascites fluid was purified, tested, and divided into portions for use (10).

**Specimen preparation for fluorescence and acid-fast methods.** Stool sediment was pretreated with 10% KOH to break up mucus, washed in 10% Formalin, and centrifuged at 300 $\times g$ for 2 min. One drop of the sediment was spread thinly on a slide, heat fixed (70°C for 10 min), and subsequently stained by the modified hot acid-fast method (5). Those smears, prepared for fluorescence staining, were heat fixed and then methanol fixed before staining (10).

**Preparation of 15-well, Teflon-coated slides.** The 15-well Teflon-coated slides were coated with a glycerol-agar adhesive. The adhesive mixture of 0.1 g of Noble agar (Difco Laboratories; any refined agar is acceptable), 0.5 ml of

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TABLE 1. Organisms used to test monoclonal antibody reagent for specificity

| Organism | No. of samples |
|---|---|
| Protozoa | |
| *Entamoeba histolytica* trophozoites, cysts | 8 |
| *Entamoeba coli* trophozoites, cysts | 14 |
| *Entamoeba hartmanni* trophozoites, cysts | 13 |
| *Endolimax nana* trophozoites, cysts | 23 |
| *Iodamoeba butschlii* trophozoites, cysts | 5 |
| *Giardia lamblia* trophozoites, cysts | 6 |
| *Chilomastix mesnili* trophozoites, cysts | 1 |
| *Dientamoeba fragilis* trophozoites | 2 |
| *Trichomonas hominis* trophozoites | 2 |
| *Balantidium coli* trophozoites, cysts | 1 |
| *Blastocystis hominis* trophozoites, cysts | 24 |
| *Isospora belli* oocysts | 8 |
| Helminth eggs and larvae | |
| *Ascaris lumbricoides* | 3 |
| *Trichuris trichiura* | 2 |
| Hookworm | 1 |
| *Strongyloides stercoralis* | 1 |
| *Taenia* spp. | 1 |
| *Hymenolepis nana* | 1 |
| *Hymenolepis diminuta* | 1 |
| *Diphyllobothrium latum* | 1 |
| *Clonorchis sinensis* | 1 |
| *Paragonimus westermani* | 1 |
| *Fasciola/Fasciolopsis* | 1 |
| *Schistosoma mansoni* | 1 |
| Bacteria | |
| *Shigella flexneri* | 27 |
| *Salmonella* group B | 1 |
| *Salmonella* group D | 1 |
| *Campylobacter jejuni* | 2 |
| *Mycobacterium avium-M. intracellulare* | 51 |
| Yeastlike fungi | |
| *Candida albicans* | 2 |
| *Candida guilliermondii* | 1 |
| *Candida tropicalis* | 1 |
| *Candida krusei* | 1 |
| *Candida pseudotropicalis* | 1 |
| *Candida parapsilosis* | 1 |
| *Candida (Torulopsis) glabrata* | 1 |
| *Cryptococcus neoformans* | 1 |
| *Cryptococcus albidus* | 1 |
| *Cryptococcus laurentii* | 1 |
| *Saccharomyces cerevisiae* | 1 |
| *Geotrichum* sp. | 1 |
| *Trichosporon cutaneum* | 1 |
| *Rhodotorula rubra* | 1 |

glycerol (analytical grade), and 100.0 ml of distilled water was boiled, dispensed in screw-cap tubes, autoclaved, and stored at room temperature. Screw-cap tubes were tightly sealed. One drop of glycerol-agar adhesive was placed on one corner of the slide, a piece of Whatman no. 40 or 41 filter paper was cut in squares (width of glass slide or a bit smaller), and a filter paper square was placed over the drop of adhesive and used to smear the adhesive over the slide. The slide was air dried for several minutes or placed at 37°C for 5 min (California Viral Laboratory, California State Department of Public Health, Berkeley, Calif.).

**Modified acid-fast staining (hot method).** The smears were placed on a staining rack and flooded with carbolfuchsin. The slides were heated to steaming and stained for 5 min. The smears were then rinsed with tap water, decolorized with 5% aqueous sulfuric acid for 30 s, and rinsed again. The smears were then flooded with methylene blue counterstain for 1 min, rinsed with tap water, drained, and air dried (5).

**Antigen detection with monoclonal antibody (fluorescence).** Mouse antibody (5 μl of 1:100 dilution) was added to each well containing the stool sample and incubated at 37°C for 20 min. The slide was rinsed three times in phosphate-buffered saline. The fluorescein isothiocyanate-labeled anti-mouse antibody (Kirkegaard and Perry Laboratory, Inc.) (5 μl) was added to each well and incubated at 37°C for 20 min. The slide was rinsed four times in phosphate-buffered saline, mounted on a glycerol-phosphate-buffered saline cover slip, and examined (4, 10).

**Method of slide examination.** The modified acid-fast-stained smears were scanned at a magnification of ×400, and oocyst identification and confirmation were made at a magnification of ×1,000. The oocysts were pink to red, measured from 4 to 6 μm, and were round to oval, with some organisms containing four crescent-shaped sporozoites.

Each well on the fluorescence slide was scanned at ×100 magnification, and organism confirmation was made at ×250 magnification. The organisms were round and easily visible (4 to 6 μm), showing apple-green to yellow fluorescence against a dark background free of nonspecific fluorescence.

## RESULTS

Sensitivity was 100% with a 3- to 4+ fluorescence on all 99 *Cryptosporidium* sp. oocysts, in both light and heavy infections. (Light infection was defined as 0 to 2 oocysts per every five fields at ×400 with the hot acid-fast method or 0 to 5 oocysts per field at ×100 with the monoclonal antibody fluorescence method. Heavy infection was defined as >5 oocysts per field at ×400 with the hot acid-fast method or >50 oocysts per field at ×100 with the monoclonal antibody fluorescence method.) Specificity was also 100%, with all 99 positive specimens exhibiting fluorescence and all 198 negative specimens showing no fluorescence. Organisms which were used to test the monoclonal reagent for specificity are listed in Table 1. None of the 115 specimens containing these organisms was positive at any level of fluorescence. Seven specimens which were previously considered negative by the acid-fast method were positive by the monoclonal antibody method. Additional smears were prepared from these specimens and examined by the modified acid-fast technique. After an additional average review of 4 to 6 smears per specimen, the presence of *Cryptosporidium* sp. oocysts was confirmed, again emphasizing the higher sensitivity of the monoclonal antibody method for detection of low numbers of organisms.

## DISCUSSION

Since there were no instances of false-positives, this confirms the ability to screen the fluorescent-antibody wells with low power (magnification of ×100). A positive diagnosis can be determined by finding one *Cryptosporidium* sp. oocyst per well. This increase in sensitivity was clearly shown by the fact that seven specimens were found to be positive by the fluorescent-antibody technique and were negative by acid-fast techniques. This ability to detect very low numbers of organisms will become more important when drugs are found to be effective in this infection, thus allowing the patient to be put on therapy earlier, and when experimental drug efficacy is being determined. The lower limit of

sensitivity of the acid-fast-staining technique is difficult to determine and would be based on the amount of time spent scanning the smear and the overall thickness of the preparation. However, based on our experience with these two techniques, we would suggest that the monoclonal antibody technique is at least 10 times more sensitive than the acid-fast method.

With a batch of seven specimens for comparison, specimen preparation time to perform each test is comparable. However, the time for examination of acid-fast smears is considerably longer as the number of organisms becomes fewer and the slides must be carefully screened. A negative acid-fast smear can take up to 60 min (normal time is 15 to 20 min), whereas review of the well for fluorescence takes 20 to 30 s. It is impossible to make an adequate comparison of cost, because the commercial reagents are not presently available. However, reading wells for fluorescence certainly saves time compared with reading acid-fast smears. Employee training time (less for the fluorescence technique) and the cost of the fluorescence microscope must also be considered.

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