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# Detection of Microsporidia by Indirect Immunofluorescence Antibody Test Using Polyclonal and Monoclonal Antibodies

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**During a screening for monoclonal antibodies (MAbs) to the microsporidian *Encephalitozoon hellem*, three murine hybridoma cell lines producing strong enzyme-linked immunosorbent assay (ELISA) reactivities were cloned twice, were designated C12, E9, and E11, and were found to secrete MAbs of the immunoglobulin M isotype. On subsequent ELISAs, the three MAbs reacted most strongly to *E. hellem*, and they reacted somewhat less to *Encephalitozoon cuniculi* and least to *Nosema corneum*, two other microsporidian species. The MAbs produced values of absorbance against microsporidia that were at least three times greater than reactivities obtained with control hybridoma supernatants or with uninfected host cell proteins used as antigens. By Western blot immunodetection, the three MAbs detected three *E. hellem* antigens with relative molecular weights ($M_r$s) of 62, 60, and 52 when assayed at the highest supernatant dilutions producing reactivity. At lower dilutions, the MAbs detected additional proteins with $M_r$s of 55 and 53. By using indirect immunofluorescence antibody staining, the MAbs, as well as hyperimmune polyclonal murine antisera raised against *E. cuniculi* and *E. hellem*, were able to detect formalin-fixed, tissue culture-derived *E. cuniculi* and *E. hellem* and two other human microsporidia, *Enterocytozoon bieneusi* and *Septata intestinalis*, in formalin-fixed stool and urine, respectively. *E. bieneusi*, however, stained more intensely with the polyclonal antisera than with the MAbs. Neither the MAbs nor the hyperimmune murine polyclonal antibodies detected *Cryptosporidium*, *Giardia*, *Trichomonas*, or *Isospora* spp. At higher concentrations, the polyclonal antisera did stain *N. corneum* and yeast cells. The background staining could be absorbed with *Candida albicans*. These results demonstrate that polyclonal antisera to *E. cuniculi* and *E. hellem*, as well as MAbs raised against *E. hellem*, can be used for indirect immunofluorescence antibody staining to detect several species of microsporidia known to cause opportunistic infections in AIDS patients.**

Microsporidia are small obligate intracellular protozoan parasites which infect a wide range of animal hosts, including all classes of vertebrates and most invertebrates (8). Awareness of microsporidia is important because the number of reported microsporidiosis cases in AIDS patients is increasing (2, 5–7, 22, 23, 29). The three most common microsporidia reported to infect individuals with AIDS are *Enterocytozoon bieneusi*, *Encephalitozoon hellem*, and the *Encephalitozoon*-like *Septata intestinalis*. *E. bieneusi* primarily infects small-intestinal enterocytes, causing diarrhea, but can also infect the biliary tract, leading to cholangitis (4, 10, 19, 23, 26). On the other hand, *E. hellem* and the *Encephalitozoon*-like *S. intestinalis* are not as tissue specific and have been reported to cause keratoconjunctivitis, sinusitis, nephritis, and enteritis (3, 15, 22, 27). Although cases of *Encephalitozoon cuniculi*-associated hepatitis and peritonitis in AIDS patients have been reported, it is possible that they were actually due to the morphologically identical *E. hellem*.

Presently, definitive identification of microsporidiosis depends upon transmission electron microscopy, which is time- and cost-consuming. In addition, transmission electron microscopy may not be sensitive enough to detect small numbers of organisms. Serological studies for detecting microsporidium-specific antibodies are reliable for antemortem diagnosis in infected laboratory animals (29, 30) but may be unreliable for AIDS patients whose immune responses are compromised (12, 13). Mammalian microsporidium spores do stain with Gram, Giemsa, calcofluor, and concentrated trichrome (25, 30, 32, 33, 35), but because these organisms are very small (measuring 0.5 to 2.0 μm by 1.0 to 4.0 μm) they are difficult to distinguish from bacteria and small yeasts. In this article, we report the use of polyclonal antibodies and monoclonal antibodies (MAbs) in detecting microsporidia in formalin-fixed stool and urine by the indirect immunofluorescence antibody test (IFAT).

## MATERIALS AND METHODS

**Parasites.** *E. hellem*, *E. cuniculi*, and *Nosema corneum* were grown in Madin-Darby canine kidney (MDCK) cells by using RPMI 1640 supplemented with 5% fetal bovine serum, streptomycin (100 μg/ml), penicillin (100 U/ml), amphotericin B (0.25 μg/ml), and L-glutamine (2 mM) (referred to as complete RPMI) as described previously (11, 14). Parasites from tissue culture supernatants were centrifuged at 400 × *g* for 15 min, washed once in 25 mM Tris-buffered saline (TBS; pH 7.4) containing 0.05% Tween 20 (TBS-Tween), resuspended in TBS, and centrifuged over 50% Percoll (Pharmacia, Piscataway, N.J.) at 14,000 × *g* for 2 min. The parasites were washed twice with TBS to remove the Percoll and stored at 4°C in bicarbonate buffer (pH 9.6) for use in the enzyme-linked immunosorbent assay (ELISA) or were stored in TBS for all other uses.

**Antibodies.** Murine hyperimmune antisera were obtained

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from female BALB/c mice (Charles River Laboratories, Wilmington, Mass.) immunized intraperitoneally with four inoculations of $5 \times 10^7$ spores of *E. hellem*, *E. cuniculi*, or *N. corneum* at 4-week intervals. Sera were collected by tail bleeding 1 week after each inoculation, pooled from mice within each group, and stored at −70°C until used. Sera from the third bleeding were used in these studies, and the ELISA titers obtained by assaying the sera against each homologous microsporidian were ≥1:12,800.

The *E. hellem*-immunized BALB/c mice used for obtaining the polyclonal antisera were then employed for producing MAbs. Four days after the fourth inoculation, spleen cells were fused with the murine myeloma cell line X63.Ag8.653 (17). Approximately 2 weeks later, and after at least three medium changes, the supernatants were screened for antibody production by ELISA by using intact *E. hellem* spores as antigens. Positive hybridomas were cloned twice by limiting dilutions and seeded at $10^5$ cells per ml of complete RPMI (containing 10% fetal bovine serum) into 25-$cm^2$ tissue culture flasks. Three-day log-phase culture supernatants were used in the assays described here. The MAb isotypes were determined with the Sigma immunotype kit by following the directions enclosed (catalog no. ISO-1; Sigma, St. Louis, Mo.).

Ascites fluid was produced in BALB/c mice by intraperitoneal injection of 0.5 ml of pristane (2,6,10,14-tetramethyldecanoic acid) followed by intraperitoneal injection of $10^6$ hybridoma cells 2 weeks later (17). The ascites fluid was tapped approximately 3 weeks later, centrifuged ($400 \times g$ for 15 min) to remove cells, aliquoted, and stored at −70°C until used.

**ELISA.** To perform the ELISA, *E. hellem*, *E. cuniculi*, and *N. corneum* in 0.2 M bicarbonate buffer (pH 9.6) were used to coat 96-well, flat-bottom polystyrene microtiter plates (Corning Glass Works, Corning, N.Y.) at a concentration of $5 \times 10^7$ spores per well as described by Hollister and Canning (18). MDCK cell proteins were adjusted to 1.0 mg/ml in bicarbonate buffer. Plates were incubated overnight at 37°C, washed with TBS three times, and blocked with 3% (wt/vol) bovine serum albumin (BSA) in TBS for 2 h at 37°C. After three washings with TBS, 100 μl of the appropriate undiluted hybridoma or control supernatant was added to each well. The plates were incubated for 2 h at 37°C, washed three times in TBS-Tween, and incubated for 1 h at 37°C with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG)-IgM-IgA (Sigma) diluted 1:4,000 in TBS containing 1% (wt/vol) BSA (TBS-BSA). After the plates were washed three times with TBS, 100 μl of the enzyme substrate *p*-nitrophenylphosphate (1.0 mg/ml of diethanolamine buffer, pH 9.6) was added, and the optical densities were read at 405 nm on an ELISA spectrophotometer (Dynatek Laboratories Inc., Chantilly, Va.) (17).

**SDS-PAGE and Western blotting (immunoblotting).** Parasite proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (20), using a 5% stacking gel, a 10 to 20% gradient resolving gel, and diallytartardiamide as the cross-linker. Parasites in sample buffer containing β-mercaptoethanol were boiled for 5 min and centrifuged at $14,000 \times g$ for 1 min to remove particulate materials. Each preparative slab gel (16 by 20 cm) was loaded with $2 \times 10^9$ parasites. After electrophoresis, the separated polypeptides were electrophoretically transferred onto Immobilon-P membranes (Millipore, Bedford, Mass.) and cut into strips. The strips were incubated with 5% (wt/vol) nonfat dry milk (Carnation) in TBS for 30 min to block unbound sites, washed in TBS-Tween for 10 min, and incubated with various dilutions of each hybridoma supernatant, positive control serum, or negative control hybridoma supernatant. After overnight incubation the strips were washed three times with TBS-Tween and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG-IgM (Sigma) diluted 1:1,000 in TBS-BSA. After incubation for 60 min at 37°C, the strips were washed with three changes of TBS and rinsed with developing buffer (50 mM Tris-HCl [pH 9.6] containing 3 mM $MgCl_2$) as described previously (1). Bands were visualized by the addition of the substrates 5-bromo-4-chloro-3-indolylphosphate (0.05 mg/ml) and nitroblue tetrazolium (0.01 mg/ml) to the developing buffer. After color development for 30 min, the strips were rinsed in distilled water, dried, and stored in the dark.

**IFAT.** The IFAT was performed as described previously (17). Thin smears were prepared on microscope slides from (i) patient stools containing *E. bieneusi* fixed with buffered formalin-stool (1:3, vol/vol); (ii) urine specimens containing *S. intestinalis*, which were centrifuged at $400 \times g$ for 10 min and resuspended in 1/50 of the original volume of buffered formalin; (iii) formalin-fixed tissue culture-derived *E. hellem*, *E. cuniculi*, or *N. corneum*; and (iv) nonfixed microsporidia derived from tissue culture. The slides were air dried, fixed in 100% methanol for 5 min, and then incubated with MAbs, polyclonal antisera, or a negative hybridoma supernatant. After incubation in a moist chamber for 30 min at 37°C, the slides were washed twice in TBS. Fluorescein isothiocyanate-conjugated goat anti-mouse IgG-IgM-IgA (Sigma) was absorbed with formalin-fixed stool sediment, diluted 1:200, and added to the slides for incubation at 37°C for 30 min. Then the slides were washed, coverslips were added with Cytoseal mounting medium (Stephens Scientific, Riverdale, N.J.), and the slides were examined under UV light microscopy (Olympus AH2 microscope) with an excitation wavelength of 380 to 490 nm, which generates an observation light wavelength equal to or greater than 515 nm.

## RESULTS

**Characterization of MAbs by ELISA.** Three hybridoma cultures, whose supernatants reacted most strongly to *E. hellem* in the ELISA, were cloned twice, designated C12, E9, and E11, and found to secrete MAbs of the IgM isotype. The MAbs were then tested again by ELISA using *E. hellem* as well as *E. cuniculi* and *N. corneum* as antigens (Table 1). The three MAbs reacted most strongly against *E. hellem*. C12 and E9 also reacted strongly to *E. cuniculi* and less strongly against *N. corneum*, a microsporidian isolated from the corneal stroma of a human immunodeficiency virus-seronegative individual (9, 31). E11 produced nearly identical low levels of reactivity against both *E. cuniculi* and *N. corneum*. In all cases, however, the MAbs produced at least threefold-higher levels of reactivity against the microsporidia than against MDCK tissue culture host cell proteins or against a negative hybridoma control supernatant.

**Characterization of MAbs by Western blot immunoassay.** Western blot immunodetection of *E. hellem* was performed to determine the molecular weights of proteins recognized by the MAbs. All three IgM MAbs detected proteins with $M_r$s of 62, 60, and 50 at the highest dilutions producing reactivity. Less diluted hybridoma supernatants also bound to proteins with $M_r$s of 55 and 53 (Fig. 1). Identical results were obtained when parasite proteins were prepared by using the protease inhibitors leupeptin (0.5 μg/ml) and phenylmethylsulfonyl fluoride (0.2 mM).

**IFAT.** Murine polyclonal antisera to *E. cuniculi* and *E. hellem*, as well as the MAbs raised against *E. hellem*, were used to detect microsporidia by IFAT. Smears of tissue culture-

TABLE 1. ELISA reactivities of MAbs raised to *E. hellem*[a]

| MAb | ELISA values for absorbance to antigen target[b] | | | |
|---|---|---|---|---|
| | *E. hellem* | *E. cuniculi* | *N. corneum* | MDCK cells |
| C12 | 1.153 ± 0.011 | 0.785 ± 0.090 | 0.555 ± 0.005 | 0.101 ± 0.006 |
| E9 | 0.953 ± 0.058 | 0.491 ± 0.014 | 0.341 ± 0.070 | 0.094 ± 0.002 |
| E11 | 0.818 ± 0.072 | 0.310 ± 0.033 | 0.318 ± 0.043 | 0.052 ± 0.013 |
| Neg. sup't. | 0.104 ± 0.007 | 0.055 ± 0.030 | 0.020 ± 0.027 | −0.045 ± 0.0001 |

[a] Undiluted culture supernatants from twice-cloned hybridomas were recovered and assayed as described in Materials and Methods.
[b] Values are the means of two replicates ± the standard deviations.

derived *E. hellem*, *E. cuniculi*, *N. corneum*, formalin-fixed stools mixed with the tissue culture-derived organisms, and stool and urine specimens with *E. bieneusi* and *S. intestinalis*, respectively, were examined. The murine antiserum to *E. hellem* (diluted 1:500) detected both native *E. hellem* (data not shown) and formalin-fixed *E. hellem* (Fig. 2A) and *E. cuniculi* (data not shown), as well as *E. bieneusi* (Fig. 2B) and *S. intestinalis* (Fig. 2C) in patient stools and urine, respectively. The murine polyclonal antiserum to *E. cuniculi* produced results identical to those obtained with the murine polyclonal antiserum to *E. hellem* (data not shown). The three MAbs producing the strongest reactivity to *E. hellem* in the ELISA also detected *E. hellem*, *E. cuniculi*, *E. bieneusi*, and *S. intestinalis* but not *N. corneum* by IFAT. Results are shown for E9 detection of *E. hellem* (Fig. 2D), *E. bieneusi* (Fig. 2E), and *S. intestinalis* (Fig. 2F). IFA staining of *E. bieneusi* with the MAbs (Fig. 2E) was not as strong as with the polyclonal antisera (Fig. 2B). The MAbs did not stain *Cryptosporidium*, *Isospora*, *Giardia*, *Candida*, or *Trichomonas* spp. in known positive patient stool specimens (data not shown). The murine hyperimmune sera raised against *E. hellem* or *E. cuniculi* did detect *N. corneum* and *Candida albicans* when used at dilutions of 1:200 or lower, and the nonspecific binding to yeast cells could be reduced by absorbing with *C. albicans* (data not shown). In addition, background staining, particularly when the polyclonal antisera were used, could be further reduced by absorbing the

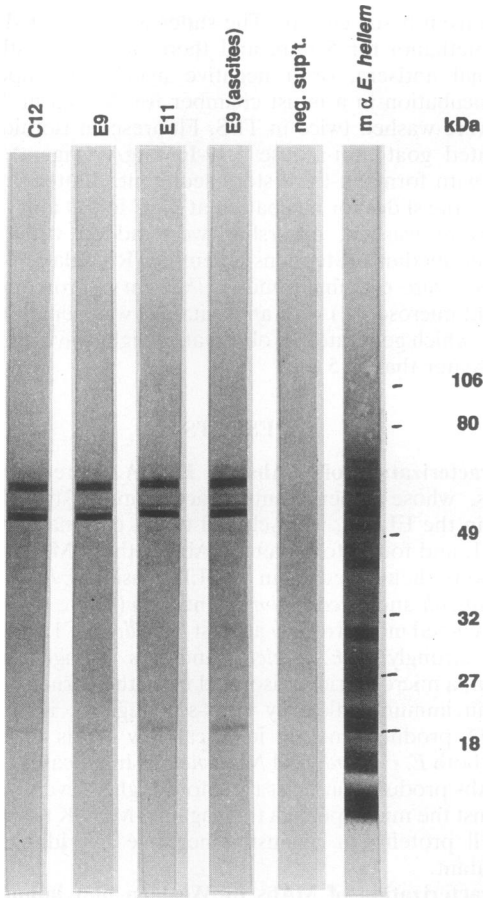


FIG. 1. Western blot characterization of MAbs to *E. hellem*. *E. hellem* proteins were separated by SDS-PAGE under reducing conditions and transferred to Immobilon-P membranes. The membrane antigen strips were incubated with MAbs (C12, E9, and E11) harvested from 3-day log-phase hybridoma cell cultures and diluted 1:10; this was followed by immunodetection using alkaline phosphatase-conjugated goat anti-mouse IgM and color development with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate. Ascites fluid from BALB/c mice inoculated with hybridoma E9 cells was diluted 1:100, and the hyperimmune murine polyclonal antiserum to *E. hellem* (m α *E. hellem*) was used at a dilution of 1:2,000. Molecular masses of standards are given at the right (in kilodaltons). Neg. sup't., negative supernatant.

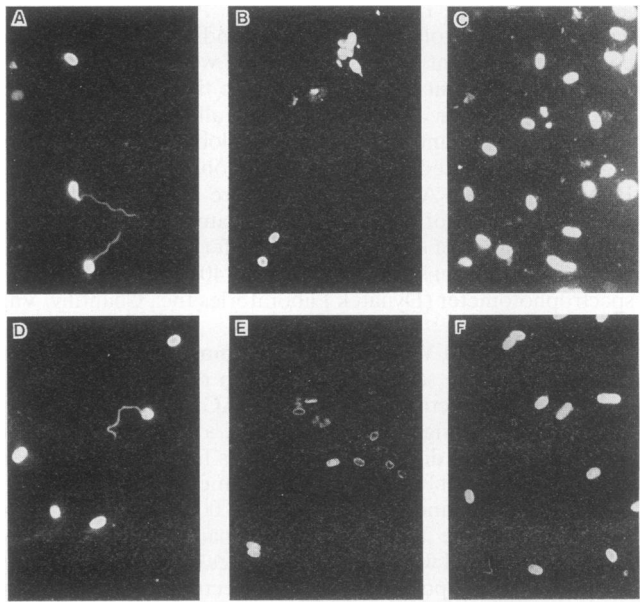


FIG. 2. IFAT for detecting microsporidia using polyclonal antibodies and MAbs. Formalin-fixed tissue culture-derived *E. hellem* (A and D), *E. bieneusi* in stool (B and E), and *S. intestinalis* in urine (C and F) were stained with murine hyperimmune polyclonal antiserum raised against *E. hellem* (A to C) or MAb E9 (D to F) as described in Materials and Methods.

fluorescein isothiocyanate-conjugated antibodies with formalin-fixed stool sediment.

## DISCUSSION

Opportunistic infections with microsporidia in AIDS patients are increasingly being reported, yet the number of microsporidiosis cases is probably greatly underreported. Microsporidium infections are difficult to diagnose, primarily because the organisms are small and difficult to distinguish from bacteria and small yeasts in tissue and stool. Giemsa stain (25, 32) and a modified trichrome stain using chromotrope 2R (35) have been used to detect microsporidia in stool, but with some difficulties. Giemsa-stained microsporidia are blue and display a purple-blue nucleus which distinguishes them from bacteria. It is difficult, however, to find microsporidia in stool smears in which most other organisms also stain blue. The modified trichrome (chromotrope 2R) staining method described by Weber et al. (35) has the advantage that most bacteria counterstain light green, leaving the microsporidia pink. The stool smear must be very thin in order to observe the internal structure of the microsporidia, so microsporidia may be missed if the parasite burden is low or if microsporidia are mixed with mucus. In addition, small yeasts and some bacteria in stool also stain pink, which can complicate the results. Finally, it is crucial to monitor the destaining step so that the microsporidia remain pink but high background levels of staining are not generated (16). A calcofluor staining method utilizing Uvetix 2B (Ciba-Geigy) or fluorescent brightener (catalog no. F-6259; Sigma) also may be useful for detecting microsporidia (33). The microsporidia display a relatively thick ring of fluorescence, and the anterior region appears concave. However, because yeasts also stain with calcofluor, further studies are needed to assess the specificity and reliability of these stains.

The use of microsporidium-specific antibodies in IFAT procedures appears to overcome some of these difficulties. In earlier studies, IFAT procedures with polyclonal antisera were used to show that several species of microsporidia demonstrated immunological cross-reactivity (21). More recently, polyclonal antisera produced against *E. cuniculi* and *E. hellem* were used to diagnose ocular and systemic *E. hellem* infections (27, 28), and polyclonal antiserum raised against *E. cuniculi* in rabbits was used in the IFAT to detect *E. bieneusi* organisms in deparaffinized tissue sections (36) and in stool (37).

In this study, we observed that polyclonal antisera raised against *E. cuniculi* and *E. hellem* in mice, as well as the three MAbs, detected formalin-fixed, tissue culture-derived *Encephalitozoon* species as well as *E. bieneusi* in formalin-fixed stool. The polyclonal antisera to *E. cuniculi* and *E. hellem* also detected a newly described microsporidian, *S. intestinalis* (3, 24), in formalin-fixed urine. The antiserum raised against *E. cuniculi* and *E. hellem* detected *N. corneum* only when used at lower dilutions, suggesting that *E. hellem* and *E. cuniculi* are more closely related to *E. bieneusi* and *S. intestinalis* than to *N. corneum*. Unlike the polyclonal antisera raised against *E. cuniculi* and *E. hellem*, the hybridoma supernatants did not stain *N. corneum* or yeast cells in the IFAT, even though they did react somewhat to *N. corneum* in the ELISA. Microsporidia of the genus *Nosema* typically infect insects, and the lack of cross-reactivity in the IFAT is most likely due to a lower degree of phylogenetic relatedness (34).

The MAbs and polyclonal antibodies provided different advantages in the IFA stain. *E. bieneusi* stained more intensely with the polyclonal antisera raised against *E. cuniculi* or *E. hellem* than with the MAbs raised against *E. hellem*. However, we found that the polyclonal antisera generated more background in formalin-fixed stool specimens with the IFA stain. The degree of background depended on the dilution of antiserum, as also described by Weiss et al. (36). We also found that the polyclonal antisera stained yeast cells, but this problem could be overcome by absorbing the antisera with *C. albicans*. Neither the *Candida*-absorbed polyclonal antisera nor the MAbs stained yeast cells, bacteria, or *Cryptosporidium*, *Isospora*, *Giardia*, or *Trichomonas* spp.; both the antisera and MAbs thus appeared to be specific for microsporidia. In addition, we found that absorbing the fluorescein isothiocyanate-conjugated antibody with sedimented stool material helped reduce the background staining.

That the most strongly reacting MAbs selected for cloning were of the IgM isotype was probably due to the pentameric structure of the IgM antibodies, which could amplify the signal in the ELISA more than monomeric IgG MAbs could. Although the three MAbs appeared to detect the same proteins on Western blots of *E. hellem*, binding by E9 and E11 (but not C12) was lost by periodate oxidation, suggesting that the epitopes recognized by E9 and E11 are carbohydrates (unpublished observations). In addition, the ELISA values for absorbance by the MAbs against the different microsporidia varied, so that it is possible that the MAbs detected different epitopes on the same proteins. The fact that several proteins were detected by each MAb could be due to the presence of the same epitopes on precursor molecules and/or breakdown products in the parasite preparations used to prepare the blots. Adding the protease inhibitors leupeptin and phenylmethylsulfonyl fluoride to the parasite preparations, however, still resulted in detection of the multiple bands. Finally, it is possible that the same epitope is found on functionally different proteins.

The availability of polyclonal antibodies to *Encephalitozoon* species and MAbs to *E. hellem* should be particularly useful in screening specimens for the presence of the most common microsporidia causing opportunistic infections in AIDS patients. Additional specimens need to be stained with these reagents to determine their feasibility and reliability for routine diagnostic and epidemiologic studies. In addition, IgG MAbs which, in combination, may prove more useful than IgM MAbs are being evaluated. Most of the MAbs generated against *E. hellem* were of the IgM isotype, and these MAbs reacted more intensely than the IgG MAbs. However, we are finding that the use of the IgM MAbs characterized in this study may not be feasible for ELISA procedures because of high-level, nonspecific reactivity. This may be due to the higher level of sensitivity in ELISA than in IFA staining. Another difficulty is that the secondary or conjugated antibodies need to be absorbed with sedimented stool to reduce background in the IFAT, and it may not be possible to remove this interference for the ELISA. To reduce this background interference, one could conjugate the MAb (e.g., with fluorescein isothiocyanate or alkaline phosphatase), which is easier with IgG than with IgM MAbs. The polyclonal antibodies and MAbs described in this study, however, provide a basis for screening specimens for the presence of microsporidia by IFA staining. Species-specific diagnosis of microsporidium-positive specimens should then become possible as species-specific immunologic and molecular probes become available.

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