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Evaluation of an Immunofluorescent-Antibody Test Using Monoclonal Antibodies Directed against *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* for Diagnosis of Intestinal Microsporidiosis in Bamako (Mali)

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A 2-month study was carried out in Mali to evaluate an immunofluorescent-antibody test (IFAT) using monoclonal probes specific for *Enterocytozoon bieneusi* or *Encephalitozoon intestinalis*. Sixty-one human immunodeficiency virus (HIV)-seropositive adult patients and 71 immunocompetent children were enrolled. Microsporidia were detected in stools from 8 of 61 patients (13.1%) seropositive for HIV. A single species, *E. bieneusi*, was identified. All the children were negative for microsporidia. The sensitivity and specificity of IFAT were 100% compared with those of PCR, which was used as the "gold standard." Moreover, species identification by IFAT was more rapid and less expensive than that by PCR. These results show the suitability of IFAT for detection of microsporidia in developing countries.

Microsporidia are widespread, obligately intracellular eukaryotic parasites infecting a diversity of hosts, including invertebrates and vertebrates. Microsporidia have emerged as important opportunistic pathogens of humans during the AIDS pandemic. *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* are the two species responsible for gastrointestinal disease in humans (6). According to different studies, 15 to 50% of chronic malabsorptive diarrhea cases in human immunodeficiency virus (HIV)-infected patients are caused by microsporidia, and *E. bieneusi* has been found in most cases (15). Microsporidia also cause infection in HIV-negative immunodepressed transplant recipients (10, 18). Since the introduction of highly active antiretroviral therapy, intestinal microsporidiosis has been less frequently reported in Europe and the United States. In developing countries, however, an increase in the impact of microsporidiosis is to be expected due to the rapid expansion of AIDS. A study carried out in Zimbabwe in 1995 showed that *E. bieneusi* was the parasite most frequently found (11.1%) in HIV-positive patients presenting with diarrhea (27). These results were confirmed with an even greater prevalence in 1999, since the percentage of HIV-infected patients with *E. bieneusi* infection reached 51% when diagnosis was performed with PCR (11). *E. bieneusi* was also detected in Niger (3), Mali (17), and more recently in Thailand (28). Only a few studies have reported intestinal infections caused by *E. intestinalis* in Africa (8, 9, 13). Some cases have been described in immunocompetent patients, notably in adult travelers and in young children (16, 12). Two systematic surveys in Niger (3) and Zambia (12) showed the low prevalence of microsporidia in children: 0.8 and 0.56%, respectively. In Argentina, of 344 toddlers hospitalized in a pediatric institution, 22 were found positive for microsporidia; curiously, no significant difference was found between diarrheic children (7.2%) and those who were nondiarrheic (8.2%) (25).

Infections caused by *E. intestinalis* are treated with albendazole (19), while fumagillin has been shown to be effective for eradicating *E. bieneusi* (20). Thus, species identification is important for defining the appropriate treatment. Chromotrope staining (29) and staining with the fluorochrome Uvitex 2B (U2B) (26) are the reference techniques for diagnosis of intestinal microsporidiosis from stool specimens. Both methods require a high level of expertise in order to be reliable (5). To date, however, species identification has been possible only by using transmission electron microscopy or PCR genomic amplification (17, 22). We recently produced monoclonal antibodies which enable identification of both species (1, 2). In the present study we evaluated an immunofluorescent-antibody test (IFAT) using these monoclonal antibodies. The reliability and suitability of this diagnostic method were compared with those of U2B staining and PCR.

MATERIALS AND METHODS

Patients. Recruitment was performed in three principal health divisions in the district of Bamako (Mali): the Hôpital National du Point G, the Hôpital Gabriel Touré, and the Centre d'Écoute, de Soins, d'Animation et de Conseils (CESAC), a psychosocial and medical support center for persons with HIV/AIDS and their families. HIV serological status was determined by using different enzyme-linked immunosorbent assays: Murex, Vironostica, and Genescreen. Adult HIV-seropositive patients and children, presumed immunocompetent, all presenting with diarrhea, were recruited among outpatients and inpatients between 21 April and 20 July 2000. HIV-seropositive patients included 29 men and 32 women (male/female ratio, 0.90), with a median age of 33 years (range, 11 to 58 years).

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TABLE 1. Reliability and predictive values of U2B and IFAT, and concordance with PCR, for HIV-positive patients ($n = 61$)

Parameter[a]	Value for:		
	U2B	IFAT	
		E. bieneusi	*E. intestinalis*
Sensitivity (%)	100	100	
Specificity (%)	98.1	100	100
PPV (%)	88.8	100	
NPV (%)	100	100	100
Expected concordance	0.76	0.77	
Observed concordance	0.98	1.00	
Kappa (95% CI)	0.93 (0.8–1)	1.00	

[a] PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval.

Immunocompetent children included 40 males and 31 females (male/female ratio, 1.29) aged 1 to 60 months (median, 6.5 months; mean, 8.37 ± 8.3 months). Informed consent was obtained from the adult patients and the children's parents. The study was approved by the Ethics Committee of the Faculté de Médecine, Pharmacie, et d'Odonto-Stomatologie of Bamako.

Stool samples. Fresh stool samples, one per patient, were first investigated for intestinal parasites by direct examination. The Henricksen Poblenz acid-fast staining technique was used for detection of *Cryptosporidium parvum*. For detection of microsporidia, stool samples were homogenized in phosphate-buffered saline (PBS), pH 7.2. Then the stool suspension in PBS was filtered through a graded series of nylon sieves with pore diameters of 300 and 100 μm. A second filtration was carried out by aspiration (pore size, 50 μm). The final filtrate was centrifuged at 800 × *g* for 5 min (Beckman GPR Centrifuge; Beckman Coulter, Roissy, France), after which the supernatant was centrifuged at 2,500 × *g* for 10 min, and the pellet was resuspended in PBS (1/3, vol/vol).

U2B staining. The U2Bmethod described by van Gool et al. was used (26). Briefly, 20 μl of ethanol-fixed stools was spread on a glass slide and air dried. The slide was covered for 15 min with 1% U2B (Biotrim, Lyon, France), rinsed with distilled water, counterstained for 5 min with 1% Evans blue (Réactifs RAL, Bordeaux, France), and then rinsed with distilled water. The slide was air dried for 10 min and then examined under a fluorescence microscope (excitation filter, 355 to 425 nm; barrier or emission filter, 460 nm; 50-W mercury bulb) at a magnification of ×1,000. Spores appeared as fluorescent blue ovoid elements on a black background. To avoid confusion with fluorescent bacteria, the slide was viewed with ordinary light, under which spores are not visible.

IFAT. We used two monoclonal antibodies, 6E52D9 and 3B82H2, directed against the spore walls of *E. bieneusi* and *E. intestinalis*, respectively (1, 2). These monoclonal antibodies were obtained after immunization of BALB/c mice by intraperitoneal injection of a lysate of microsporidian spore proteins, followed by lymphocyte hybridization (1). A slide (18-well) was prepared by depositing 2 μl of pelleted stool onto each well; then it was air dried at room temperature and fixed in ice-cold acetone (−20°C) for 10 min. The supernatant of the hybridoma culture was transferred to the 18-well slide (20 μl per well). After incubation at room temperature for 30 min in a moist chamber, the slide was washed three times in PBS; then each well was covered with 20 μl of fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin G (IgG)-IgM-IgA (Sigma-Aldrich Chimie, St Quentin-Fallavier, France) at a dilution of 1/100 containing Evans blue as a counterstain (1/200) before incubation in a moist chamber for 30 min at room temperature. After a wash, as described above, the slide was mounted with glycerol fluid (Immu-mount; Shandon Inc.), and spores were visualized with a Leitz Laborlux epifluorescence microscope (450 nm; magnification, ×1,000). Controls consisted of stool specimens from patients tested by PCR for the presence or absence of either *E. bieneusi or E. intestinalis.*

DNA extraction and PCR amplification. Stool pellets were conserved at +4°C until DNA extraction. Microsporidian DNA was extracted by using the QIAamp protocol (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions and was then stored at −20°C. The region of DNA to be amplified was that encoded by the small subunit of the rRNA of *E. bieneusi* or *E. intestinalis* (4). Primers common to both species and their sequences were INBI (also called V1) (5′-CAC CAG GTT GAT TCT GCC TGA C-3′) and PMP2 (5′-CCT CTC CGG AAC CAA ACC CTG-3′) (21, 32). Primers V1 and EB450 (5′-ACT CAG GTG TTA TAC TCA CGT C-3′) were used to amplify *E. bieneusi* DNA (32), whereas primers V1 and SI500 (5′-CTC GCT CCT TTA CAC TCG −3′) were used to amplify *E. intestinalis* DNA (31). For a final volume of 50 μl, the mixture was composed of 27.35 μl of distilled water, 5 μl of buffer solution (10×), 5 μl of $MgCl_2$ (25 mM for a final concentration of 2.5 mM), 0.4 μl of deoxynucleoside triphosphates at 25 mM each (final concentration, 200 μM), 1 μl of each nucleotide at 10 pmol/ml (final concentration, 0.4 μM), and 0.25 μl of *Taq* polymerase (5 U/μl). Ten microliters of the solution containing the DNA extract was then added. The amplification procedure consisted of an initial denaturation at 94°C for 5 min; 35 cycles of amplification with denaturation at 94°C for 45 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s; and a final phase of extension at 72°C for 10 min. Each series to be amplified was completed by controls. Sterile water and all other reagents were used for negative controls. Positive controls consisted of spores in PBS (pH 7.4). Amplified products were visualized as previously described by Ombrouck et al. (22).

Gold standard. For evaluating the reliability of our IFAT, we selected the PCR method as the "gold standard" due to its high specificity and sensitivity compared to those of other techniques including U2B, chromotrope staining, and transmission electron microscopy (7, 24, 30).

Statistical analysis. Sensitivity, specificity, and positive and negative predictive values were calculated for each technique. The kappa statistic was used to measure the levels of agreement (concordance) between IFAT and PCR as the gold standard.

Technical considerations. Labor time, which corresponds to the approximate duration of technician labor, was compared to the total time required to obtain a result.

RESULTS

Prevalence of intestinal parasites. Of 61 HIV-seropositive patients, 8 (6 men, 2 women) were infected with microsporidia (13.1%). Only one species, *E. bieneusi*, was identified in these patients. All the children recruited in the study were negative for microsporidia. Other pathogens were identified in the group of HIV-positive patients. They included *Blastocystis hominis* (6.55%), *Pentatrichomonas hominis* (3.27%), *C. parvum* (1.63%), and *Strongyloides stercoralis* (1.63%).

Prevalence of microsporidia in HIV-seropositive patients by the various diagnostic techniques. Of the 61 HIV-positive patients presenting with diarrhea, 8 (13.1%) were found positive for microsporidia by IFAT. These eight patients were also positive by PCR. Nine patients (14.8%) were positive by U2B staining. *E. bieneusi* was identified in the eight cases detected by IFAT and PCR.

Sensitivity, specificity, and predictive values of U2B and IFAT, and concordance with PCR, for HIV-positive patients. A perfect concordance was observed between the sensitivity and specificity of IFAT and those of PCR (100%). A loss of specificity, as well as a decrease in positive predictive value (PPV), was observed for the U2B technique (specificity, 98.1%; PPV, 88.8%) due to the detection of one case that was not confirmed by IFAT or PCR. Kappa values were 0.93 and 1 for U2B and IFAT, respectively (Table 1).

Technical considerations for diagnosis of intestinal microsporidiosis. IFAT permitted an accurate diagnosis in a much shorter time than PCR: 3 h 5 min versus 8 h (Table 2).

TABLE 2. Summary of total time and labor time required by U2B, IFAT, and PCR for diagnosis of intestinal microsporidiosis

Test	Labor time[a]	Total time[b]
U2B	1 h 0 min	1 h 25 min
IFAT	1 h 2 min	3 h 5 min
PCR	1 h 21 min	8 h 0 min

[a] Approximate duration of technician labor.
[b] Time required to obtain a result.

DISCUSSION

In this study we evaluated the reliability of an IFAT using the monoclonal antibodies recently developed by our group (1, 2). The results thus obtained were compared to those of two techniques currently employed for diagnosing intestinal microsporidiosis, U2B staining and PCR.

The eight cases of microsporidiosis detected by IFAT were confirmed by PCR. In addition, both methods identified the species *E. bieneusi* in the eight cases. PCR has been shown to be the most reliable method due to its specificity and sensitivity (7). Thus, the perfect concordance between the two techniques demonstrated the reliability of IFAT. Besides, IFAT permitted an accurate diagnosis in a much shorter time than PCR (3 h 5 min versus 8 h). Furthermore, the procedure requires less-expensive reagents and consumables than those used for PCR.

Nine cases were detected by U2B staining, suggesting that this method was more sensitive than the other techniques. The high sensitivity of U2B has also been shown by other studies (5, 7). However this fluorescent stain labels the chitin, a component present in the spore walls of microsporidia and also in numerous intestinal microorganisms such as yeast, alimentary spores, and even some bacteria (7, 14). This lack of specificity suggests that the U2B-positive case found negative by both PCR and IFAT was a false positive. Thus, although U2B remains the simplest and most rapid method of diagnosis, the presence of microsporidia in specimens found positive with this staining technique must be confirmed by other methods. For this purpose the IFAT used in the present study appears to be the most suitable technique.

The results reported here confirmed that microsporidia are the most prevalent intestinal parasites in Mali. However, the prevalence rate of 13.1% is much lower than that (32.4%) reported in a previous study (17). The lower number of cases could be due to the short duration of the present study compared to the former, which was performed over a 2-year period. Only *E. bieneusi* was identified in this study, confirming that this species is a more frequent cause of diarrhea than *E. intestinalis* in Mali, as well as in other developing countries (3, 11, 27, 28). *C. parvum* was detected in only 1 of 61 adult patients (1.63%). This result confirms those of other studies showing that cryptosporidia were not the primary cause of diarrhea in HIV-positive patients in Mali (17) or Zimbabwe (11, 27). Likewise, only one patient had hookworm infection, and no cases of coinfection were observed. More generally, the results presented here tend to indicate that intestinal parasites were less frequent in Bamako in 2000 than in 1997 (17). Although determination of the stage of immunodepression (by CD4 titer) and the viral load, for reasons of cost, are not systematically carried out in Mali, it may be presumed that our patients were less immunocompromised than those in the previous study (17). All samples from the pediatric population that were examined were negative for microsporidia. Diarrhea in this population was not related to parasitic infection, since no enteropathogenic parasite was identified. Diarrhea could be related to viral, bacterial, or toxin infections, but those infections were not investigated for reasons of cost. For the same reason, HIV status was not determined for the children recruited for this study; the seroprevalence in pregnant women, estimated as 1 to 4% in Bamako (Programme National de Lutte contre le SIDA, 1997), reduces the probability of maternal-fetal transmission. Our observations corroborate the results of studies carried out with children presumed to be immunocompetent. In Niger, only 0.8% (8 of 990) of children from the age of 1 month to 6 years presented with microsporidian infections (3), and only one asymptomatic case has been reported to date in Zambia (12). The higher percentage of children with microsporidia in Tucuman (7%) could be due to the inclusion of undernourished children in both groups studied (with and without diarrhea) (25). Indeed, malnutrition has been shown to be a cause of immunosuppression in children (23). Infection by microsporidia generally occurred in immunocompetent children in the form of an asymptomatic or subsymptomatic infection consisting of a transitory diarrhea, and might pass unnoticed. A serologic survey to determine the seroprevalence of the disease and to identify exposed subjects would therefore be better adapted to this at-risk subgroup, but serologic studies are not possible at this time.

In conclusion, our data show the reliability of IFAT for the specific identification of *E. bieneusi*. This evaluation, however, needs to be extended to *E. intestinalis* in order to fully validate the method. The lower cost of IFAT compared to that of PCR is a decisive argument in the choice of a technique for routine application in developing countries.

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