Prevalence of Microsporidiosis Due to Enterocytozoon bieneusi and Encephalitozoon (Septata) intestinalis Among Patients with AIDS-Related Diarrhea: Determination by Polymerase Chain Reaction to the Microsporidian Small-Subunit rRNA Gene

Christina M. Coyle, Murray Wittner, Donald P. Kotler, Charles Noyer, Jan M. Orenstein, Herbert B. Tanowitz, and Louis M. Weiss

Microsporidia are emerging as opportunistic pathogens in patients with AIDS. Enterocytozoon bieneusi and Encephalitozoon (Septata) intestinalis have been implicated in enteric infections in AIDS patients with chronic diarrhea, a wasting syndrome, and malabsorption. We used the polymerase chain reaction (PCR) and primers that amplify the conserved regions of the small-subunit rRNA (SSU-rRNA) gene of E. bieneusi and E. intestinalis in tissue specimens from HIV-infected patients with and without diarrhea to examine the association between microsporidia and diarrhea in patients with AIDS. Tissue specimens were obtained from 68 patients with AIDS and diarrhea (mean CD4 lymphocyte count, 21/mm³) and 43 AIDS patients without diarrhea (mean CD4 lymphocyte count, 60/mm³). By means of PCR with use of the SSU-rRNA primers specific for E. bieneusi and E. intestinalis, we found that 44% of patients with diarrhea were infected with microsporidia, whereas only 2.3% of the patients without diarrhea were infected with microsporidia (P < .001). There was a clear association between the presence of microsporidia and diarrhea. In addition, the SSU-rRNA primers proved to be sensitive and specific when used in this clinical setting.

Microsporidia are obligate, intracellular, spore-forming parasites that are emerging as opportunistic pathogens in the setting of AIDS [1–3]. They are sufficiently unique to be classified in a separate phylum, the Microspora, which contains dozens of genera and hundreds of species [1, 2]. These organisms are defined by their small resistant unicellular spores that contain a nucleated sporoplasm, a coiled polar tube, and an anchoring disk [1–3].

Human disease has been associated with the following genera [1, 2]: Nosema (N. corneum has been recently renamed Vittaforma corneae [4]), generally found in insects; Pleistophora, a pathogen of fish and insects; Encephalitozoon, found in many mammals; Enterocytozoon, reported from patients with AIDS [5] and several species of fish [6]; and Septata (Septata intestinalis, which has recently been renamed Encephalitozoon intestinalis [7, 8]), isolated from patients with AIDS [9]. The genus Microsporidium has been used to designate microsporidia of uncertain taxonomic status [1].

Enterocytozoon bieneusi was first described in 1985 and has been associated with malabsorption and diarrhea in patients with AIDS [5]. The recovery of E. bieneusi from an immunocompetent patient with diarrhea has recently been described [10]. E. intestinalis causes enteric and disseminated infections in patients with AIDS [11]. It is found in enterocytes and in the cells of the lamina propria as well as in epithelial cells of the biliary tree, in tubular kidney cells, in bronchial epithelial cells, and in nasal epithelial cells [9, 11, 12].

A diarrheal and wasting syndrome has been reported as the most common manifestation of microsporidial infection in HIV-infected patients [1, 2]. However, the association between microsporidia and diarrhea has been called into question by some researchers [13]. We therefore used sensitive diagnostic techniques to examine the association between microsporidia and diarrhea in patients with AIDS.

The diagnosis of intestinal microsporidiosis has depended on detection of the parasite by light microscopy and transmission electron microscopy (TEM) [1, 2, 14, 15]. The sensitivity and specificity of these techniques is dependent on the experience of the microscopist. For patients with enteric infection, the small intestine has provided the highest diagnostic yield [16]. Serology has not proved useful in the diagnosis of human microsporidial infections [1, 17]. Taxonomic studies have shown that the small-subunit (SSU) rRNA genes of microspori-
dia share limited homology with the SSU-rRNA genes of other eukaryotic organisms; therefore, these sequences have proved useful as gene probes in hybridization and PCR assays [18–20]. With use of the conserved SSU-rRNA sequences and the known microsporidian rRNA sequence of *Vairimorpha necatrix*, we amplified and subsequently cloned and sequenced the SSU-rRNA of *E. bieneusi* and *E. intestinalis* [18–20]. These cloned genes were used to develop probes for the detection of *E. bieneusi* and *E. intestinalis* in tissue [18–20].

In the present study, we report the utility of these primers as diagnostic and epidemiological tools in the evaluation of microsporidiosis in HIV-infected patients with and without diarrhea. In addition, we examine the prevalence of microsporidia among AIDS patients with and without diarrhea.

**Materials and Methods**

**Clinical material.** One hundred eleven biopsy specimens from AIDS patients with and without diarrhea were studied by PCR for the presence of microsporidia. Multiple specimens were obtained by endoscopy from the small intestine (duodenum); these specimens were frozen at −20°C for assay by PCR, or they were fixed for examination by means of light and electron microscopy. Specimens were available from 68 patients who were seen at St. Lukes-Roosevelt Hospital Center (New York) and Bronx Municipal Hospital Center (Bronx, NY) for the clinical evaluation of chronic diarrhea. In addition, specimens were obtained from 43 patients without diarrhea who were infected with HIV-1 and who underwent endoscopy at one of the two hospitals for other reasons including weight loss, odynophagia, vomiting, abdominal pain, rectal pain, and rectal bleeding. Control tissue (i.e., that from eight non-HIV-infected patients without diarrhea) was also available for study.

All specimens were examined by light microscopy, and 95 of 111 specimens were examined by TEM, as described previously [9, 12, 19, 21]. The 16 specimens examined only by light microscopy included 12 specimens from HIV-1-infected patients without diarrhea and four specimens from HIV-1-infected patients with diarrhea. All microsporidia-positive specimens were confirmed by TEM, and the parasites were identified as either *E. bieneusi* (25 cases) or *E. intestinalis* (five cases). No unclassifiable microsporidia were identified.

**Microsporidiosis.** RK (rabbit kidney)-13 cells were maintained in minimal essential medium containing 10% fetal bovine serum, penicillin (1,000 U/mL), streptomycin (1,000 µg/mL), and fungizone (2.5 µg/mL; Gibco-BRL, Gaithersburg, MD). Cells were infected with microsporidia of the family *Encephalitozoonidae* at a multiplicity of 1:1. After 7–14 days, infection of cells was clearly visible, and spores were present in the medium. Cells were subpassaged every 10–20 days. The supernatant that contained spores was harvested and stored at 4°C. (*Encephalitozoon cuniculi* was obtained from Dr. A. Cali, Rutgers University, Newark, NJ, and *Encephalitozoon hellem* and *E. intestinalis* were obtained from Dr. E. Didier, Tulane Primate Center, New Orleans, LA).

*V. necatrix* was grown in *Heliothis zea* larvae fed an artificial diet and/or obtained from stock [22]. *Glugea stephani* was field collected in feral winter flounder and subsequently grown in vivo according to the methods of Cali et al. [23]. *Nosema locustae* was established and maintained in grasshoppers (provided by Dr. J. Henry, U.S. Dept of Agriculture, Bozeman, MO), and *Nosema bombycis* was established and maintained in silkworms. The spores were stored at 4°C when not in culture. *Pleistophora* was isolated from feral ocean pout. The organisms were purified by differential centrifugation [22]. *Ameson michaelis* (gift of Dr. E. Weidner, Louisiana State University, Baton Rouge, LA) was harvested from feral blue crabs.

**Extraction.** DNA was prepared from frozen tissue specimens by incubation overnight at 37°C in TE buffer (10 mM Tris [pH 7.4] and 0.1 mM EDTA) containing 1% SDS and proteinase K (20 µg/mL) followed by phenol-chloroform extraction and ethanol precipitation. DNA prepared by ethanol precipitation was resuspended in TE buffer for use in the PCR assay. DNA was prepared from microsporidian spores by glass bead (425–600 µm; Sigma, St. Louis) spore disruption for 2 minutes with a mini bead beater (BiorSpec Products, Bartlesville, OK) in TE buffer, followed by centrifugation for 5 minutes at 13,500g and then phenol-chloroform extraction and ethanol precipitation of DNA from the supernatant. DNA prepared by ethanol precipitation was resuspended in TE buffer for use in PCR. All sample preparations for PCR assays were prepared with either aerosol-guard pipette tips or positive-displacement tips to prevent contamination. Control reactions containing no DNA were run for all PCR assays.

**PCR.** PCR was carried out with use of standard buffer and conditions (per Perkin Elmer Cetus, Norwalk, CT: pH 8.3; 10 mM Tris HCl, 50 mM KCl; 1.5 mM MgCl2; 0.01% [wt/vol] gelatin, with 8 mM dNTP, 5 units of Taq DNA polymerase, and 100 picomoles of each primer). A total of 40 cycles were run, with a cycle at 94°C for 1 minute, an annealing temperature of 48°C or 58°C for 2 minutes, and 72°C for 3 minutes. A 10-minute 72°C extension was used following the 35 cycles. For the amplification, 0.1–1 µg of sample DNA was used. The annealing temperature was changed to optimize the reaction for the different primer pairs. All samples were run in triplicate, each repeat being done on a separate day.

On the basis of the SSU-rRNA sequence of *E. bieneusi*, the primer pair V1:5′CACCAAGTTTATTCTCGCTGA3′ and EB450 5′ACTCAGGTGTTATACTCACGTC3′ (primer 450 is antisense and is located at position 450 based on the alignment of *E. bieneusi* with *Escherichia coli*) gave consistent amplification from infected tissue and a plasmid containing *E. bieneusi* SSU-rRNA [18, 19]. PCR was performed under the above conditions with an annealing temperature of 48°C. A 353-bp fragment was amplified from samples containing *E. bieneusi* with use of these primers. The internal 30-mer oligonucleotide EB150:5′TGGTCCCCTATTTGTCTCTGTGTGAAA3′
was used to confirm by Southern blotting that the amplified fragment was from *E. bieneusi* [18].

On the basis of the obtained SSU-rRNA sequence of *E. intestinalis*, the primer pair V1 and S1500 5′CTCGCTCCT-TTACACTCGAA3′ (primer 500 is antisense and is located at position 500 based on the alignment of *E. intestinalis* with *Escherichia coli*) gave consistent amplification from infected tissue and DNA from spores with use of an annealing temperature of 58°C. The primer pair V1 and S1500 amplified a 375-bp fragment [18]. The internal primer S160: 5′TGTTGATGACCTTGTGG3′ was used to confirm by Southern blotting that the amplified fragment was from *E. intestinalis* [18].

**Southern blotting.** After agarose gel electrophoresis was performed, the separated amplified fragments were transferred to a Nytran (Schleicher and Schuell, Keene, NH) membrane by capillary action. The membrane was air dried and placed in a vacuum oven at 80°C. The membrane was then prehybridized for 15 minutes at 62°C with QuikHyb buffer (Stratagene, La Jolla, CA). EB150 was labeled with (γ-32P)ATP and T4 polynucleotide kinase to 10⁶ cpm/pmol, added at 1.5 × 10⁶ cpm/mL to the QuikHyb buffer; incubation was continued for 2 hours at 62°C. The blot was then washed at 65°C for 1 hour in 2 × standard saline citrate (SSC) (300 mM NaCl and 30 mM sodium citrate [pH 7.0]) and 0.5% SDS, washed for 1 hour at 65°C in 0.1 × SSC (15 mM NaCl and 1.5 mM Na₃ citrate [pH 7.0]) and 0.2% SDS, and then autoradiographed overnight at −70°C with an intensifier screen.

For *E. intestinalis*, the membrane was hybridized for 15 minutes at 42°C with QuikHyb buffer. SI60 was labeled with (γ-32P)ATP and T4 polynucleotide kinase to 10⁶ cpm/pmol, added at 1.5 × 10⁶ cpm/mL to the QuikHyb buffer; incubation was continued for 2 hours at 42°C. The blot was then washed twice at room temperature for 15 minutes and once at 42°C in 6 × SSC/0.1% SDS. The membrane was autoradiographed at −70°C overnight with an intensifier screen.

The data were organized in 2 × 2 tables and analyzed with Fisher’s exact test (with Yates’ correction) and the χ² test.

**Results.**

Amplification with the primers V1 and EB450 produced a 353-bp DNA fragment on ethidium bromide–stained gels with control DNA from a plasmid with the SSU-rRNA sequence of *E. bieneusi* or from a biopsy specimen known to contain *E. bieneusi* based on TEM results. The primer set V1::EB450 was specific for *E. bieneusi*. The primer set did not amplify DNA from *E. intestinalis*, *V. necatrix*, *E. hellem*, *E. cuniculi*, *N. locustae*, *N. bombycis*, *G. stephani*, *Pleistophora*, *Sacccharomyces cerevisiae*, or *E. coli*. In addition, no amplification was seen in biopsy specimens containing *Mycobacterium avium* complex, *G. lamblia*, Kaposi’s sarcoma cells, lymphoma cells, or cytomegalovirus. Zhu et al. [19] previously reported that V1::EB450 could amplify DNA from *E. hellem*; however, the SSU-rRNA sequence of *E. hellem* (Genbank accession L39108 [8]) does not suggest that this would occur, and we did not demonstrate amplification from DNA prepared from *E. hellem*. It is likely the amplification observed by Zhu et al. was due either to contamination of the original *E. hellem* culture with spores of *E. bieneusi* (which were being used in the same incubator) or to contamination of the culture with a plasmid containing the cloned *E. bieneusi* SSU-rRNA gene.

The 353-bp fragment could be amplified from 25 of the biopsy specimens from the 68 HIV-infected patients (table 1). This amplification product was confirmed with use of Southern blotting. On TEM, we found *E. bieneusi* in 24 of the 25 PCR-positive samples. Examination of one biopsy specimen did not reveal microsporidia. This specimen was positive by Southern blotting, a finding that suggests the presence of a low number of microsporidia. The positive reaction was unlikely due to contamination, as repeated independent samples were persistently positive. It is of interest that the patient from whom this specimen was obtained also had cryptosporidiosis and that abundant cryptosporidia were present in the biopsy specimen.

Because other specimens from patients with cryptosporidiosis did not amplify, we doubt this was a cross-reaction between the primers and the SSU-rRNA gene of *Cryptosporidium parvum*. Of the specimens obtained from the 43 HIV-infected patients without diarrhea, one amplified with these primers; light microscopy did not reveal any microsporidia in this specimen. During follow-up the patient from whom this specimen was obtained developed biliary tract disease requiring cholecystectomy. Examination of the gallbladder by means of light microscopy and TEM demonstrated *E. bieneusi*. These data, summarized in table 1, demonstrate a significant association between diarrhea and the presence of *E. bieneusi*, as determined by PCR (RR = 16.6; *P* < .001).

Amplification with the primers V1 and S1500 produced a 375-bp DNA fragment on ethidium bromide–stained gels with control DNA obtained from *E. intestinalis* spores. This DNA fragment could be amplified from five of the 68 HIV-infected patients’ specimens (table 2). Review of these five specimens by TEM confirmed the presence of *E. intestinalis*; these specimens amplified with the primers specific to *E. intestinalis*. There was no amplification in specimens obtained from the 43 patients without diarrhea, which was confirmed on microscopic examination. These data, while not statistically significant because of the small number of cases, suggest that *E. intestinalis*,

<table>
<thead>
<tr>
<th>Patient group</th>
<th>No. (%) of patients with positive PCR result</th>
<th>95% CI*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diarrhea (n = 68)</td>
<td>25 (36.8)</td>
<td>25.3-49.3</td>
</tr>
<tr>
<td>Asymptomatic (n = 43)</td>
<td>1 (2.3)</td>
<td>0-12</td>
</tr>
</tbody>
</table>

* RR = 16.6; *P* < .001.
Discussion

We examined tissue specimens from patients with HIV infection and diarrhea. Amplification of a 353-bp DNA fragment was achieved with \textit{E. bieneusi}–specific primers in 25 of these specimens, and amplification of a 375-bp DNA fragment was achieved with \textit{E. intestinalis}–specific primers in five. One tissue specimen from a patient with diarrhea was positive on Southern blotting with the \textit{E. bieneusi} primers, but no microsporidia–specific band was visualized on ethidium-bromide staining. One of the specimens from a patient with HIV infection who did not have diarrhea, demonstrated amplification with the \textit{E. bieneusi}–specific primers. The patient from whom this specimen was obtained absorbed D-xylose at an extremely low level, which could not be explained. While TEM initially failed to demonstrate microsporidia in this patient, examination of the patient’s gallbladder confirmed the diagnosis of microsporidiosis, which was suggested by the results of PCR.

Review of the TEM findings for 31 tissue specimens revealed that microsporidia were found in 30 specimens that amplified with either primer set. No cross-reactions were demonstrated with these primer sets and other microsporidia, common opportunistic pathogens, or normal bowel organisms. Recently, Franzen et al. [20] confirmed the utility and specificity of primer set V1:EB450 for demonstrating \textit{E. bieneusi} in duodenal tissue samples. These observations indicate that our primers appear to be specific and highly sensitive when used in the clinical setting, and they should prove particularly useful for epidemiological studies of microsporidia.

There have been several studies on the association of microsporidiosis and diarrhea. In several studies of patients with HIV infection and diarrhea, the prevalence of microsporidiosis ranged from 10% to 40% [2, 21, 25–30]. Among our patients, the prevalence of microsporidia was 44.1% (the prevalence of \textit{E. bieneusi} was 36.8%, and that of \textit{E. intestinalis} was 7.3%). Many of our patients with diarrhea were seen initially at a referral center for AIDS-related diarrhea; therefore, before they were referred to our center, many of these patients with other causes of diarrhea had been excluded from our study population. The higher prevalence of microsporidia that we found probably reflects the select population studied.

In a prospective study, Rabeneck et al. [13] found microsporidia in 29% of patients seen at an HIV primary care clinic; however, this finding was not associated with the presence of diarrhea (18 of 55 patients with diarrhea and 13 of 51 patients without diarrhea had microsporidia detected in duodenal biopsy specimens). The mean CD4 lymphocyte count among patients with microsporidiosis in their study was 113/mm$^3$ [13]. During the 15 months that these patients were followed up, two of the 13 asymptomatic patients developed diarrhea [31]. Infection with microsporidia was demonstrated by microscopy in both of these studies.

Our data, based on both the results of PCR and TEM, do not support such a high carriage rate in patients without diarrhea. In our study, the 95% confidence interval for the prevalence of microsporidiosis in AIDS patients without diarrhea was 0–12%, a range that was significantly different from that observed by Rabeneck et al. [13].

In a previous study of patients presenting to a gastroenterology clinic, Kotler and Orenstein [21] found that 39% of patients with HIV infection and diarrhea had microsporidiosis and that the presence of microsporidia was associated with wasting, a mean CD4 lymphocyte count of 28/mm$^3$, and abnormal absorption of D-xylose [21]. In contrast, only one (2.6%)
of their HIV-infected patients without diarrhea had microsporidiosis, and this patient subsequently developed diarrhea. The findings in our study also support the concept that microsporidiosis is associated with diarrhea. We found that 44% of patients with diarrhea were infected with microsporidia and that only 2.3% of the asymptomatic patients were infected with these parasites. Thus, there was a clear association between the presence of microsporidia in biopsy specimens and the presence of diarrhea.

Microsporidia are clearly emerging as important pathogens in the immunocompromised host and may be important pathogens in immunocompetent hosts as well [10]. The epidemiology of microsporidiosis has not yet been determined. With use of primers to the SSU-rRNA genes of microsporidia, we have demonstrated an association between microsporidiosis and diarrhea in patients with AIDS. By using these primers to analyze stool and environmental samples, it may be feasible to further elucidate the epidemiology of microsporidiosis.

References