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Detection of Microsporidia (*Enterocytozoon bieneusi*) in Intestinal Biopsy Specimens from Human Immunodeficiency Virus-Infected Patients by PCR

CASPAR FRANZEN,* ANDREAS MÜLLER, PETRA HEGENER, BERND SALZBERGER, PIA HARTMANN, GERD FÄTKENHEUER, VOLKER DIEHL, AND MATTHIAS SCHRAPPE

Department of Internal Medicine I, University of Cologne, D-50924 Cologne, Germany

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Intestinal microsporidiosis has been implicated as a major cause of chronic diarrhea in human immunodeficiency virus (HIV)-infected patients. So far diagnosis depends on direct visualization of the parasites by light and transmission electron microscopy. We evaluated the diagnostic value of microsporidian DNA amplification by PCR on duodenal biopsy specimens obtained from patients with and without intestinal microsporidiosis caused by *Enterocytozoon bieneusi*. Thirteen HIV-infected patients (all CDC stage C3) were studied. Eight patients had intestinal microsporidiosis caused by *E. bieneusi* ($n = 6$), *Septata intestinalis* ($n = 1$), and *Encephalitozoon cuniculi* ($n = 1$); microsporidioses were diagnosed by light microscopy of stool samples and confirmed by light and electron microscopy of intestinal biopsy specimens. Five patients had no microsporidia in their stool samples or in their intestinal biopsy specimens, as examined by light and electron microscopy. Additionally, DNA prepared from *Toxoplasma gondii* derived from mouse ascites was used as a further control. A 353-bp DNA fragment of the small-subunit rRNA gene could be amplified from all six biopsy specimens infected with *E. bieneusi*, and the nature of the PCR products was confirmed by Southern blot hybridization. No amplification of DNA fragments was seen by using DNA extracted from biopsy specimens with *S. intestinalis* or *E. cuniculi* infection or without microsporidian infection and with template DNA extracted from *T. gondii*. The results suggest that PCR testing of intestinal biopsy specimens may be a useful approach to diagnosing microsporidiosis in HIV-infected patients.

Microsporidia are obligate intracellular protozoan parasites which infect a broad range of vertebrates and invertebrates. They are increasingly recognized as human pathogens, especially in human immunodeficiency virus (HIV)-infected patients (1, 7, 8). Intestinal microsporidiosis has been implicated as a major cause of chronic diarrhea in HIV-infected patients, and so far diagnosis depends on detection of the parasites by light and transmission electron microscopy (1, 8, 9, 12). These standard methods may lack sensitivity, and species differentiation, which requires ultrastructural analysis of spores and tissue stages, can be missed (17). Mono- and polyclonal antisera are not yet available outside research laboratories, in part because the most important microsporidian species, *Enterocytozoon bieneusi*, could not be propagated in cell culture systems (10, 13). The different species of microsporidia which infect humans show variable responses to therapy, and species differentiation is also important for epidemiological research (2, 3, 6).

Taxonomic studies have shown that the small-subunit (SSU) rRNA genes of microsporidia shared only limited homology with the SSU rRNA genes of other eukaryotic organisms, so that these sequences may be useful as gene probes in hybridization and PCR assays (4, 5, 14–16, 18–22).

We therefore evaluated the diagnostic value of amplification of microsporidian DNA by PCR with template DNA extracted from duodenal biopsy specimens from HIV-infected patients with and without intestinal microsporidiosis.

* Corresponding author. Mailing address: Department of Internal Medicine I, University of Cologne, Joseph-Stelzmann Str. 9, D-50924 Cologne, Germany. Phone: 49-(0)221-478-4433. Fax: ++49-(0)221-478-6456.

MATERIALS AND METHODS

Thirteen HIV-infected patients (all CDC stage C3) were studied. Eight patients had intestinal microsporidiosis caused by *E. bieneusi* ($n = 6$), *Septata intestinalis* ($n = 1$), and *Encephalitozoon cuniculi* ($n = 1$). Infections were diagnosed by stool examinations with a fluorescent stain with Uvitex 2B (11, 12) and were confirmed by transmission electron microscopy (1–3). For the remaining five patients no microsporidia were found in their stool samples or intestinal biopsy specimens by transmission electron microscopy (Table 1). Additionally, *Toxoplasma gondii* derived from mouse ascites was used as a further control.

At least three biopsy specimens were obtained from the distal duodenum of each patient by flexible fiberglass endoscopy. One biopsy specimen was used for routine histology, and one was processed for transmission electron microscopy as described previously (1). The third biopsy specimen was embedded in O.C.T. compound Tissue-Tek (Miles Inc., Elkhart, Ind.), snap frozen in liquid nitrogen, and stored at −80°C until processing.

DNA was prepared from frozen tissue by using the QIAamp Tissue Kit (Qiagen, Hilden, Germany). Biopsy specimens were incubated in digestion buffer with 400 μg of proteinase K at 55°C for 2 h, and DNA was prepared by using QIAamp spin columns (Qiagen) in an Eppendorf microcentrifuge.

Amplification was done in 50-μl reaction mixtures under the following conditions: 25 pmol of each primer, 200 μM (each) deoxynucleoside triphosphate, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer, Norwalk, Conn.). A total of 0.1 to 1.0 μg of genomic DNA and 40 μl of mineral oil was used. The reactions were run in a Perkin-Elmer thermocycler by using a step cycle program. After initial denaturation of the DNA at 94°C for 3 min, 35 cycles were run, as follows: 94°C for 1 min, 48°C for 2 min, and 72°C for 3 min, with a 10-min extension at 72°C after the 35 cycles (22).

The primers V1 (5′-CACCAGGTTGATTCTGCCTGAC-3′) and EB450 (5′-ACTCAGGTGTTATACTCACGTC-3′) described by Zhu et al. (22) were used to amplify a 353-bp DNA fragment of the SSU rRNA gene of *E. bieneusi* (22). A 10-μl aliquot from each reaction mixture was run on a 3% NuSieve 3:1 electrophoresis-grade agarose gel (FMC Corp., Philadelphia, Pa.) in 1× TAE buffer (0.04 M Tris, 0.001 M EDTA) with ethidium bromide (0.5 mg/ml) to visualize the amplified PCR products under UV illumination.

After gel electrophoresis the PCR products were denaturated and neutralized by soaking the gel in denaturation buffer (0.4 N NaOH, 0.6 M NaCl) and neutralization buffer (1.5 M NaCl, 0.5 M Tris [pH 7.5]), respectively, for 30 min. The separated amplified DNA fragments were transferred to positively charged

TABLE 1. Intestinal pathogens and method of diagnosis of the 13 HIV-infected patients studied

Patient no.	Intestinal pathogen	Diagnostic method[a]	PCR result
1	*E. bieneusi*	EM, LM	+
2	*E. bieneusi*	EM, LM	+
3	*E. bieneusi*	EM, LM	+
4	*E. bieneusi*	EM, LM	+
5	*E. bieneusi*	EM, LM	+
6	*E. bieneusi*	EM, LM	+
7	*E. cuniculi*	EM, LM, antibody staining	−
8	*S. intestinalis*	EM, LM	−
9	*Cryptosporidium* sp.	EM, LM	−
10	*G. lamblia*	LM	−
11	*M. avium*	LM, culture	−
12	*C. albicans*	EM, culture	−
13	No pathogen		−

[a] EM, electron microscopy; LM, light microscopy.

nylon membranes (Hybond-N$^+$; Amersham, Buckinghamshire, United Kingdom) by using a vacuum blotter (Appligene, Illkirch, France) at 55 mbar for 1 h and in 20× SSPE (3 M NaCl, 0.2 M $NaH_2PO_4 \cdot H_2O$, 1 M EDTA) and were coupled to the membranes by using a UV cross-linker (Appligene) with 1.2 J/qcm. Membranes were washed in 2× SSC (300 mM NaCl, 30 mM trisodium citrate) and were prehybridized for 1 h at 55°C with hybridization buffer (Amersham). An internal 30-mer oligonucleotide EB150 (5′-TGTTGCGGTAATT TGGTCTCTGTGTGTAAA-3′) was 3′ labeled with fluorescein-11-dUTP by using terminal deoxynucleotidyl transferase (Amersham), and 15 pmol of the labeled probe was added to the hybridization buffer. Hybridization was carried out overnight at 55°C. The blot was then washed twice in 2× SSC with 0.1% sodium dodecyl sulfate (SDS), two times in 1× SSC with 0.1% SDS, and in 0.1× SSC with 0.1% SDS at 57°C.

Bound fluorescein-11-dUTP-labelled oligonucleotides were visualized by using an enhanced chemiluminescence detection kit (Amersham). Blots were washed in buffer 1 (0.15 M NaCl, 0.1 M Tris [pH 7.5]), blocked with buffer 1 containing 0.5% blocking agent (Amersham), incubated with 1:1,000-diluted anti-fluorescein-horseradish peroxidase conjugate in buffer 2 (0.4 M NaCl, 0.1 M Tris, 0.5% bovine serum albumin [pH 7.5]) for 30 min, washed thoroughly in buffer 2, and then incubated with detection solution containing luminol for 1 min. When the probe bound there was peroxidase-catalyzed oxidation of luminol to 3-aminophthalate and subsequent enhanced chemiluminescence (428 nm), which was autoradiographed on Hyperfilm ECL (Amersham) for 1 h.

RESULTS

Amplification with the two primers V1 and EB450 produced a 353-bp DNA fragment on ethidium bromide-stained gels. This 353-bp DNA fragment could be amplified from all six biopsy specimens obtained from HIV-infected patients with electron microscopically confirmed intestinal *E. bieneusi* infection. No amplification of DNA fragments was seen with DNA extracted from intestinal biopsy specimens obtained from patients without intestinal microsporidiosis. The two primers did not amplify DNA from tissues infected with other microsporidia (*S. intestinalis* and *E. cuniculi*) or from tissues infected with other parasites like *Cryptosporidium* sp. or *Giardia lamblia* or other pathogens like *Mycobacterium avium* and *Candida albicans*. Also, no amplification of DNA was seen with template DNA prepared from *T. gondii* (Fig. 1).

The nature of the PCR products was confirmed by Southern blot hybridization with the internal probe EB150. The probe hybridized with all 353-bp fragments amplified from *E. bieneusi*-infected tissue, indicating that the amplified 353-bp fragments were derived from the SSU rRNA gene of *E. bieneusi*. By using the internal oligomer hybridization with EB150, no signal could be obtained from Southern blots of the PCR products from tissues from *S. intestinalis* and *E. cuniculi* infection, from tissues not infected with microsporidia but with other pathogens, or from *T. gondii* template DNA.

DISCUSSION

After the description of the SSU rRNA gene of *E. bieneusi* the sequence data for this gene were used by Zhu et al. (21, 22) to define a primer pair for the use in PCR to amplify DNA fragments of the SSU rRNA gene. The foreward primer V1 is

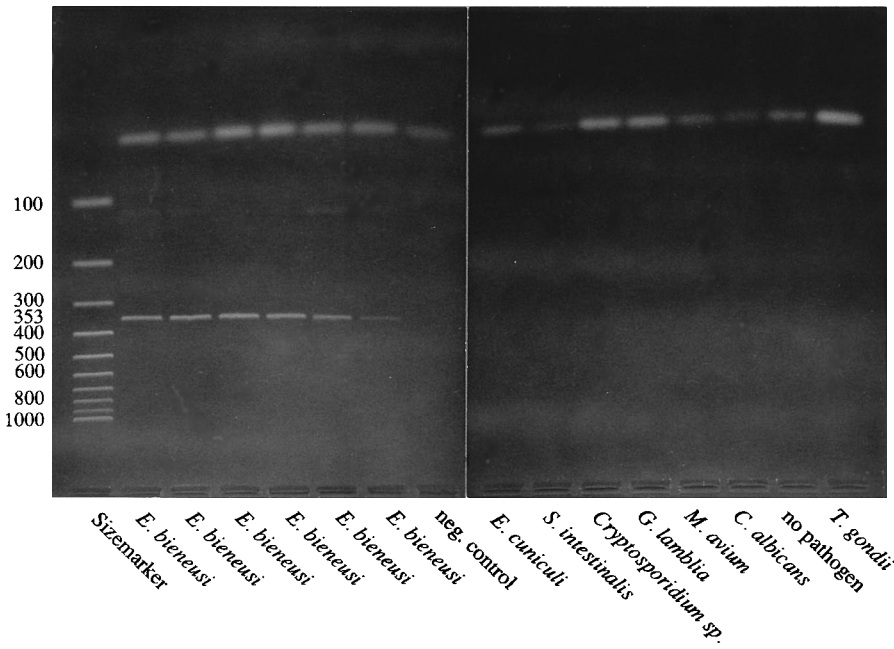

FIG. 1. Amplification of a 353-bp DNA fragment of the SSU rRNA gene of *E. bieneusi*. Molecular size markers (in bases), DNAs prepared from intestinal biopsy specimens with *E. bieneusi* infection, negative (neg.) control, DNAs prepared from intestinal biopsy specimens without *E. bieneusi* infection but infection with other organisms, as indicated, and DNAs prepared from *T. gondii* are shown.

based on the conserved sequence of the SSU rRNA gene at the 5′ end, and the reverse primer EB450 is located at position 450 of the SSU rRNA gene on the basis of the alignment of *E. bieneusi* and *Escherichia coli* (14, 21, 22). The primer pair reliably amplifies a 353-bp DNA fragment of the SSU rRNA gene from all tissues infected with *E. bieneusi* but not from uninfected control tissues. Hybridization with the internal probe EB150, which is located at position 150 of the SSU rRNA gene of *E. bieneusi* on the basis of the alignment of *E. bieneusi* and *E. coli* (21, 22), verifies that the amplified PCR product is really derived from the SSU rRNA gene of *E. bieneusi*. Although the foreward primer V1 is not specific for *E. bieneusi*, Zuh and colleagues (18, 22) found that the primer pair did not amplify DNAs of most other microsporidian species except of *Encephalitozoon hellem*. We tested tissues infected with *E. cuniculi* and *S. intestinalis* and with other parasites and bacteria such as *Cryptosporidium* sp., *G. lamblia*, *C. albicans*, and *M. avium* and DNA isolated from *T. gondii*, and no amplification was seen. This suggests that the primer pair V1 and EB450 only amplifies DNA from the microsporidian species *E. bieneusi*.

The described patient population was very well defined. For the diagnosis of intestinal microsporidiosis three different diagnostic methods apart from PCR were used (stool examinations, histology, and transmission electron microscopy of intestinal biopsy specimens). Although the specimen numbers presented here are small, this is the first study to show the conformity of DNA amplification by PCR and standard methods for the diagnosis of intestinal microsporidiosis. In the only previous studies (18, 22), no transmission electron microscopy data were available for all patients.

The results of the present study demonstrate the presence of microsporidian DNA in intestinal biopsy specimens from patients infected with HIV and suggest that PCR testing of intestinal biopsy specimens may be a useful approach for the diagnosis of intestinal microsporidiosis in HIV-infected patients. Differentiation of species of microsporidia is important because some species (*S. intestinalis*, *Encephalitozoon* spp.) respond very well to albendazole therapy, whereas for other species like *E. bieneusi*, no convincing therapy is available (1–3, 6). DNA amplification with the described primer pair was species specific for *E. bieneusi* and other primer pairs, and hybridization probes may be useful for differentiating between species of microsporidia (13).

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