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Detection of the Microsporidian Parasite *Enterocytozoon bieneusi* in Specimens from Patients with AIDS by PCR

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Microsporidia are protozoa parasites responsible for significant gastrointestinal disease in patients infected with human immunodeficiency virus. We evaluated a PCR assay of stool samples, duodenal aspirates, and biopsy specimens from patients with *Enterocytozoon bieneusi* infection. A 210-bp DNA fragment of the unique rRNA intergenic spacer could be amplified from all samples infected with *E. bieneusi*, but no amplification was seen by using DNA purified from samples with *Septata intestinalis* or other parasites and from negative control human cells. These results suggest that the PCR in stool samples may be a useful tool for the diagnosis of intestinal microsporidiosis in patients with AIDS.

Members of the phylum Microspora are primitive eukaryotic protozoa that lack mitochondria (5, 19, 21). Microspora, obligate intracellular parasites, are known to infect invertebrates and vertebrates (5). The genera within this phylum which have been recognized in humans include *Encephalitozoon*, *Nosema*, *Pleistophora*, *Enterocytozoon*, and *Septata* (2, 4). *Enterocytozoon bieneusi* has been associated with persistent diarrhea, cholangitis, and acalculous cholecystitis in human immunodeficiency virus-infected patients (7, 12, 14, 16). Recently, another microsporidian species, *Septata intestinalis*, gen. nov., sp. nov., has been identified as the causal agent of intestinal and extraintestinal infections in patients with AIDS (4).

The standard methods of diagnosis depend on detection of the parasites in fluids and feces by light and transmission electron microscopy (6, 16, 22). It is possible to identify species by ultrastructural analysis of spores and tissue stages in transmission electron micrographs (3, 18). Accurate identification and differentiation of microsporidian species by novel diagnostic techniques is vital for treatment assessment and prognosis and to reduce dissemination risks (11, 13, 14).

The small subunit rRNA genes of microsporidia have been used as probes in hybridization and PCR assays (8, 9, 20, 24).

The purpose of this report is to evaluate a PCR technique that amplifies the intergenic spacer sequence of the rRNA genes for diagnosis of microsporidiosis caused by *E. bieneusi* in stool samples, duodenal aspirates, and biopsy specimens.

The unique rRNA intergenic spacer sequence of *E. bieneusi* available from the GenBank database (accession number L20290) was employed to design species-specific primers that amplify a 210-bp DNA fragment. The forward primer named Eb.gc (5′-TCAGTTTTGGGTGTGGTATCGG-3′) and the reverse primer named Eb.gt (5′-GCTACCCATACACACAT CATTC-3′) were based on nucleotides 1 to 22 and 189 to 210, respectively, of the above-mentioned sequence. The amplification procedure included 30 s of denaturation at 98°C in the first cycle (94°C afterwards), followed by 30 s of annealing at 49°C and 90 s of extension at 72°C for 31 cycles. All PCRs were carried out using a Gene Ataq thermocycler (Pharmacia, Uppsala, Sweden) in a total volume of 100 μl. The PCR mixture consisted of 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 9.0), 50 μM (each) deoxyribonucleotide triphosphate, 0.2 μM (each) primer, and 2.0 U of *Taq* DNA polymerase (Pharmacia, Uppsala, Sweden).

Stool samples, duodenal aspirates, and intestinal biopsy specimens were obtained from 10 human immunodeficiency virus-infected patients, all CDC stage C4, with chronic diarrhea. All stool and duodenal aspirate samples were examined for ova and parasites by standard techniques (10). Microsporidia infections were diagnosed by using duodenal aspirate in 5% formaldehyde saline solution sediment and uncentrifuged stool suspensions (22) stained by the trichrome-blue technique (17). Duodenal biopsy specimens were stained with hematoxylin-eosin, Giemsa, and methylene blue Azur II and processed for transmission electron microscopy (3, 6, 16). Six of 10 patients had intestinal microsporidiosis caused by *E. bieneusi* ($n = 2$) and *S. intestinalis* ($n = 4$). Three patients had other intestinal parasites: *Isospora belli* ($n = 1$), *Cryptosporidium* sp. ($n = 1$), and *Giardia lamblia* ($n = 1$). No pathogens were found in samples from one patient.

DNA was purified from the samples as follows. Two milliliters of stool sample in formaldehyde saline solution was passed successively through 16-, 21-, and 25-guage needles and concentrated by centrifugation at 18,000 × *g* during 10 min. Aliquots of 200 μl of duodenal aspirates and biopsy specimens of about 10 mm^3 were used for DNA extraction. All samples were incubated for 2 h at 56°C in 200 μl of lysis buffer containing 100 mM Tris-HCl (pH 8.0), 100 mM EDTA (pH 8.0), 2% sodium dodecyl sulfate, 150 mM NaCl, and 200 μg of proteinase K per ml. After digestion, DNA was purified by ethanol-sodium acetate precipitation after phenol-chloroform extraction.

The 210-bp DNA fragment could be amplified from samples obtained from two patients with microsporidiosis caused by *E. bieneusi* that had been confirmed by electron microscopy (Fig. 1). This PCR product was seen with DNA extracted from biopsy specimens, feces, and duodenal aspirates. No amplification fragment was obtained with control negative DNA from human white blood cells (Fig. 2). When DNA template was purified from the three kinds of samples from four patients infected with the other Microsporidia, *S. intestinalis*, and one patient without pathogens, no amplification product was de-

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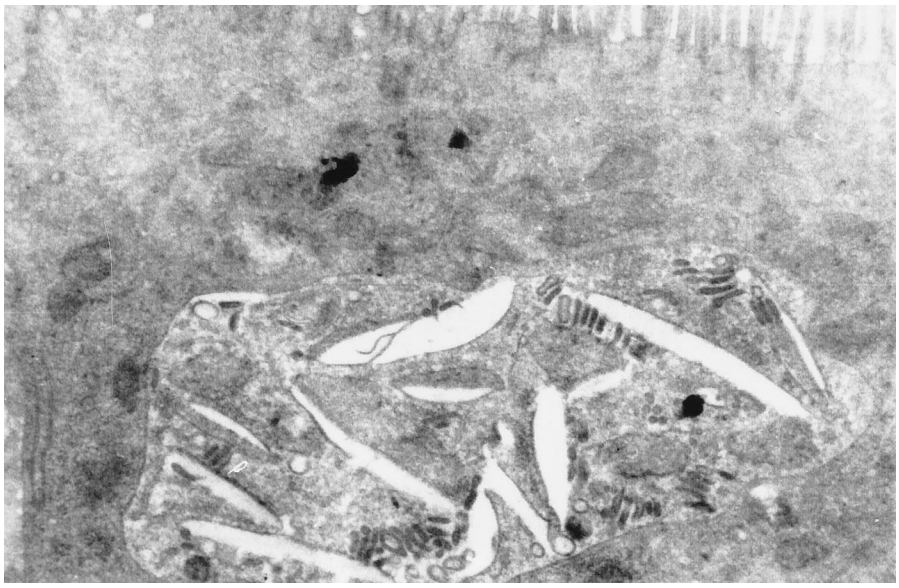

FIG. 1. Transmission electron micrograph showing duodenal epithelium from one of the patients parasitized with *E. bieneusi* in the sporogonial plasmodial stage with advanced polar tube formation. Fused electron-dense disks are seen in coiled, stacked, and cross-sectional profiles throughout the cytoplasm. The electron-lucent inclusions are seen in elongated and cross-sectional views. Anterior anchoring disks and associated polaroplast membranes are present at this stage.

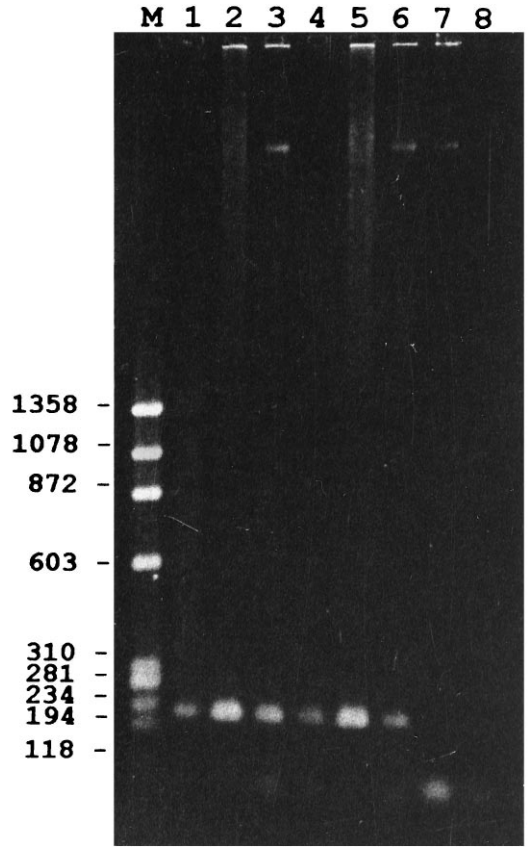

FIG. 2. Agarose gel electrophoresis of PCR-amplified products. Lanes 1 to 6, DNA prepared from patients S and R with *E. bieneusi* infection. Lanes: 1, patient S stool sample; 2, patient S duodenal aspirate; 3, patient S biopsy specimens; 4, patient R feces; 5, patient R duodenal aspirate; 6, patient R biopsy specimens; 7, negative control; 8, reaction mixture. Molecular size markers (II X 174 RF/*Hae*III fragments) are shown on the left.

tected on agarose gels after PCR assay. The same negative results were obtained with DNA template from samples with other parasites like *Cryptosporidium* sp., *I. belli*, and *G. lamblia.*

Although the number of cases studied here is small, this is the first report that describes the use of the PCR technique with a novel primer pair from stool samples and duodenal aspirates instead of biopsy specimens. The results of the present study demonstrate the amplification of a *E. bieneusi* DNA fragment in feces with a length identical to that of the PCR product from duodenal aspirates and tissues. The main advantages of employing the PCR procedure in feces are the differentiation of species of Microsporidia which is helpful in instituting therapy (1, 23), because dissemination risks and treatment response are not identical for *S. intestinalis* and *E. bieneusi*; the use of a noninvasive method for sample collection; and the simple and common storage conditions, allowing the use of the same stool sample for routine parasitological examination and PCR protocol. These technical characteristics suggest that the PCR in feces may be a very useful approach for the diagnosis of intestinal microsporidiosis in patients with AIDS.

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