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Development and Application of Genetic Probes for Detection of *Enterocytozoon bieneusi* in Formalin-Fixed Stools and in Intestinal Biopsy Specimens from Infected Patients

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The microsporidium *Enterocytozoon bieneusi* is closely linked to wasting and diarrhea in a high proportion of individuals with AIDS. However, its relative contribution to disease is uncertain because diagnosis until recently depended on procedures involving endoscopy. A sensitive PCR technique which amplifies a fragment of the small-subunit rRNA gene of *E. bieneusi* from formalin-fixed stool samples was developed. Of 80 formalin-fixed stool samples collected from 74 Zimbabweans and 6 U.S. patients who were human immunodeficiency virus positive, 50% tested positive for *E. bieneusi* by PCR, whereas 24% tested positive for *E. bieneusi* by light microscopy of trichrome-stained fecal smears. In addition, we describe an in situ hybridization technique which detected and identified *E. bieneusi* as the causative agent in all six intestinal biopsy specimens tested. Both the PCR and in situ hybridization procedures are sensitive diagnostic tools which will complement currently available techniques and enable the differentiation of *E. bieneusi* from other microsporidia to be made.

Microsporidia are obligate intracellular parasites capable of infecting many invertebrate and vertebrate hosts and a variety of cell types. The first case of human microsporidiosis was reported in 1959 (13). However, not until 1985, with the emergence of AIDS, was microsporidiosis recognized as a significant cause of morbidity in the AIDS population (5, 11, 14–16, 21, 23). Five genera of microsporidia are currently recognized in association with AIDS, namely, *Encephalitozoon*, *Enterocytozoon*, *Septata*, *Pleistophora*, and *Trachipleistophora* (1, 9), of which *Enterocytozoon bieneusi* is the most frequently encountered and hence probably the most significant. *E. bieneusi* infection in the immunodeficient host appears to be closely linked with chronic diarrhea and wasting (11, 15, 16). Unlike other microsporidia, infection appears to be primarily confined to the intestinal epithelium, in which organisms proliferate. Infection presumably leads to cell death with little or no inflammation of the surrounding mucosa. There have also been occasional reports of *E. bieneusi* infection of the gallbladder and biliary tree (17). Because of a lack of suitable models and appropriate diagnostic reagents, the diagnosis of *E. bieneusi* has depended in large part on the identification of parasite forms in duodenal aspirates and in intestinal biopsy specimens (5, 14, 20). Examination of intestinal biopsy specimens or aspirates by light or electron microscopy (6, 20), which normally yields good results, requires the use of an invasive procedure and is very expensive. However, by light microscopy it is not possible to identify the type of microsporidia, and electron microscopy is lengthy, requiring sophisticated equipment for diagnosis. Recently, staining techniques with either trichrome chromatrope 2R or fluorescent chitin stains were described. These techniques allowed for the identification of microsporidium spores in stools (3, 18). Identification of spores in stool smears is, however, not very sensitive, and these stains cannot distinguish *E. bieneusi* from other microsporidia.

A sensitive PCR technique for detecting *E. bieneusi* in duodenal aspirates (4, 22), intestinal biopsy specimens (8), and fresh stools (7) was recently described. We sought to develop a PCR method that can be applied to formalin-fixed stools, which are safer to handle and which can readily be transported or stored for longer periods. In addition, we describe an in situ hybridization technique which can be used to detect even low-grade infections with *E. bieneusi* from paraffin-embedded tissue. Both of these techniques are sensitive, should enable a more accurate and specific diagnosis of *E. bieneusi* infection, and should help in the assessment of the role of *E. bieneusi* in gastrointestinal tract disease seen in AIDS patients, prospectively and retrospectively.

Using the PCR technique, we compared the rate of identification of *E. bieneusi* DNA in 80 formalin-fixed stools from Zimbabwean and U.S. AIDS patients by trichrome staining of fecal smears.

MATERIALS AND METHODS

Samples. Formalin-fixed stools were obtained from six AIDS patients at St. Lukes-Roosevelt Hospital (New York, N.Y.) with diarrhea who were infected with *E. bieneusi* as confirmed by transmission electron microscopy of biopsy specimens. Another 74 formalin-fixed stool samples were collected from human immunodeficiency virus (HIV)-positive (as determined by enzyme-linked immunosorbent assay) Zimbabwean patients, 45 of whom had diarrhea (defined as two or more loose bowel movements/day) and wasting. The formalin fixation was carried out by adding 2 ml of 10% buffered formalin to 2 ml or 0.5 g of stool, vortexing this mixture extensively, and storing the mixture at 4°C. After fixation, the samples were stored for between 2 and 4 months prior to extraction for PCR. For the PCR run, a negative stool sample from an immunocompetent person without diarrhea was obtained and formalin fixed in the same manner for use as a negative control. For the PCR run, a negative stool sample from an immuno-

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competent person without diarrhea was obtained and formalin fixed in the same manner for use as a negative control. For the in situ hybridization assay, formalin-fixed, paraffin-embedded jejunal biopsy specimens were obtained between 2 and 3 years prior to their use from six AIDS patients with *E. bieneusi* infection, two AIDS patients with *Septata intestinalis* infection, one AIDS patient with *Cryptosporidium parvum* infection, and one apparently healthy HIV-positive individual not infected with microsporidia.

Light microscopy. Fecal smears were heat fixed and stained by the modified trichrome technique of Weber et al. (20). Stained slides were examined by light microscopy under oil immersion (magnification, ×100).

DNA extraction and PCR. DNA was extracted from stools by a technique modified from that of Weiss et al. (22). Briefly, 1 ml of feces was preincubated for 2 h at 37°C in 3.5 ml of 10 mM Tris (pH 7)–2 mM EDTA–20 mM NaCl–0.6 mg of collagenase per ml. This was then supplemented with 440 μl of 10% sodium dodecyl sulfate (SDS)–20 μl of 20 mg of proteinase K (Sigma Chemical, St. Louis, Mo.) per ml–0.5 M NaCl (final concentration)–5 μl of β-mercaptoethanol–14 μl of 1 M dithiothreitol–500 μl of 10× hexadecyltrimethylammonium bromide. After incubating this mixture overnight at 37°C in a shaking incubator at 85 rpm, a 1-ml aliquot was transferred to vials containing 500-μm-diameter glass beads, and the vials were agitated in a Mini Bead beater (Biospec Products Inc., Bartlesville, Okla.) for 2 min, followed by centrifugation at 14,000 × *g* for 5 min. A total of 600 μl of the supernatant was then extracted twice with an equal volume of Tris-HCl-buffered phenol (pH 7.4; Gibco BRL, Grand Island, N.Y.), followed by one chloroform-isoamyl alcohol (24:1) extraction. The DNA was precipitated with ethanol-sodium acetate, dried, and resuspended in 30 μl of TE (10 mM Tris [pH 7.5], 1 mM EDTA). This procedure concentrates the fecal DNA approximately fourfold.

The amplification primer V1 (5′-CAGGTTGATTCTGCCTGAC-3′) (24) and the reverse primer Mic3 (5′-CAGCATCCACCATAGACAC-3′) located at positions 445 to 427 (GenBank accession no. L07123) were used to amplify a 446-bp fragment from the *E. bieneusi* small-subunit (SSU) rRNA gene. Amplification of 2 μl of DNA was carried out in 50 μl of a mixture containing 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.2 mM (each) deoxynucleoside triphosphates 400 pM (each) primer, and 2.5 U of *Taq* polymerase. We used a Touchdown PCR technique in which initial denaturation occurred at 94°C (2 min) and then the annealing temperature was decreased in 2°C steps from 62 to 54°C over eight cycles, each of which had an elongation temperature of 72°C (1 min). The remaining 32 cycles were carried out with denaturing at 94°C (40 s), annealing at 54°C (1 min), and elongation at 72°C (1 min), followed by a 10-min extension at 72°C (1 min). In all the reactions performed, *E. bieneusi* DNA, which had kindly been donated by L. M. Weiss (Albert Einstein College of Medicine, Bronx, N.Y.), was used as a positive control and reaction mixtures containing no DNA were used as a negative control. The 446-bp amplification product obtained from DNA in the stool of an experimentally infected monkey (20) was cloned (TA cloning kit; Invitrogen, Carlsbad, Calif.) and sequenced (Sequenase, version 2.0; U.S. Biochemicals, Cleveland, Ohio) to verify that its sequence corresponded to that of *E. bieneusi*. To further exclude the possibility that the PCR product originated from *S. intestinalis*, another intestinal microsporidium, we also carried out with all fecal samples the PCR with primers V1 and Sep1 (5′-CCTGCCCG CTTCAGAACC-3′), located at positions 876 to 859 (GenBank accession no. U09929) in the SSU rRNA of *S. intestinalis.*

In situ hybridization. The in situ hybridization assay was performed with formalin-fixed, paraffin-embedded human jejunal biopsy samples by a modification of the method previously described by Ilyinskii (10). Briefly, this method entailed labeling 3.0 μg of the 446-bp *E. bieneusi* PCR product with digoxigenin-11-dUTP in a randomly primed labeling reaction with 10 U of DNA polymerase I (Klenow enzyme, large fragment; Boehringer Mannheim, Indianapolis, Ind.) per μl in a total reaction volume of 100 μl. Hybridization was carried out with 5-μm-thick sections of biopsy specimens which had been placed on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, Pa.) and baked at 60°C for 1 h. After rehydration in alcohol, the slides were placed for two 5-min washes in 1× phosphate-buffered saline–5 mM $MgCl_2$ followed by digestion with 2 to 3 drops of prediluted proteinase K (70 μg/ml; Dako Corporation, Carpinteria, Calif.) in a humidified chamber for 5 min at room temperature. The slides were then washed for 10 min in 0.1 M Tris-HCl–0.1 M glycine followed by two 5-min washes in 1× phosphate-buffered saline–5 mM $MgCl_2$. Thirty microliters of the labeled probe was then applied to the biopsy specimens at a concentration of 0.05 ng/μl, and coverslips were applied. Overnight hybridization in a humidified chamber at 37°C was carried out under denaturing conditions to localize both DNA and RNA (10). Slides were washed twice for 30 min each time in 1× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 37°C, during which time the coverslips were removed. Sections were then immunostained with an avidin-biotin-horseradish peroxidase complex technique (Vector Laboratories, Burlingame, Calif.) by using a DAB chromogen (Sigma), followed by counterstaining with Mayer's hematoxylin. As negative controls, tissue sections were hybridized to a similarly labeled pUC19 plasmid probe following RNase digestion. A jejunal biopsy section from an HIV-positive, microsporidium negative individual was included in the protocol as a negative control.

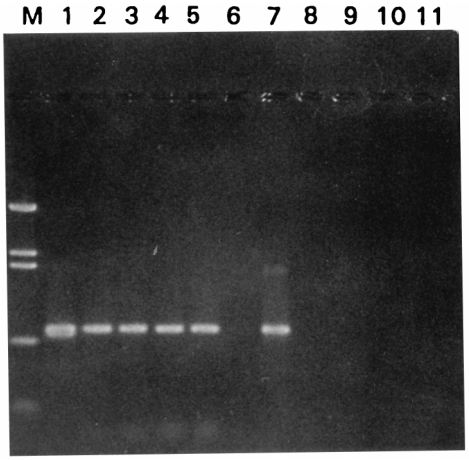

FIG. 1. PCR analysis with primers V1 and Mic3 of stool samples obtained from AIDS patients and a control subject without AIDS. Lane M, molecular size marker (size marker is *Bst*NI-digested pBR322 DNA; fragment sizes are 1,857, 1,058, 929, 383, and 121 bp); lane 1, *E. bieneusi* plasmid-positive control; lanes 2 and 3, stools from patients with endoscopy-confirmed *E. bieneusi* infections; lane 4, stool from a patient with *E. bieneusi* infection confirmed by microscopy of a stool specimen; lanes 5 and 6, stool negative for *E. bieneusi* by microscopy; lane 7, the stool specimen in lane 6 spiked with *E. bieneusi* plasmid DNA to ensure that inhibition was not occurring; lane 8, the stool specimen in lane 4 amplified with primers V1 and Sep1 (*S. intestinalis*); lane 9, the stool specimen in lane 6 amplified with primers V1 and Sep1 (*S. intestinalis*); lane 10, a stool specimen from an immunocompetent patient; lane 11, negative control.

RESULTS

PCR and microscopy. Formalin-fixed fecal samples from 80 individuals with AIDS and from 1 HIV-negative person were examined both by light microscopy and by PCR for the presence of *E. bieneusi*. The characteristic pink, birefringent, ovoid, 1.5- by 1-μm spores described by Weber et al. (20) were noted in 19 (24%) of the 80 stool samples. No spores were detected in the stool sample from the control individual. In contrast, a 446-bp fragment was amplified by PCR from 40 (50%) of the fecal samples from HIV-positive individuals. All stool samples which were positive by microscopy were also positive by PCR. The 446-bp fragment corresponded in size to that amplified from the *E. bieneusi* DNA which was incorporated into the protocol as a positive control. When we spiked five randomly selected negative samples with *E. bieneusi* plasmid DNA, the PCR always yielded a 446-bp fragment, indicating that there were no agents in these feces inhibiting the reaction. The presence of inhibitory material in unspiked samples is theoretically possible. No products were detected when amplification was carried out with the conserved primer V1 and the reverse primer Sep1 specific for *S. intestinalis* (Fig. 1). The uninfected stool specimen was PCR and microscopy negative.

In situ hybridization. Initially, the jejunal biopsy sections were stained with both hematoxylin-eosin and Gram stains for examination by light microscopy. In the human biopsy sections, occasional *E. bieneusi* organisms were identified either as bluish grey bodies (hematoxylin and eosin staining) or dark blue bodies (Gram staining) in the apical region of the enterocyte, consistent with previous descriptions in the literature (16). The control jejunal biopsy specimen appeared to be negative. After hybridization of the biopsy sections to the *E. bieneusi* probe, characteristic supranuclear staining of epithelial cells (Fig. 2) was readily apparent in the six sections obtained from patients with confirmed *E. bieneusi* infection but not in those obtained from the HIV-positive, microsporidium-negative patients or the *S. intestinalis*-positive or *C. parvum*-positive patients. Spe-

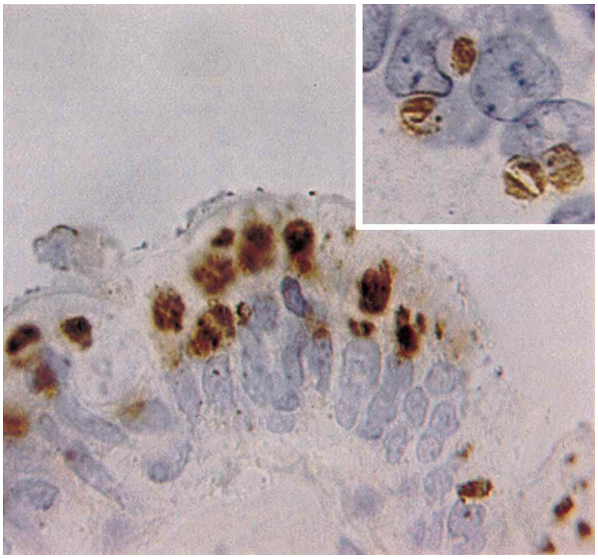

FIG. 2. In situ hybridization of a duodenal biopsy specimen from a patient with AIDS infected with *E. bieneusi* as confirmed by electron microscopy (magnification, ×80). The section was reacted with digoxigenin-labeled probe against *E. bieneusi* SSU rRNA (3,3′-diaminobenzidine tetrahydrochloride [DAB] chromogen). (Inset) Note the perinuclear location and nonstaining cleft-like spaces.

cific staining was also absent in the tissues which had been hybridized either after RNase digestion or to the nonspecific pUC19 plasmid probe.

DISCUSSION

E. bieneusi infection is one of the few new human infections to be described with the emergence of AIDS (2). Indeed, clinically apparent *E. bieneusi* infection, first described in 1985 (5, 14), is primarily found in the immunodeficient population, although a few cases have also been reported in immunocompetent individuals (18). The association of *E. bieneusi* with chronic diarrhea and wasting remains circumstantial because of the lack of animal models and appropriate diagnostic reagents for extensive epidemiological studies. Until recently, diagnosis was based entirely on endoscopy, a highly invasive and expensive procedure. The technique of staining fecal smears which was recently described (20) is able to detect infections in which spores are present in stools in moderate numbers. The microscopy technique is incapable of accurately determining the parasite genera.

The PCR technique described in this report is noninvasive, safe, sensitive, and specific. It detected double the number of cases of a microsporidium infection compared with the number detected by microscopy and was able to establish that they were due to *E. bieneusi*. We describe in some detail a technique for the extraction and subsequent amplification of *E. bieneusi* DNA from formalin-fixed fecal samples. Without sequencing the DNA in majority of stool samples, using a Touchdown PCR method we were able to detect *E. bieneusi* in 50% of the 80 samples tested, whereas by microscopy we detected *E. bieneusi* in 24% of the 80 samples. By the PCR technique, the incidence of *E. bieneusi* among patients with AIDS may prove to be higher than was previously thought. Our data suggest that the PCR technique may help to detect infections when the number of excreted spores is minimal and not readily detected by microscopy. Because the assay uses formalin-fixed stools, retrospective studies like those done with the Zimbabwean stool samples can now be performed. The PCR technique will also help determine the occurrence of mixed microsporidium infections and the relative contribution of each species to symptoms.

The in situ hybridization technique described here was able to detect many more infected cells than were otherwise apparent after staining with routinely used stains. In situ hybridization probably detects the *E. bieneusi* DNA present in the multiple developmental stages of the parasite. Thus, this procedure will enable the accurate identification of *E. bieneusi* in biopsy specimens even if infection is mild and present only in sporadic cells. Also, this technique can be used to differentiate *E. bieneusi* infection from that caused by other microsporidia. In addition, it will make an assessment of the role of *E. bieneusi* in lesions seen in mixed infections possible. In situ hybridization will also allow for retrospective studies, as was done with the nine biopsy sections received from the archives of St. Luke's-Roosevelt Hospital, because the DNA is stable in paraffin-embedded samples for extended periods of time.

In summary, we describe two molecular biology-based techniques which will help diagnostic laboratories detect microsporidiosis more readily and at the same time identify the genera involved. The assays will help establish more accurately the incidence of *E. bieneusi* infection in AIDS and other populations, including animals, and reflect its true etiological role in diarrhea and wasting in the immunodeficient host.

ADDENDUM

Since the submission of the manuscript, both techniques, PCR and in situ hybridization, were used extensively to confirm the transmission and establishment of persistent infections of human *E. bieneusi* in rhesus monkeys (19). They were also vital tools in the identification and characterization of *E. bieneusi* isolates originating from simian immunodeficiency virus-infected and healthy monkeys (12). The two studies described above further confirm the sensitivity and application of the two assays.

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