CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Mar. 1999, p. 243–246 Vol. 6, No. 2
1071-412X/99/$04.00+0

A Powerful DNA Extraction Method and PCR for Detection of Microsporidia in Clinical Stool Specimens

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Received 29 June 1998/Returned for modification 7 October 1998/Accepted 23 November 1998

The diagnosis of intestinal microsporidiosis has traditionally depended on direct visualization of the parasite in stool specimens or intestinal biopsy samples by light and/or electron microscopy. Limited information about the specificity and sensitivity of PCR for the detection microsporidia in clinical stool specimens is available. To establish a sensitive and specific method for the detection of microsporidia in clinical samples, we studied clinical stool specimens of 104 randomly selected human immunodeficiency virus-infected patients with diarrhea to compare light microscopy and PCR. Fluorochrome Uvitex 2B staining was used for light microscopy. To raise the sensitivity of PCR, we used a powerful and fast DNA extraction method including stool sedimentation, glass bead disruption, and proteinase K and chitinase digestion. PCR was performed with primer pairs V1-PMP2, V1-EB450, and V1-SI500, and the nature of the PCR products was confirmed by Southern blot hybridization. Microsporidiosis was diagnosed by light microscopy in eight patients. Ten patients tested positive for microsporidiosis by PCR. *Enterocytozoon bieneusi* was found in seven cases, and *Encephalitozoon intestinalis* was found in four cases. In one case a double infection with *E. bieneusi* and *E. intestinalis* was diagnosed by PCR, whereas light microscopy showed only *E. bieneusi* infection. PCR testing of stool specimens is useful for diagnosis and species differentiation of intestinal microsporidiosis in HIV patients.

Microsporidia are obligate intracellular, spore-forming protozoa which infect a broad range of vertebrates and invertebrates (19). Since the advent of the human immunodeficiency virus (HIV) pandemics, they are increasingly recognized as human pathogens. Now up to six genera of microsporidia have been reported to infect humans. Microsporidia of the genera *Enterocytozoon*, *Encephalitozoon*, *Nosema*, *Pleistophora*, *Trachipleistophora*, and *Vittaforma* and unclassified microsporidia were primarily detected in immunocompromised hosts with a broad variety of clinical presentations (19). *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* have been identified as important agents for chronic diarrhea and wasting syndrome in patients with AIDS (1, 19). The diagnosis of intestinal microsporidiosis has traditionally depended on direct visualization of the parasites by light and/or electron microscopy, but the sensitivity and specificity of these techniques are not known, and for exact species differentiation ultrastructural observations by transmission electron microscopy are necessary (16, 18, 19). PCR for the detection of microsporidian DNA in different biological samples, including gastrointestinal biopsy specimens and feces (1–7, 9–11, 17, 20, 21), has been developed. Many such studies used stool samples spiked with microsporidian spores or stool specimens with known microsporidial infection previously diagnosed by light microscopy (2, 3, 7, 9–11, 17). Limited information is available about the specificity and sensitivity of PCR in clinical stool specimens. The aim of this study was to determine the accuracy of PCR for detection of microsporidian DNA in clinical stool specimens for diagnosis and species differentiation of intestinal microsporidiosis in a large number of HIV-infected patients and to compare the PCR results with light microscopic findings. A powerful DNA extraction method was used to enhance sensitivity.

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MATERIALS AND METHODS

To determine the threshold of detection for light microscopy and PCR, stool specimens from healthy, asymptomatic volunteers without travel history during the last 3 months were spiked with culture-derived microsporidian spores of *E. intestinalis* at different concentrations (10^2, 10^3, 10^4, and 10^6/g). Probes were examined by PCR and light microscopy.

Stool samples were obtained from 104 HIV-infected patients with diarrhea between January 1996 and September 1997. All patients had confirmed HIV serology with a mean CD4 cell count of 26/μl (range, 1 to 552/μl). All stool specimens were examined by light microscopy and PCR.

Stool was concentrated by a modified water-ether sedimentation procedure (16). Feces (0.5 to 1.0 g) was homogenized in 8 ml of distilled water and filtered through a 300-μm-mesh sieve. Three microliters of ether was added, and the water-ether mixture was vortexed for 1 min and then centrifuged at 700 × *g* for 2 min. After decanting the supernatant, the pellet was diluted in 100 μl of water, and a part (20 μl) of the suspension was used for preparing smears (about 15 mm^2) (16).

Smears were fixed in methanol for 2 min and stained with 1% Uvitex 2B in phosphate-buffered saline (Fungiqual A; R&R, Kandern, Germany) for 15 min, counterstained with 0.5% Evans blue (Sigma, St. Louis, Mo.) in phosphate-buffered saline for 1 min, and examined by two independent investigators at ×1,000 magnification under a fluorescence microscope (Zeiss, Oberkochen, Germany) with a 50-W mercury high-pressure lamp, an excitation filter with a transmission rate of 355 to 425 nm, and a supression filter of 460 nm (14).

DNA was prepared from the rest of the stool sediment by using a QIAmp tissue kit (Qiagen, Hilden, Germany). Specimens were incubated in digestion buffer with 400 μg of proteinase K (Qiagen) and 0.4 U of chitinase (Sigma) at 55°C for 2 h. Glass beads (500 μg; diameter, 425 to 600 μm; Sigma) were added, and the mixture was vortexed every 15 min for 1 min. DNA was prepared from the solution by using QIAmp spin columns (Qiagen) in an Eppendorf microcentrifuge following the manufacturer's instructions.

As described in detail elsewhere, small-subunit (SSU) rRNA gene fragments of *E. bieneusi* and *E. intestinalis* were ligated into a pMOS*Blue* T vector (Amersham International, Amersham, England) and cloned in *Escherichia coli* (4). Clones were used as positive controls during all amplifications.

TABLE 1. Light microscopy and PCR results for 10 patients with intestinal microsporidiosis

Patient	Organism detected by light microscopy	Amplicon size(s) (bp) by PCR using primer pair V1-PMP2	Result of PCR using primer pair:	
			V1-EB450	V1-SI500
1	*E. bieneusi*	250	Positive	Negative
2	*E. bieneusi*	250	Positive	Negative
3	*E. bieneusi*	250	Positive	Negative
4	*E. bieneusi*	250, 270	Positive	Positive
5	*E. bieneusi* or *Encephalitozoon* sp.	250	Positive	Negative
6	*Encephalitozoon* sp.	270	Negative	Positive
7	*Encephalitozoon* sp.	270	Negative	Positive
8	*Encephalitozoon* sp.	270	Negative	Positive
9	Negative	250	Positive	Negative
10	Negative	250	Positive	Negative

Amplifications were done in 50-μl reaction mixtures under the following conditions: 25 pmol of each primer, 200 μM concentrations of 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.). Reactions were run in a Perkin-Elmer thermocycler using a step cycle program. After initial denaturation of DNA at 94°C for 3 min, 35 cycles were run—94°C for 1 min, 48 to 60°C for 2 min, and 72°C for 3 min—with a 10-min 72°C extension after the 35 cycles (4–6). Primers V1 (5′-CACCAGGTTGATTCTGCCTGAC-3′) and PMP2 (5′-CCTCTCCGGAACCAAACCCTG-3′) were used to amplify a 250-bp DNA fragment of the SSU rRNA gene of *E. bieneusi* and a 270-bp DNA fragment of *E. intestinalis* at an annealing temperature of 60°C (3). A second primer pair, V1 and EB450 (5′-ACTCAGGTGTTATAC TCACCTG-3′), amplified a 353-bp DNA fragment of the SSU rRNA gene of *E. bieneusi* at an annealing temperature of 48°C (4–6, 21) and was used for species confirmation as *E. bieneusi* for samples which were positive for microsporidia by the first PCR. Primers V1 and SI500 (5′-CTCGCTCCTTTACACTCG-3′) were used for amplification of a 375-bp DNA fragment of the SSU rRNA gene of *E. intestinalis* at an annealing temperature of 58°C and were used for species confirmation as *E. intestinalis* for samples which were positive for microsporidia by the first PCR (4–6, 20).

A 10-μl aliquot from each reaction mixture was run on a 3% NuSieve 3:1 electrophoresis-grade agarose gel (FMC Bioproducts, Rockland, Maine) in 1× TAE buffer (Tris acetate, 0.04 mol/liter; EDTA 0.001 mol/liter) with ethidium bromide (0.5 g/ml) to visualize the amplified PCR products under UV illumination.

As described in detail elsewhere, an internal 30-mer oligonucleotide, EB150 (5′-TGTTGCGGTAATTTGGTCTCTGTGTGTAAA-3′), and an internal 18-mer oligonucleotide, SI60 (5′-TGTTGATGAACCTTGTGG-3′), were used for Southern blot hybridization (4–6).

Procedures for avoiding contamination were strictly followed. DNA isolation, preparation of reaction mixtures, and amplification and analysis were physically separated and performed in three different rooms. Positive displacement tips were used for all manipulation, and negative controls containing reaction mixtures without DNA were used during all amplifications.

RESULTS

When stool samples spiked with microsporidian spores at various concentrations were used, the detection limit of light microscopy varied between 10^4 and 10^6 spores per g of stool, but reliable detection of spores was achieved only at spore concentrations of 10^6/g of stool. By PCR, spore concentrations as low as 10^2/g of stool were detected in all examined probes.

Examination of clinical stools samples by light microscopy showed microsporidian spores in stool samples of eight patients. In four cases *E. bieneusi* was identified, and in three cases an *Encephalitozoon* sp. was identified. In one case it was impossible to differentiate between *E. bieneusi* or *Encepalitozoon* sp. (Table 1).

Amplification with the primer pair V1 and PMP2 produced 250-bp DNA fragments, indicating infection with *E. bieneusi*, from stool specimens of seven patients, including the specimen that was indeterminate after light microscopical examination, as well as from the plasmid with the SSU rRNA sequence of *E. bieneusi*. Two of these patients were negative for microsporidia by light microscopy. A 270-bp DNA fragment, indicating infection with *E. intestinalis*, was amplified from stool specimens of four patients as well as from the plasmid containing the SSU rRNA gene of *E. intestinalis*. One of these patients was diagnosed by light microscopy as having only *E. bieneusi* infection.

The results were controlled by PCR with primer pairs V1-EB450 and V1-SI500. Amplification with the primer pair V1 and EB450 produced a 353-bp DNA fragment with stool samples from seven patients, including the two with negative results by light microscopy as well as from the plasmid with the SSU rRNA sequence of *E. bieneusi* (Table 1). The nature of these PCR products was confirmed by Southern blot hybridization with the internal probe EB150 in all cases. Amplification with the primer pair V1 and SI500 produced a 375-bp DNA fragment from stool samples of four patients, including the patient who was positive for only *E. bieneusi* by light microscopy, as well as from the plasmid containing the SSU rRNA gene of *E. intestinalis* (Table 1). These PCR products hybridized with the internal probe SI60 in all cases.

No stool specimens positive by light microscopy showed negative results by PCR. Two cases were found to be positive by PCR, but examination by light microscopy failed to demonstrate microsporidian spores in these samples. In one case, light microscopy detected only infection with *E. bieneusi*, whereas PCR showed additional infection with *E. intestinalis*.

DISCUSSION

Light microscopic visualization of spores has been the standard technique to diagnose intestinal infections with microsporidia. The sensitivity of this approach is probably limited, and species differentiation is impossible although it is necessary for clinical management and epidemiological studies (12).

In this study we have demonstrated that PCR is a sensitive and specific method for diagnosing intestinal microsporidial infections based on clinical stool specimens of HIV-infected patients. PCR for the detection of microsporidian DNA in stool specimens has already been performed by different investigators (2, 7, 9–11, 17), but most of these studies used stool samples spiked with microsporidian spores or stool specimens from patients with known microsporidial infection previously diagnosed by light microscopy. Little is known about the usefulness of PCR assays using clinical stool samples. In the few studies utilizing such samples, only limited data on light microscopy were available (2, 11).

To the best of our knowledge the present study included the largest group of randomly selected HIV-infected patients with diarrhea, whose stool specimens were examined for microspo-

ridia by light microscopy and PCR. Our study demonstrates identical results by both techniques for 102 of 104 (98%) specimens. No microscopically positive samples showed negative results by PCR. Two other studies compared light microscopy and PCR for the detection of microsporidia in clinical stool samples (8, 11). One study indicated that PCR is as sensitive as standard staining methods, but many samples in this study were known to be positive for microsporidia by previous microscopic analysis (11). Another study demonstrated identical results by both techniques for 28 of 34 (82%) specimens (8). In this study four samples with positive PCR results were microscopically negative, but surprisingly, two samples were microscopically positive and PCR negative (8). Based on the low number of positive specimens, the present study is not able to detect a true difference between results by light microscopy and PCR.

The prevalence of intestinal microsporidiosis in our cohort amounted to 9.6%, but if only light microscopical results were taken into consideration the prevalence was only 7.4%. Since the threshold of detection for light microscopy seems to be between 10^4 and 10^6 spores per g of stool, epidemiological studies utilizing only light microscopy may produce prevalence data which do not reflect the true prevalence of microsporidia. Application of PCR to stool samples offers an approach that can be adapted for processing large numbers of specimens to define the true prevalence of intestinal microsporidiosis in human populations and to determine whether microsporidia are present in the general population (12). Furthermore, we identified an additional case of double infection with *E. intestinalis* and *E. bieneusi*, in which *E. intestinalis* was detected only by PCR, as we and other groups have reported previously for several HIV-infected patients (4, 5, 11, 15).

Several methods for DNA isolation from microsporidian spores in stool samples have been reported (2, 3, 7, 9–11, 17). This step is critical because microsporidian spores are very resistant and many PCR inhibitors are usually present in stool samples. One technique was based on treatment of samples with sodium hypochlorite to remove PCR inhibitors followed by both mechanical and chemical disruption in a laborious 4-day procedure (3). Others used glass bead treatment followed by digestion with proteinase K and final purification with the RapidPrep Micro genomic DNA isolation kit, which requires 2 days to perform (2), whereas boiling of the samples with subsequent dilution of the fecal solution to nullify PCR inhibition may influence sensitivity (11). Other investigators have used alkaline or guanidinium thiocyanate cell lysis, and both methods seem to be suitable procedures (7, 10). We used a fast and simple DNA extraction method in combination with a modified water-ether sedimentation procedure to increase sensitivity by raising the number of spores which were inserted for DNA extraction. Spore concentration to higher numbers by water-ether sedimentation has been shown previously by van Gool et al. (14), but we did not compare our extraction method with previously published methods. Furthermore, we used the enzyme chitinase for hydrolysis of the natural substrate chitin (13) occurring in microsporidian spore walls in addition to mechanical disruption by glass beads to improve the DNA extraction. The resulting extract was subsequently purified by silica membrane adsorption using QIAmp spin columns to remove inhibitors from stools so that no further treatment with hypochlorite or formalin was necessary. No testing for inhibitors was done in our study, so we cannot exclude the possibility of presence of inhibitors in our samples. However, the detection limit of at least 10^2 spores per g of stool seems to indicate the absence of inhibitors after DNA isolation. The procedure needs less than 3 h to perform and provides sufficient amounts of pure DNA for PCR testing.

DNA amplification with the primer pair V1 and PMP2 is useful for identification of microsporidial infections, and the primer pairs V1-EB450 and V1-SI500 are species specific for *E. bieneusi* and for *E. intestinalis*, as has been shown previously (1, 4–6, 11, 20, 21). The primer pair V1-PMP2 probably amplifies SSU rRNA from several microsporidia, but species other than *E. bieneusi* or *Encephalitozoon* spp. are not likely to occur in stool samples of HIV-infected patients. Amplification of other species was further excluded by subsequent amplification with the species-specific primer pairs V1-EB450 and V1-SI500. Although there seems to be some polymorphism in the SSU rRNA genes of microsporidia, the primer pairs V1-EB450 and V1-SI500 are the best that have been evaluated for diagnosis of microsporidiosis due to *E. bieneusi* and *E. intestinalis*, and several researchers have published studies using these primer pairs, usually with excellent results (1, 4–6, 11, 20, 21).

The results of the present study demonstrate that PCR testing of stool samples with a powerful DNA extraction method is a sensitive and useful approach for the diagnosis and species differentiation of intestinal microsporidiosis.

ACKNOWLEDGMENTS

We thank Britta Franzen for editorial assistance during preparation of the manuscript.

This work was supported by the Köln Fortune Program from the Faculty of Medicine, University of Cologne, Cologne, Germany.

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