Genetic Evidence for Latent Septata intestinalis Infection in Human Immunodeficiency Virus–Infected Patients with Intestinal Microsporidiosis

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Sequence data of the small subunit rRNA (SSU-rRNA) gene were used to identify Septata intestinalis in biopsies of human immunodeficiency virus–infected patients by polymerase chain reaction (PCR), southern blot hybridization, cloning, and comparative genetic sequencing. DNA products of correct size could be amplified from all examined tissues with S. intestinalis infection but also from 2 biopsies with Enterocytozoon bieneusi and from 1 biopsy with Encephalitozoon cuniculi infection. Southern blot hybridization with an S. intestinalis–specific probe and partial sequencing of the DNA fragments showed high homology with published S. intestinalis sequences and confirmed that the amplified PCR products really derived from the SSU-rRNA gene of S. intestinalis. PCR testing can detect very light infections with S. intestinalis. Thus, S. intestinalis seems to occur more frequently in the form of latent infections and the true prevalence of the parasites may be much higher than previously reported.

Microsporidia are primitive eukaryotic, obligate intracellular, protozoan parasites. They infect members of almost every major animal group, including primates [1]. Microsporidia of five genera (Enterocytozoon, Encephalitozoon, Septata, Nosema, and Pleistophora) are known to infect humans [1]. More than 400 cases of microsporidiosis have been documented, most of them caused by Enterocytozoon bieneusi [1, 2], and ~35 documented cases of infection with Encephalitozoon cuniculi or Encephalitozoon hellem have occurred in human immunodeficiency virus (HIV)–infected patients [1]. However, intestinal infections with the microsporidian species Septata (Encephalitozoon) intestinalis are increasingly recognized as the cause of chronic diarrhea in HIV-infected patients [3]. First described in 1992 as a microsporidium with ultrastructural similarities to the genus Encephalitozoon [4], S. intestinalis was later named as a new genus and species on the basis of ultrastructural differences [5]. New sequence data now suggest that S. intestinalis should be placed in the genus Encephalitozoon and called Encephalitozoon intestinalis [6].

Until now, diagnosis of microsporidiosis has depended on direct visualization of the parasites by light and transmission electron microscopy (TEM), and for exact species differentiation, ultrastructural analysis of spores and tissue stages has been necessary [1, 2, 5]. With use of conserved sequences, the small subunit rRNA (SSU-rRNA) genes of E. bieneusi, S. intestinalis, E. cuniculi, E. hellem, and other microsporidian species were amplified by polymerase chain reaction (PCR), cloned, and sequenced [6–9]. These sequences were used to develop primers and probes for PCR and hybridization assays to diagnose infections with microsporidia [9, 10]. We used these sequence data to identify S. intestinalis in intestinal biopsies of HIV-infected patients.

Materials and Methods

Microsporidia. Intestinal biopsies from HIV-infected patients were obtained from the distal duodenum by flexible fiberglass endoscopy. Samples were snap-frozen in liquid nitrogen and stored at −80°C until processing. Four biopsies were available from patients with TEM-confirmed S. intestinalis infection, 1 from a patient with TEM- and antibody-confirmed E. cuniculi infection, and 6 from patients with TEM-confirmed E. bieneusi infection.

PCR. DNA isolation and PCR amplification were done as described [10]. Two primers, VI (5′-CACCAGGTGGATTCTGAGAC-3′) and 1492 (5′-GGTTACCTTGGTACGACTT-3′), were used for amplification of the entire SSU-rRNA genes of E. bieneusi and S. intestinalis at an annealing temperature of 50°C. This primer pair was designed to amplify a fragment of ~1.2 kb from E. bieneusi– and S. intestinalis–infected tissue [7, 9].

The primer pair VI and SI500 (5′-CTCGCTCTTTA-CACTCG-3′) was used for amplification of a 375-bp DNA fragment of the SSU-rRNA gene of S. intestinalis at an annealing temperature of 58°C, and the 20-mer oligonucleotide SI60 (5′-TGTGATGACACCTGTGG-3′) was used to confirm by Southern blot hybridization that the amplified fragments were derived from S. intestinalis [11].

The primer pair V1 and EB450 (5′-ACTCAGGTGTTACT- CAGTTCG-3′) was used for amplification of a 353-bp DNA fragment of the SSU-rRNA gene of E. bieneusi at an annealing temperature of 48°C [9–11].
the expected 1.2-kb DNA fragments on ethidium bromide-stained intestinalis was used as template (figure 1). After ligation of the DNA fragments into the pMOSBlue vector, species-specific sequences could be detected by PCR amplification with either the primer pair VI and EB450 using plasmids containing the 1.2-kb DNA fragment from E. bieneusi-infected tissue or the primer pair V1 and S1500 using plasmids containing the 1.2-kb DNA fragment from S. intestinalis-infected tissue as template. No amplification with V1 and S1500 was seen when plasmids containing the 1.2-kb DNA fragment from E. bieneusi-infected tissue were used as template. Likewise, no bands of the correct size were detected using the primers V1 and EB450 and plasmids containing the 1.2-kb DNA fragment from S. intestinalis-infected tissue as template (figure 1).

With DNA prepared from tissue with TEM-confirmed S. intestinalis infection, strong bands of correct size (375 bp) could be amplified with the primer pair V1 and S1500 from all 4 biopsies. With use of this primer pair with DNA prepared from biopsies with TEM-confirmed E. bieneusi infection, DNA fragments of correct size (375 bp) could be amplified from 2 of the 6 biopsies. A DNA fragment of correct size (375 bp) could be amplified also by using DNA prepared from a biopsy with TEM- and antibody-confirmed E. cuniculi infection (figure 1).

After Southern blotting and hybridization with the probe S160, 375-bp DNA fragments could be visualized from all 7 biopsy samples, including the 2 from E. bieneusi and the 1 from E. cuniculi-infected tissues (data not shown).

Partial sequencing of the three different DNA fragments that were amplified from tissue infected with E. bieneusi and E. cuniculi obtained 228 bases for comparison (data not shown, GenBank accession no. U39297). Alignment of this partial sequence against the GenBank data base showed high genetic homology with previously published sequences of S. intestinalis. Other matches against E. hellem, E. cuniculi, and E. bieneusi showed only limited homology. Sequences under accession numbers U09929 and L19567 showed 100% and 95.6% similarity, respectively. Both are described as the SSU-rRNA gene of S. intestinalis. Sequences under accession numbers L16866 and L16867 showed 100% and 99.6% similarity, respectively. These are described as the SSU-rRNA gene of an Encephalitozoon species that was later confirmed as S. intestinalis [7]. Comparison with sequences from E. hellem (accession no. L19070), E. cuniculi (accession nos. L17072, L07255, Z19563), and E. bieneusi (accession no. L07123) showed a similarity of only 85.2%, 82.6%, and 55.7%, respectively.

Discussion

The most frequent intestinal microsporidian species, E. bieneusi, occurs in ~10%–30% of HIV-infected patients with diarrhea [1, 2], but S. intestinalis is increasingly recognized as a pathogen in HIV-infected patients [1, 3, 4], and the parasite has been found in intestinal biopsies of ~3% of patients with AIDS [1, 3].

The data presented here show that the primer pair V1 and S1500 can be used for species-specific amplification of DNA.
fragments of the SSU-rRNA gene of *S. intestinalis*. The forward primer V1 is based on the conserved sequence of the SSU-rRNA gene of *Vairimorpha necatrix* at the 5′ end and the reverse primer S1500 is located at position 500 of the SSU-rRNA gene based on the alignment of *S. intestinalis* and *Escherichia coli* [9, 11]. Strong bands of correct size were obtained using this primer pair with DNA from all tissues with TEM-confirmed *S. intestinalis* infection as well as with plasmids containing the 1.2-kb DNA fragments of the SSU-rRNA gene of *S. intestinalis_. In opposition, the primer pair did not amplify DNA with the plasmid containing the 1.2-kb DNA fragment of the SSU-rRNA gene of *E. bieneusi* as template.

Surprisingly, the two primers also amplified DNA fragments of correct size from tissue with *E. bieneusi* and *E. cuniculi* infection. The forward primer V1 is not specific for *S. intestinalis* but is directed against a conserved sequence similar to those of many microsporidian species [11]. Several confirmation techniques were used to determine the specificity of the primer pair V1 and S1500 and to exclude the possibility of an amplification of the three DNA fragments as a result of unspecific annealing of the primer S1500. Hybridization with the *S. intestinalis*–specific internal probe S160, which is located at position 60 of the SSU-rRNA gene of *S. intestinalis* based on the alignment of *S. intestinalis* and *E. coli* [11], and partial sequencing of the DNA fragments showing high homology with published *S. intestinalis* sequences confirmed that the amplified PCR product really derived from the SSU-rRNA gene of *S. intestinalis*.

Because PCR testing is susceptible to cross-contamination, procedures for avoiding contamination were strictly followed. Pre- and post-PCR handling were physically separated and done in different rooms, and positive-displacement tips were used for all manipulations. Negative controls containing reaction mixtures without DNA were always done and PCR was done twice to check for false-positive and false-negative results. The three DNA fragments that were amplified using the primers V1 and S1500 from the 2 *E. bieneusi*– and the 1 *E. cuniculi*–infected biopsies were amplified during the first use of the primer pair V1 and S1500, and in former amplifications, tissue with confirmed *S. intestinalis* infection as template had not been used at any time. Thus, we can take for granted that the results were not caused by cross-contamination.

Double infections with two different types of microsporidia were reported previously [12], but subclinical infection with *S. intestinalis* was not expected until similar results were obtained in 1994 by van Gool et al. [13], who cultivated *S. intestinalis* from stool of 5 HIV-infected patients with TEM-confirmed *E. bieneusi* infection [13]. Like our group, they were not able to visualize the parasites by light or electron microscopy.

The results presented herein show that the primer pair V1 and S1500 and the hybridization probe S160 can detect very light infections with *S. intestinalis*. *S. intestinalis* seems to occur more frequently in the form of latent infections, and our results suggest that the true prevalence of the parasites may be much higher than the previously reported 2%–4%. Studies using the molecular techniques described herein are underway to evaluate the true prevalence of intestinal infections due to *S. intestinalis*.