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Utility of microsporidian rRNA in diagnosis and phylogeny: a review*

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Abstract. This paper summarizes work done in this laboratory over the last two years on the cloning of microsporidian rRNA by homology PCR and its subsequent use in diagnostic tests and phylogenetic studies. Using highly conserved primers in the 16S or small subunit rRNA (SSU-rRNA) these genes were cloned from human intestinal biopsies with transmission electron microscopy proven *Enterocytozoon bieneusi* and *Septata intestinalis*. The SSU-rRNA genes were then used to design and test several primer pairs for the diagnosis of microsporidian infection. Utilizing the polymerase chain reaction and primers V1 and EB450 *Ent. bieneusi* infected duodenal aspirates or intestinal biopsies could be detected. Using V1 and SI500 infection with *S. intestinalis* could be detected. In addition to diagnostic tests, phylogenetic relationships were examined using sequence data from the fragment amplified by PCR by primer 530f in the SSU-rRNA and primer 580r in the large subunit rRNA. This data supported the placement of *S. intestinalis* in the family Encephalitozoonidae. In addition, it confirmed that *Encephalitozoon cuniculi, E. hellem* and *S. intestinalis* are distinct organisms. These techniques have broad applications to the study of other microsporidia and the development of a molecular phylogeny.

Microsporidia are obligate intracellular, spore forming parasites infecting every major animal group, especially insects, fish and mammals (Sprague and Vávra 1977, Bryan et al. 1991). They are sufficiently unique to be classified in a separate phylum, the Microspora (Sprague and Vávra 1977, Bryan et al. 1991). Within the phylum, there are dozens of genera and hundreds of species. Microspora are defined by their small unicellular spores containing a nucleated sporoplasm, an extrusion apparatus with a coiled polar tube and an anchoring disk within the resistant spore (Sprague and Vávra 1977). All of the microsporidia contain an extrusion apparatus with a polar tube which coils around the sporoplasm. This tube serves as an unique vehicle for transmission of infection in that the polar tube can pierce an adjacent cell inoculating the sporoplasm directly into that cell. Currently, the classification of microsporidia is based upon spore morphology and developmental life cycle as demonstrated by electron microscopy (Cali et al. 1993, Wittner et al. 1993). Within their hosts, they have been reported from every tissue and organ type. The majority infect the digestive tract and/or related organs but reproductive, excretory and nervous system infections are well documented as well as those in connective and muscle tissues (Wittner et al. 1993).

Five microsporidia genera have been associated with human disease: *Nosema*, generally found in insects; *Pleistophora*, a pathogen of fish and insects; *Encephalitozoon*, found in many mammals; *Enterocytozoon* reported from AIDS patients and Chinook salmon and *Septata* reported from AIDS patients (Bryan et al. 1991, Wittner et al. 1993). A sixth genus, *Microsporidium* has been used to designate microsporidia of uncertain taxonomic status. These organisms appear to be important opportunistic pathogens in patients with human immunodeficiency virus type 1 infection (AIDS).

Enterocytozoon bieneusi was first described in 1985, in an AIDS patient with intestinal malabsorption and diarrhea and thus far has been described only in humans (Desportes et al. 1985, Modigliani et al. 1985). Based on its morphologic characteristics, it was placed in a new family, Enterocytozoonidae (Cali et al. 1990). *Septata intestinalis* was recently reported to cause enteric infection in AIDS patients (Cali et al. 1993). Although it is found in enterocytes it also infects cells of the *lamina propria*. Its unique morphology justified the establishment of a new genus and species for this organism (Cali et al. 1993). On morphologic analysis

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litozoonidae, which contains only the genus *Encephalitozoon*, therefore it has been placed in this family (Cali et al. 1993). Recent studies on SSU-rRNA have shown that *S. intestinalis* and *Encephalitozoon cuniculi* are closely related (Zhu et al. 1993a).

Currently, species diagnosis of microsporidiosis requires biopsy and transmission electron microscopic (TEM) study of tissue stages and spore ultrastructure. The establishment of techniques that would allow the specific identification of microsporidia in stool or biopsy tissue without TEM are urgently needed to facilitate diagnostic and epidemiologic studies on *Ent. bieneusi*. Taxonomic studies (Vossbrinck et al. 1987) have shown that microsporidian small subunit-rRNA (SSU-rRNA) displays limited homology with other eukaryotic organisms suggesting these organisms are an early branch point in evolutionary development. Relman (Relman et al. 1990) has described techniques for the identification of the non-cultivatable organism responsible for bacillary angiomatosis using conserved SSU-rRNA (16S rRNA) gene oligonucleotide primers and the polymerase chain reaction (PCR). Utilizing the conserved SSU-rRNA sequences and the known microsporidian rRNA sequence of *Vairimorpha necatrix* (Vossbrinck et al. 1987) we were able to amplify by PCR and subsequently clone and sequence the SSU-rRNA of *Ent. bieneusi* (Zhu et al. 1993b) and *S. intestinalis* (Zhu et al. 1993a). These cloned genes were used to further develop probes for the detection of *Ent. bieneusi* (Zhu et al. 1993b) and *S. intestinalis* (Zhu et al. 1993b) in tissue. Such probes may also be useful for diagnosis from stool specimens of infected patients.

Vossbrinck has recently described a phylogenetic construction of several microsporidia based on ribosomal RNA sequence analysis (Vossbrinck et al. 1993). We have sought to expand this approach to include *Ent. bieneusi* and *S. intestinalis*, the microsporidia that account for most of the described human infections (Wittner et al. 1993). Both of these microsporidia are non-cultivatable, however the SSU-rRNA, intergenic spacer region and a fragment of the large subunit rRNA were cloned from infected human tissue using phylogenetically conserved rRNA primers in the SSU-rRNA gene (530f and 228f) and in the large rRNA subunit (580r) (Vossbrinck et al. 1993, Zhu et al. 1993a,b,c,d). Since these organisms have only been found in humans, the determination of their phylogenetic relationships may be of importance in suggesting the reservoir hosts for these opportunistic pathogens. In addition, our analysis confirms that *S. intestinalis* and *Encephalitozoon hellem* are distinct organisms (Zhu et al. 1993a). This method allows the establishment of phylogenetic relationships on limiting material where culture and electron microscopy are difficult if not impossible. This method also can be applied to archival material to expand the molecular phylogenetic analysis of the phylum Microspora.

This paper reviews data generated in our laboratory on the use of microsporidian rRNA sequence data obtained by homology PCR cloning in the diagnosis and phylogenetic reconstruction of microsporidia.

MATERIALS AND METHODS

Microsporidia

Enterocytozoon bieneusi and *Septata intestinalis*: As part of a study of the effectiveness of octreotide on Human Immunodeficiency Virus (HIV-1) associated diarrhea (Cello et al. 1991) tissue samples were obtained with informed consent by endoscopy from HIV patients with diarrhea (provided by Dr. D. Simon, Albert Einstein College of Medicine, New York, New York). Samples were frozen, fixed for TEM and fixed for routine histology. In addition, tissue specimens were also available from patients seen for the clinical evaluation of chronic diarrhea in HIV-1 infection (provided by Dr. D. Kotler, St Lukes Roosevelt Hospital, New York, New York). Nine intestinal biopsies were available from patients with TEM confirmed *Ent. bieneusi* infection, 4 from patients with TEM confirmed *S. intestinalis* infection and 10 biopsies from HIV-1 positive patients with diarrhea of other etiologies (2 with cryptosporidiosis and 8 idiopathic [no etiologic organism identified]). Control tissue, i.e. HIV positive no diarrhea (2 patients) and HIV negative no diarrhea (2 patients), was also available.

Encephalitozoon cuniculi was maintained and passaged as previously described (Weiss et al. 1992).

Encephalitozoon hellem was obtained (Visvesvara et al. 1991) from the Centers for Disease Control (courtesy of Dr. G. Visvesvara) and grown as described (Visvesvara et al. 1991) in Vero cells in DME containing 10% FBS (Hyclone, Logan, UT), 1X Trace element mix (Gibco-BRL, Gaithersburg, MD), 1% Penicillin/Streptomycin (Gibco-BRL, Gaithersburg, MD) and 10 ng/ml Epidermal Growth Factor (Collaborative Biomedical Products, Bedford, MA). Cells were subpassaged every 20 days using Trypsin/EDTA (Gibco-BRL, Gaithersburg, MD). Cells were fed every 5 days and the supernatant which contained the spores harvested and stored at 4° C.

Variomorpha necatrix was grown in *Heliothis zea* larvae on an artificial diet and/or obtained from stock (Langley et al. 1987, Weiss et al. 1992). *Glugea stephani* was field collected in feral winter flounder and subsequently grown *in vivo* as described (Cali et al. 1987). *Nosema locustae* was established and maintained in grasshoppers (provided by Dr. J. Henry, U.S. Dept. of Agriculture, Bozeman, MT) and *N. bombycis* in silkworms. The spores were stored at 4° C when not in culture. *Pleistophora* was isolated from feral ocean pout. The organisms were purified by differential centrifugation (Langley et al. 1987). Spores of *Ameson michaelis* (formerly called *N. michaelis*) were purified from the muscle of blue crabs (*Callinectes sapidus*) (provided by Dr. E. Weidner, LA) (Zhu et al. 1993c).

Polymerase Chain Reaction (PCR)

DNA was prepared from frozen tissue specimens by incubation overnight at 37° C in TE buffer (10mM Tris, pH 7.4, 0.1mM EDTA) containing 1% SDS and proteinase K (20 µg/ml) followed

0.1mM EDTA) containing 1% SDS and proteinase K (20 µg/ml) followed by phenol-chloroform extraction and ethanol precipitation (Weiss et al. 1991). DNA was prepared from microsporidia spores by glass bead (425–600 µM, Sigma, St.Louis, MO) disruption for 2 minutes with a Mini bead-beater (BioSpec Products Inc., Bartlesville, OK) of spores in TE buffer, followed by centrifugation for 5 minutes at 13,500 *g* and then phenol-chloroform extraction and ethanol precipitation of DNA from the supernatant. DNA prepared by ethanol precipitation was resuspended in TE buffer for use in PCR. All sample preparations for PCR reactions were prepared with either aerosol guard pipette tips or positive displacement tips to prevent contamination. Control reactions containing no DNA were run for all PCR reactions.

The PCR reaction was carried out using standard PCR buffer and conditions (Weiss et al. 1992) (Perkin Elmer Cetus, Norwalk, CT) pH 8.3, 10mM Tris HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, with 8mM dNTPs, 5 units Taq DNA polymerase and 100 picomoles of each primer. A total of 35 cycles were used with a cycle of 94° C for 1 min, an annealing temperature of 42–58° C for 2 min and 72° C for 3 min. A 10 min 72° C extension was used following the 35 cycles. 0.1 to 1 µg of sample DNA was used for the amplification. The annealing temperature was changed to optimize the reaction for the different primer sites as described in the text.

The two primers used for amplification of the entire SSU-rRNA gene were: V1:5'CACCAGGTTGATTCTGCCTGAC3' based on the sequence of small subunit rRNA of *V. necatrix* at the 5' end (Vossbrinck et al. 1987) and 1492:5'GGTTACCTT-GTTACGACTT3' (suggested by Dr C. R. Vossbrinck, University of Illinois, Urbana, Illinois, as a modification of primer PC5 of Wilson et al. [Wilson et al. 1990]) based on highly conserved rRNA sequences across many organisms at the 3' end. This primer pair amplified a fragment of about 1.2 kb from *Ent. bieneusi* or *S. intestinalis* infected but not uninfected human intestinal tissue at an annealing temperature of 50° C.

For amplification of a region of rRNA containing known conserved and variable regions on the large and SSU-rRNA genes and the intergenic spacer region as described by Vossbrinck (Vossbrinck et al. 1993) the following primers were used: the forward primer 530f (5'-GTGCCATCCAGCCGCGG-3') is in the SSU-rRNA and the reverse primer 580r (5'-GGTCCGTGTTTCAAGACGG-3') in the LSU-rRNA. The primer set generated a fragment of about 1350 Bp for *S. intestinalis*, *Enc. cuniculi*, and *A. michaelis* and about 1550 Bp for *Ent. bieneusi*.

Based on the obtained SSU-rRNA sequence of *Ent. bieneusi* several primer pairs were evaluated by PCR for their ability to amplify only microsporidian DNA. The primer pair V1 and EB450 5'ACTCAGGTGTTATACTCACGTC3' (primer 450 is antisense and is located at position 450 based on the alignment of *Ent. bieneusi* and *Escherichia coli* (Vossbrinck et al. 1987) gave consistent amplification from infected tissue and plasmid with *Ent. bieneusi* SSU-rRNA. PCR was performed using the above conditions with an annealing temperature of 48° C. Utilizing this primer set a 348 bp fragment should be amplified from samples containing *Ent. bieneusi*. The internal 30mer oligonucleotide EB150:5'TGTTGCGGTAATTTGGTCTCTGT-GTGTAAA3' was used to confirm by southern blotting that the amplified fragment was from *Ent. bieneusi*.

Based on the obtained SSU-rRNA sequence of *S. intestinalis* several primer pairs were evaluated for their ability to selectively amplify TEM confirmed biopsy material. The primer pair V1 and SI500 5'CTCGCTCCTTTACACTCGAA3' (primer 500 is antisense and is located at position 500 based on the alignment of *S. intestinalis* with *E. coli* [Zhu et al. 1993a]) gave consistent amplification from infected tissue only using an annealing temperature of 58° C. In addition the primer set SI60 5'TGTTGATGAACCTTGTGG3' and SI500 also amplified infected tissue at an annealing temperature of 58° C.

Southern Blotting

After agarose gel electrophoresis the separated amplified fragments were transferred to Nytran membrane by capillary action (Weiss et al. 1991). The membrane was air dried and UV cross-linked for 5 min. The membrane was then prehybridized for 15 minutes at 62° C with QuikHyb buffer (Stratagene, LaJolla, CA). EB150 was labeled with (gamma-^{32}P)ATP and T4 polynucleotide kinase to 1 x10^6 cpm/pmol (9), added at 1.5 x10^6 cpm/ml to the QuikHyb buffer and incubation continued for 2 hours at 62° C. The blot was then washed at 65° C for 1 hour in 2X SSC(300mM NaCl, 30mM Na_3Citrate, pH 7.0) /0.5% SDS, washed for 1 hour at 65° C in 0.1X SSC(15mM NaCl, 1.5 mM Na_3 Citrate, pH 7.0)/0.2% SDS and then autoradiographed for 15 min at 25° C (Weiss et al. 1991).

Purification

The amplification products were purified using Magic PCR Preps™ (Promega, Madison, WI). If the amplification product had only a single band evident on 1.2% agarose gel electrophoresis then purification was conducted directly as follows. A volume of 100 µl of PCR product was added to 100 µl of purification buffer (50 mM KCL, 10 mM Tris HCl, pH 8.8, 1.5 mM $MgCl_2$, 0.1% Triton X-100) and 1000 µl of resin (Magic PCR Prep, Promega, Madison, WI) added. This was pushed through a minicolumn using a syringe, the column washed with 2000 µl 80% isopropanol, centrifuged at 15,800 *g* for 20 seconds and allowed to dry for 15 minutes. The DNA was eluted by adding 50µl of deionized water followed by centrifugation at 15,800 *g* for 20 s. If the PCR products had more than one band evident on gel electrophoresis, as was the case for *S. intestinalis* and *Ent. bieneusi*, then the desired bands were excised under a UV lamp from ethidium bromide stained low melting point agarose gels after electrophoresis. The gel fragments containing the bands of interest were melted and 1 ml of Magic PCR Preps resin DNA purification resin was added to the melted agarose slice and the purification carried out as described above.

Restriction Digests

For each organism, purified fragments were digested with 3 units of EcoRI, Hind II, Hinf I, DraI and Sau3A (Promega, Madison, WI) respectively at 37° C, overnight with the corresponding buffer solution. The digests were then electrophoresed on a 1.2% agarose gel and visualized with ethidium bromide.

Sequencing and Subcloning

The amplified SSU-rRNA PCR products were fractionated by 1% agarose gel electrophoresis and the presumptive SSU-rRNA

gene fragments of *Ent. bieneusi* and *S. intestinalis* were eluted from the gel and ligated into a *Sma*I-cut pBluescript II KS+ vector (Stratagene, La Jolla, CA) in the presence of T4 DNA ligase and *Sma*I at 20° C overnight. The plasmids were transfected into XL1-blue and plated on tetracycline/ampicillin/IPTG/X-gal agar plates. White colonies were selected and screened for those containing inserts of the correct size (Liu and Schwartz 1992).

Three independent SSU-rRNA clones of each microsporidian were sequenced to eliminate PCR misincorporation artifacts. Both strands of the SSU-RNA clones were sequenced. Clones were sequenced by the standard Sanger dideoxy method using two techniques: in the first, double stranded DNA (dsDNA) cycle sequencing using Taq polymerase (AmpliTaq cycle sequencing kit, Perkin Elmer Cetus, Branchburg, NJ) is used. In this technique 2 pmoles of primer were end-labeled using T4 polynucleotide kinase and (gamma-32P) ATP. 10–100 fmole of purified DNA were then sequenced using the end labeled sequencing primer, ddNTP termination mixes with 7-deaza-dGTP, and Taq polymerase. Sequencing reactions were analyzed on a 40cm 8% urea polyacrylamide gel. Each reaction was run for 6 hours at a constant power of 50 watts. In the second, single stranded DNA (ssDNA) sequencing using Sequenase version 2 (United States Biochemical, Cleveland, OH) to resolve areas of ambiguity seen with dsDNA sequencing was employed. Single stranded DNA was prepared from pBluescript plasmids by using the helper phage R408. T7 and T3 promoter primers were employed for sequencing plasmid in both directions using both sequencing methods. Internal primers for sequencing were designed based on *Ent. bieneusi* sequences obtained as sequencing progressed to enable sequencing of the entire SSU-rRNA *Ent. bieneusi* rRNA gene. Internal primers used for sequencing *Ent. bieneusi* were: V234:5'TCCGGAGAAGGAGCCTGAGA3' (based on *V. necatrix* sequence), V235:5'TCTCAGGCTCCTTCTCCGGA3' (antisense of V234), V785:5'GAGTTAAATTAAGCAGCAC3' (based on *Ent. bieneusi* sequence), V806: 5'GACTCAACGCGA-CCTAACT3' (based on *V. necatrix*) and V1059:5'CCGTTACT-AGGAATTCC3' (based on *Ent. bieneusi* sequence).

Direct sequencing of a PCR fragment amplified by primers 530f and 580r and purified by Magic PCR Preps™ (Promega, Madison, WI) without subcloning was accomplished by using the AmpliTaq cycle sequencing kit (Perkin Elmer Cetus, Branchburg, NJ). Sequence was obtained using the primers 530f, 580r and 228r (5'-GTTAGTTTCTTTTCCTCC-3'). Sequences obtained using primer 228r overlapped with the sequences on the 3'-end SSU-rRNA. In addition, primer EB2S (5'-ATCCAACCATCA-CGTACC-3') was used to further sequence the *Ent. bieneusi* spacer region to make its sequence overlap with that on 3'end. Sequences of the SSU-rRNA segments was compared with full length sequences that we or others had obtained by previously cloning these genes (Zhu et al. 1993a,b,c,d, Vossbrinck et al. 1993, Hartakeerl et al. 1993).

Sequence accession numbers are:

A. michaelis SSU-rRNA L15741, IGS/LSU-rRNA L20293.
Ent. bieneusi SSU-rRNA L07123, IGS/LSU-rRNA L20290.
S. intestinalis SSU-rRNA L19567, IGS/LSU-rRNA L20292.
Enc. cuniculi SSU-rRNA Z19563.

Homology of the obtained SSU-rRNA sequences was compared to the DNA sequences present in Genbank using Wordsearch and Segment programs. Alignment of the SSU-rRNA genes of *Ent. bieneusi* and *S. intestinalis* to that of *V. necatrix* and *E. coli* was based on the model of Noller and Woese (Noller and Woese 1981) using the published alignment of *V. necatrix* and *E. coli* (Vossbrinck et al. 1987).

Phylogenetic analysis

Using the alignments given in Fig. 1, maximum parsimony analysis was run on each region separately and all regions combined on a Macintosh II computer using PAUP version 3.1.1 (Swofford 1993). The branch and bound option was used to guarantee finding the shortest tree.

RESULTS

Diagnosis

Employing primers V1 and 1492 amplification of an rRNA gene fragment was obtained from *Ent. bieneusi* or *S. intestinalis* infected intestinal tissue (confirmed by TEM) but not from uninfected control tissue. This reaction was capable of detecting infection in 0.1 μg or less of starting DNA extracted from *Ent. bieneusi* infected intestinal biopsy material (data not shown). The amplified fragment was approximately 1.2 kb, consistent with the predicted size of the SSU-rRNA gene microsporidia based on *V. necatrix* 16S sequence data. Amplification of fragments of several sizes was also seen with DNA from *Escherichia coli*, *Saccharomyces cerevisiae*, and *Toxoplasma gondii* (data not shown). This is consistent with the highly conserved nature of the SSU-rRNA primers employed. No amplification at 1200 Bp was present in uninfected human tissue.

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   1 caccaggttg attctgcctg acgtagatgc tagtctctga gattaagcca tgcatgtcag tgaagcctta cggtggaacg gcgaacggct cagtaatgtt
 101 gcggtaattt ggtctctgtg tgtaaactaa ccacggtaac ctgtggctaa aagcggggaa taaggcgcaa ccctatcagc ttgttggtag tgtaaaggac
 201 taccaaggcc atgacgggta acgggaaatc agggtttggt tccggagagg gagcctgaga gatggctccc acgtccaagg acggcagcag gcgcgaaact
 301 tgtccactcc ttacaggaga gacagtcatg agacgtgagt ataacacctg agtgtaaaga ccttagggtg aagcaattgg agggcaagct ttggtgccag
 401 cagccgcggt aactccaact ccaagagtgt ctatggtgga tgctgcagtt aaagggtccg tagtcgtgaa atgcgttgtt caatagcgat gagtttgcta
 501 atctttgcgg aacggatagg gagtgtagta tagactggcg aagaatgaaa tctcaagacc cagtttagga ctacgagcga aggcgacact cttagacgta
 601 tcttaggatc aaggacgaag gcaggagtac cgaaagtgat tagacaccgc tgtagttcct gcagtaaact atgccgacag cctgtgtgtg agaatacgtg
 701 ggcgggagaa atcttagtgt tcgggctctg gggatagtac gctcgcaagg gtgaaactta aagcgaaatt gacggaagga cactaccagg agtggattgt
 801 gctgcttaat ttaactcaac gcgggaaaac ttaccagggt caagtcattc gttggatcga atacgtgaga atggcaggag tggtgcatgg ccgttggaaa
 901 ttgatgggca cctttagctt aaatgcttaa ccagtgagac ctccttgcac ggtgttctgt aacacaggag ggtggaggct ataacaggtc cgtgatgccc
1001 ttagatatcc tgggcagcaa gcgcaataca atactcttca gtagacaaag tgatttgaga tgagtaggat ctacgtttgt aaatacgtag tgaataagga
1101 attcctagta acggtgcctc atcaaggcat tgtgaatgtg tccctgttct ttgtacacac cgcccgtcac tatttcagat ggtcataggg atgaagagct
1201 tcggctctga atatctatgg ctagataaag tacaagtcgt aacaaggtaa
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Fig. 1. Sequence of the SSU-rRNA gene of *Enterocytozoon bieneusi* (Genbank L07123).

Cloning and sequencing of the amplified *Ent. bieneusi* gene provided the resultant sequence shown in Fig. 1 (Genbank accession number L07123). No sequence identical to this *Ent. bieneusi* SSU-rRNA gene was present in the Genbank database. On homology searching the *Ent. bieneusi* SSU-rRNA sequence has the highest homology with the SSU-rRNA gene of *V. necatrix*. Limited homology was present to yeast, bacteria, plasmodium, and mammalian rRNAs in the database. We believe, therefore, that this sequence is the SSU-rRNA sequence of *Ent. bieneusi*. In a similar fashion sequencing of the cloned SSU-rRNA of *S. intestinalis* and direct sequencing of the fragment amplified by 530f and 580r provided the resultant genbank sequence L19567.

Several primer sets were evaluated for their ability to identify *Ent. bieneusi* infected human tissue based on the predicted rRNA sequence and structure. Of these, the most useful set was V1 and EB450. As can be seen in Fig. 2A in infected tissue and in the control plasmid containing the rRNA clone of *Ent. bieneusi* amplification of the predicted 348 Bp fragment is evident on ethidium stained gels. Using an internal oligomer hybridization is present strongly to all of these samples (Fig. 2B). In addition lane 8 from an HIV-1 patient with nausea but without diarrhea also demonstrated a weak signal. This may represent a microsporidian infection in this patient that was not seen on microscopic examination of the tissue. Unfortunately TEM examination of this tissue was not possible. In all other negative controls (10 HIV-1 patients with diarrhea, 1 HIV-1 patient without diarrhea and 2 HIV-1 negative patients) no amplification was seen by ethidium staining or detected by hybridization. No amplification of DNA prepared from *Toxoplasma gondii, Trypanosoma cruzi, E. coli, Saccharomyces cerevisiae*, RK-13 (rabbit kidney cells), *V. necatrix, Enc. cuniculi, S. intestinalis, Glugea* or *Nosema* was seen with this primer pair. This primer pair was also able to detect *Ent. bieneusi* in two duodenal aspirates from infected patients.

In addition, primer pairs were designed based on the obtained *S. intestinalis* sequence and evaluated for their ability to amplify only intestinal biopsy material from patients with TEM proven *S. intestinalis*. Primer pair V1 and SI500 amplified a 370 Bp fragment from infected but not from uninfected tissue. No amplification was seen with *Ent. bieneusi* infected tissue samples or with *Enc. cuniculi*.

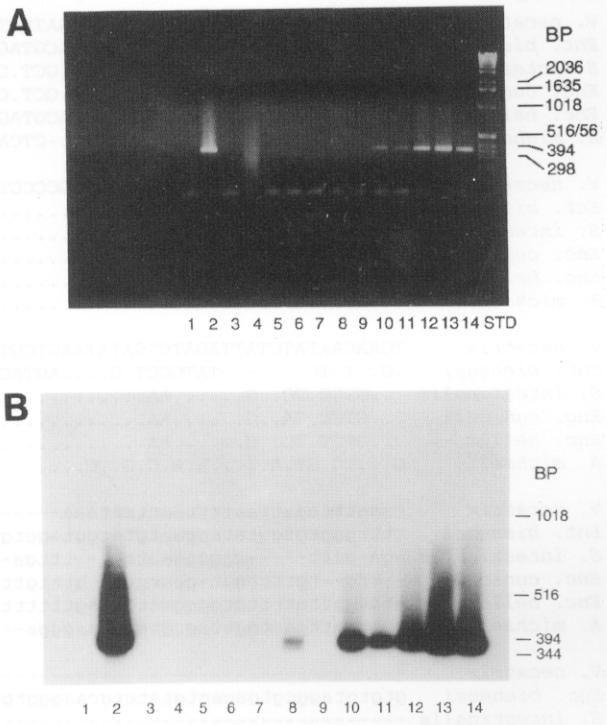

Fig. 2. Amplification of *Enterocytozoon bieneusi* SSU-rRNA 348 bp fragment by primer V1 and EB450 from various tissue samples. **A:** Ethidium bromide stained gel of various samples amplified with primer set V1 and EB450 as described in materials and methods. Lane 1: Blank. Lane 2: EB8 (Bluescript KS+ containing *Ent. bieneusi* SSU-rRNA). Lane 3: *Escherichia coli*. Lane 4: *Saccharomyces cerevisiae*. Lane 5: Human immunodeficiency virus (HIV-1) negative intestinal biopsy. Lanes 6–9: HIV-1 positive intestinal biopsies with no known microsporidia. Lanes 10–14: HIV-1 patients with TEM confirmed *Ent. bieneusi* infection.
B: Southern blot of ethidium bromide stained gel in Fig. 2A probed with EB150 as described in materials and methods. Lane 8 is weakly positive (more evident on longer exposure). This is an HIV-1 patient with nausea and no microsporidia by light microscopy (no TEM available). All other signals are in known positive patients except lane 2 which is the plasmid containing the cloned *Ent. bieneusi* SSU-rRNA gene. Lanes as indicated in Fig. 2A. This hybridization to EB150 confirms that primer set V1/EB450 amplifies the SSU-rRNA gene from *Ent. bieneusi* infected human tissue. Thus these primers may prove useful in diagnosis and epidemiologic studies.

Phylogeny

Figure 3 (Zhu et al. 1993e) shows the alignment of six microsporidian species rRNA sequences on the amplified region containing SSU-rRNA, IGS and LSU-rRNA. The sequences of *Enc. cuniculi*, *Enc. hellem* and *V. necatrix* are as previously published (Vossbrinck et al. 1987, 1993, Hartakeerl et al. 1993, Zhu et al. 1993d). The complete SSU-rRNA sequences of *Ent. bieneusi* (Zhu et al. 1993b), *S. intestinalis* (Zhu et al. 1993a) and *A. michaelis* (Zhu et al. 1993c) are published. Sequencing of the amplified segments was done using the 580r and 228r primers. Primer 228r reads LSU-rRNA, through the IGS region and into 3'-end SSU-rRNA. Therefore, the sequences obtained by primer 228r overlap with those by 580r and 1492. Because of the longer length of *Ent. bieneusi* spacer (232-bp), primer EB2S was designed to allow overlap with the 3'-end sequence. The highly variable, moderately variable and

228r region

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V. necatrix      ATCTTTATGGGATAATATTTTGTAAGAGATATTTGGAACTTGGAATTGCTAGTAAATTTTATTAAATAAGTAGAATTG
Ent. bieneusi    .GA.GAG.A....CTACG.......ATACGTAG.-....TAA......C.......CGG.GCC.C.TC...GCATTG..
S. intestinalis  GAC.G.GA.....G.AGC.G.....TG.GCT.C-.....G.......C.......TAACAT.G..CC....T.TT...
Enc. cuniculi    C.-G.G.G.....G.AGC.G......G.GCT.C-.....G.......C.......TAGCGGC.G.CG...CT.CT...
Enc. hellem      C.-G.G....T..CTACG.......TTACGTAG-....TAA......C.......GAA.G...G..C....TATT...
A. michaelis     ..AGA..G..AT.G.GGGC.-.A...C.-CTCA-.-...AC.....A........CG.CAG..C....TACG.CGA..

V. necatrix      AATGTGTCCCTGTTCTTTGTACACACCGCCCGTCGCTATCTAAGATGA-TATGTGTTGTGAAATTAGTGAAAACTACT
Ent. bieneusi    ..............................A....T.C.....GTC..A..GA-....GAGCT.-CG---GCTC
S. intestinalis  ....G........C.............................CGCA...GAAC...GA.TGG-..---GGTC
Enc. cuniculi    .............C.............................CGCAC..GAAC...GA.CCG...---GGTC
Enc. hellem      .............C.............................CGCAG..GAAC...GA.TTGAG.---GGTC
A. michaelis     ...A.............................T....G......G-AG..AT..T...GTCA.T.AT..--.TGG

V. necatrix      TGAACAATATGTATTAGATCTGATATAAGTCGTAACATGGTTGCT-GTTGGAGAACCATTAGCAGGATCATAAtaagt
Ent. bieneusi    .G..T.TC-------TATGGCT.G....AGTAC--..A....C.AG...CC..TGAG.CC...T.A.GACATTt-cag
S. intestinalis  ...GTCC.TC..G......AA.....................C....-............GC..........GT.t-ttg
Enc. cuniculi    ...GTCC.GA..G......AA.....................C....-............GC..........GT.tgttg
Enc. hellem      ...GTCT.TC..G......AA.....................C....-............GC..........GT.tgttg
A. michaelis     C...TG.GT.A.TC..A.A.C.G..C...........A..C.A.G-....A....T..GC..T.....T.GCGtgttt

V. necatrix      taaatttaaattaattttaattattaaa----------
Ent. bieneusi    ttttgggtgtggtatcggaatgtatggtaggtgatgtgtgtgtgtatggggatgccgagggaccagcagtgcggtggt
S. intestinalis  aga-gatt-----ggggggaatttt--tttga----------
Enc. cuniculi    --ttg--tgttttgat-ggatgttt-gtttgtttgtgg----------
Enc. hellem      atttgattatttttgtggggatttttagttttttagttttc----------
A. michaelis     -gttaattgaatggttagggtctacaaggga----------

V. necatrix      ----------
Ent. bieneusi    gtgtgtaggcgtgagagtgtatctgcaagggtgagggatgtgggtgcagtgagttagaggtggttccatgtggaatag
S. intestinalis  ----------
Enc. cuniculi    ----------
Enc. hellem      ----------
A. michaelis     ----------

V. necatrix      ----------TTTACCC
Ent. bieneusi    tgggatggtacgtgatggttggatgggggaatgatgtgtgtatgggtagcaaacggaggttgcggtcacggCAGTAGG
S. intestinalis  ----------...GAGG
Enc. cuniculi    ----------...CT.T
Enc. hellem      ----------...CT.T
A. michaelis     ----------.CA.TTG

V. necatrix      ACACATGGGATCAATAGGATACCATAACGATG-AAGGTCGTAATAGAATACGA----AAGTATATTATTT-ACC-TAA
Ent. bieneusi    GT-GCCCATCAAG.GGT.TATTTGG..AT.CCC..-.A..GGCACTTGG...CGCGATGCG.GCGCGAAAG.A..CG.
S. intestinalis  .TT..C.....TG.....CATTAGC.G...G.-....A...GTAG..GG....TATGC-C.T.T.-----G.GGAAGT
Enc. cuniculi    GTT..C.....TG..T..CATTAGCGG.....-....A...GCAG..GG.-..TATGC-..TGT.GT.G.G..GG.GT
Enc. hellem      .TC........TG..T..CATTATCTG.....-....A...GC.G..GG....TATGCC..T.T..T.A-GCGGA.GT
A. michaelis     GTT.-----T..GG.T.AGA.AGGATGG.CAC-....GACC.CA.TTGGT.TCTTGGG..A.AGGATGGG-.GAG..C

V. necatrix      -TTAAT-ATAT-T
Ent. bieneusi    AAAGTCCTCTCT.
S. intestinalis  C.G...GTAGCGG
Enc. cuniculi    C.G...TG.G.GC
Enc. hellem      C.G...T.GG.G.
A. michaelis     CCAC.A-TGGAT.
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530 region

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V. necatrix      AAGAGTGTGTATGATGATTGATGCAGTTAAAAAGTCCGTAGTTTATA----------TT----------
Ent. bieneusi    ........C....G..GA..C..........GG.........CGTG.----------AATGCGTTGTTCAATAGC
S. intestinalis  ..T.....C....G...A..C.....................C.T.G---------TATGTCTTTGTTTG-GGGGATT
Enc. cuniculi    .GT..A..C....G..GA..C..........T.........C.G.T------GTGTATGTCTTTGTGTGTGATGTTT
Enc. hellem      .GT..A.......G...A.............T..........-G.T-------TGTATGTCTTT-TGAGTGATG-TT
A. michaelis     .G...CT.A.TC...--A..T...G.......C.........CGCGGCTGGGACTGACCTGTAATCTATTTGGTCAAC

V. necatrix      -------------------------TAAGAAGCAATATGAGGTGTACTGTATAGTTGGGAGAGAGATGAAATGTGACG
Ent. bieneusi    GATGAGTTTGCTAAT----------CTTTGC.G..CGGATA.G.AGTGTAG..TAGACTG.C..AGAATG.AA.CTCA
S. intestinalis  ATGTCCTGATGTGGATGTAA-GAGG.TT.GCAG.GG.C....G.C..C.G.........C...G.G......AC..A.
Enc. cuniculi    GTGGTTGTGTGTGGATGTAGTGATG.GT.GCAG.GG.C....G.C....G.........C.....G.......C..A.
Enc. hellem      GGTTTTCTGTGGATGTAGTTTTAT-.GT.-TAGC.G.N....G.C....G.........C.....GG.TG.AA.A.A.
A. michaelis     AGATAGATAGGGGCAGTAGCAAGTTGG.A...AGCA..TT.....CAGC..AT.G.AT.G.GAG.GGT...GTCTGA.
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"(Continued)"

"(Continued)"

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V. necatrix     ACCCTGACTGGACGAACAGAAGCGAAA-GCTGTACACTTGTATGTATT-TTTTGAACAAGGACGTAAGCTGGAGGAGC
Ent. bieneusi   .GA.CC.G.TT.G..CT.CG......G-..GAC..T...AG.C....C-..AG.AT....G...A.G..A....T.C.
S. intestinalis ..............G..........G.....G.T....G.CT...-G.GAC..TG.......A.G..TA......T
Enc. cuniculi   ..............G..........G-.....G.T....G.CTA..G-..GC..TG.......A.G...A.....T.
Enc. hellem     ..............G.........G-.....GTT....G.CT.T.G-.GG...TG.......A.G..TA.....T.
A. michaelis    GAT.CTG.A...G..G..A.G...T..-..AC.GACAAA.AT..AT.C-.G....T.......AG.G...A.....T.

V. necatrix     GAAGATGATTAGATACCATTGTAGTTCCAGCAGTAAACTATGCCGACGATGTGATATGATA-TTA--ATTGTATTAGA
Ent. bieneusi   ...AG........C...GC.........T..................AGCC..TC-..TG.------GAATACG.G.G
S. intestinalis CG.A..CGA........G..T......T..........G........TGACG.GAC.-...-.AG----...TG.GC.
Enc. cuniculi   ...A.C...........G..T......T..........G........TGGAC.GG-.C.GT-------G...G..GCC
Enc. hellem     ...ATC...........G..T......T..........G........TGGACAGGGACTGT-..T--.G...TG.CCG
A. michaelis    ...T.C...........G.A.......T......G.C.G...AT..TTT..CCT....CA.----------..-G...

V. necatrix     TGATAGAAATTTGAGTTTTTTGGCTCTGGGGATAGTATGATCGCAAGATTGAAAA--TTAAAGAAATTGACGGAAGAA
Ent. bieneusi   C.GG......C.T...G..CG................C.C.......GG.....CTTAA.GC.............G.
S. intestinalis ...G.-..TC......A.G.G..T................C.T......G.....C--..G..................
Enc. cuniculi   ATGAGA..TC......A.GCG..T................C........G.....C--..G....G...........G.
Enc. hellem     AA.AGA..TC..A...A.G.N..T................C........G.....C--..G....G...........G.
A. michaelis    AA-.CA....---..AT------...C......--G..CATG.....---CA.G..C--..-...-...........CT

V. necatrix     TACCAGAAGGAGT
Ent. bieneusi   C..T.CC......
S. intestinalis C....C.......
Enc. cuniculi   C....C.......
Enc. hellem     C....C.......
A. michaelis    AG...C....GT.
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580r region

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V. necatrix     -ACC-TAA-TTAAT-ATAT-TTATAATGTATTGATAACCCTTTGAACTTAAGCATATCTTTAAA-GGAGGA-AAGAAA
Ent. bieneusi   GGGGAATGC.CT.AT-...G-..-.TCTC..G....CT..C.-.G.............A..-....A..A.-......
S. intestinalis G.GGAAGTC.G...G.AGCG-G.-.GC.CT.G.-GG....C..TG...........C.AA.....AAG.AGG......
Enc. cuniculi   G..GG.GTC.G...TG.G.GCG-GTG..C.CG.-GG....C...G............A-....-.A..A.G......
Enc. hellem     GCGGA.GTC.G...T.GG.G..TGTGC.CC-..TGG....C..TG............TAC....G....A.GN.....
A. michaelis    TGAGTGC.AACT..G.G.A-.CCGTC.C.GACA.GT..T.C.....T.G....C.C.TAG...........A......

V. necatrix     CTTACTTGGATTTCTTTAGTAGCAGCGAGTGAACTAGAAATTACCCTCCNTTGTAATCCTTAATTG-GAGNTGTAAAT
Ent. bieneusi   ..A...A.....C.G.......-T....A......C.G.GGAG.T.CGAA.G....AG.C...G..-CT.T.....TG
S. intestinalis ACA.AG-.....GTC.G......G.........AGGAC.GGAG-.TCGAA.G....CTGCGTT.G.C.G.T...-TT.
Enc. cuniculi   .CA.A.-.....G.C.G......G.........A.G.CATGAG-...GTG.G......GCGTT.G.C.G.T...GTTT
Enc. hellem     ..A.A.A.....ATC.G..............AGGAT.....-...GTG.G......ACGTG..T-.A.T...GAAT
A. michaelis    ..A..AA....AC.CG......-T....AC.....G..T.-G.GT..G.TA........GC..--.-...A...T..G

V. necatrix     --GGTTTATTCTATTNATTATTTTA-TAGAGAATATTATA-TTCGTTA-TTAATA------G-AATAGAAT----AAT
Ent. bieneusi   TA.CC.CCGAAACA.CTGG.AAGG.TG.CCC.CAG.GGGTGA.A.G.GA.A-G.C-----.C-C.G..G.GGAT-GTA
S. intestinalis GA.-.AGGAGGAGACTGAGC.AGGGA.T.---TC-CCGATC..A.AGG-GA.TGGCCTGT-AAGTATCCGGACCTTGA
Enc. cuniculi   GT..CAGCAG.GGCGAG.G--CG-.-.T..A.TC-GG..GCGGA.AGG-GCG..GTGCCT-.T.GGC.CGGTCGGC..
Enc. hellem     TA..A.G.CGA.GC.G.GGCACGG.-.T.GA.TCTG.T.TC..A.GGG-GTG...GGCCT-.T..GT.TTGTCTTTT.
A. michaelis    --.C.GC.AAGG.AGTCAGT.GGA.TGCTGCG.A.GATA.-GGT.A..TCCCT..-----.A-..CGTT.G----..A

V. necatrix     --GTACTTGAGTAGAGCTGCTTGGTNGTGCAGTTTGAATAAAGGTAGAATGAAATATCTAAGGTTAAATATA-ATGGT
Ent. bieneusi   AA.G.A.A......T.........A.......A....G.GGA.--.T.-....CT.....A.C......C.G.C..-
S. intestinalis GA.A.GAA...GGTGT...G.A.TGCCGC.TTGA.T-GGGTG.TGTTCT.TTC.C.GAA.GCTAA.T...AGCTAAC.
Enc. cuniculi   TTT.TG.-......T.T........A......CC-....G-C...G.T-G..TGC.C.G....CN.....TG-GCT.A
Enc. hellem     TT..GA.-......G.T..T.....A......CC.....GTG...G.T...-TGC...C....CT.....TGTGAT.A
A. michaelis    A-------A.....CCA.A......A...G.TGG...--.GC...G.TT.TTTCC...G...AC......C.GGAA..

V. necatrix     ATACCGATGGAAAATAAGTACTGCGAAGGAACTTGTGAAAATGTGTGG-AGT
Ent. bieneusi   ........A..G..G.....G......C...AG.-......GA.C.CATCTA
S. intestinalis GA-.....A.CG..G.....GA.T..TT...-A........A.ACAA.AT.AA
Enc. cuniculi   TG......A.CGG.......GA.T..TT...-G........G.A.-.ATGA.
Enc. hellem     TG......A.CG........GA.T..TT...-G........-.AT.AATGA.
A. michaelis    .G......A.CG........GA.T..TC...AG.......T...AGACTTA
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Fig. 3. Alignments of six microsporidia rRNA sequences on small subunit, large subunit and intergenic spacer region. Numbering is based on *Escherichia coli* (Vossbrinck et al. 1987).

highly conserved regions seen in *Enc. cuniculi*, *Enc. hellem* and *V. necatrix* were also observed in *Ent. bieneusi*, *S. intestinalis* and *A. michaelis*. Thus, the sequences on these 3 regions are appropriate for comparison with other species. Restriction digestion of the amplified fragments of *S. intestinalis*, *Ent. bieneusi*, *A. michaelis* and *Enc. cuniculi* by EcoRI, HindIII, HinfI, DraI and Sau3A were conducted (data not shown). The fragments conformed to that predicted by the sequence data. The restriction patterns for these organisms were unique in these regions allowing discrimination among these species. These studies agree with those of Vossbrinck (Vossbrinck et al. 1993) suggesting that restriction digests of these amplified regions may be useful for the identification of microsporidia as more data on the rRNA of the phylum become available.

Table 1 (Zhu et al. 1993e) shows the percent sequence differences among the six species sequenced for all these regions separately and combined according to the Macintosh PAUP version 3.1.1 data distance program. In all regions combined, *Enc. cuniculi / Enc. hellem* revealed the smallest distance, 0.169, *S. intestinalis / Enc.hellem* was 0.268 and *S. intestinalis / Enc. cuniculi* was 0.227 suggesting that *Enc. cuniculi* and *Enc. hellem* belong to the same genus and that *S. intestinalis* belongs to the Encephalitozoonidae family and likely is a separate genus as classified by morphology. Much larger differences exist between the other microsporidia thereby supporting their classification into different families. Similar data are obtained from examining each region separately. For example, in examining all of the regions combined the distances are 0.426 between *V. necatrix* and *A. michaelis*, 0.432 between *Ent. bieneusi* and *V. necatrix*, 0.463 between *S. intestinalis* and *Ent. bieneusi*. *V. necatrix* and *Enc. cuniculi / Enc. hellem* showed a distance of 0.339 / 0.334. These distances differ from those reported by Vossbrinck (12) perhaps due to slight differences in alignment or to the version of PAUP used. However it does not affect the subsequent phylogenetic analysis.

Fig. 4 (Zhu et al. 1993e) demonstrates an unrooted cladogram of the six species examined. Sequence alignments on each region and all regions combined were done by PAUP version 3.1.1 equally using a Branch and Bound search. The consensus tree is the same from each individual region and all of the regions combined. On an analysis of all regions (228r, 530f, 580r) separately or combined *S. intestinalis*, *Enc. hellem*, and *Enc. cuniculi* are clustered. *Ent. bieneusi* is distantly related to the other microsporidia, thereby supporting its current classification.

DISCUSSION

The data presented here represent a summary of work done in this laboratory for the past two years on the use of rRNA from microsporidia for the identification and phylogeny of the microsporidia. We have described the small subunit rRNA sequence of the non-cultivatable microsporidia *Ent. bieneusi* and *S. intestinalis* which were obtained by homology PCR cloning from intestinal biopsies of patients with HIV infection and intractable diarrhea presumably caused by these pathogens (cases documented by transmission electron microscopy). This technique of using conserved rRNA sequences to identify non-cultivatable human pathogens from tissue has been used previously to identify the bacteria involved in bacillary angiomatosis (Relman 1990) and Whipple's disease (Relman 1992), but to our knowledge, has not been used previously to identify non-cultivatable eukaryotic organisms in human tissue. The identification of the correct amplified gene fragment was aided by the difference in size of SSU-rRNA of microsporidia (1,250 Bp) versus mammalian 18S rRNA (1,870 Bp). This method is applicable not only to human microsporidia but to any microsporidia for which limited material exists and can be used on archival material to build a molecular phylogeny of the phylum.

The SSU-rRNA sequence of *Ent. bieneusi* has been used to define a primer pair for use in a PCR reaction. This primer pair could identify tissue infected with *Ent. bieneusi* as well as duodenal fluid from infected patients. Interestingly one biopsy from an HIV-1 positive patient without diarrhea used as a control was positive on PCR. Unfortunately no further tissue was available on this patient for TEM or additional light microscopic examination. It is possible this patient had an undiagnosed microsporidial infection as all of the other control tissue did not amplify.

The primer pair that reliably amplified *Ent. bieneusi* from infected tissue samples consisted of V1 which is a primer based on the conserved sequence at the 5' end

Table 1. Percent sequence difference among six species of microsporidia. Sequences are compared for all regions (530f, 228r and 580r) combined. Below the diagonal is the absolute number of different bases and above the diagonal is the percent differences.

Species	1	2	3	4	5	6
1. *V. necatrix*	-	0.432	0.385	0.339	0.334	0.426
2. *Ent. bieneusi*	428	-	0.463	0.434	0.425	0.505
3. *S. intestinalis*	377	469	-	0.227	0.268	0.505
4. *Enc. cuniculi*	335	446	234	-	0.169	0.449
5. *Enc. hellem*	337	444	281	179	-	0.455
6. *A. michaelis*	416	503	505	456	467	-

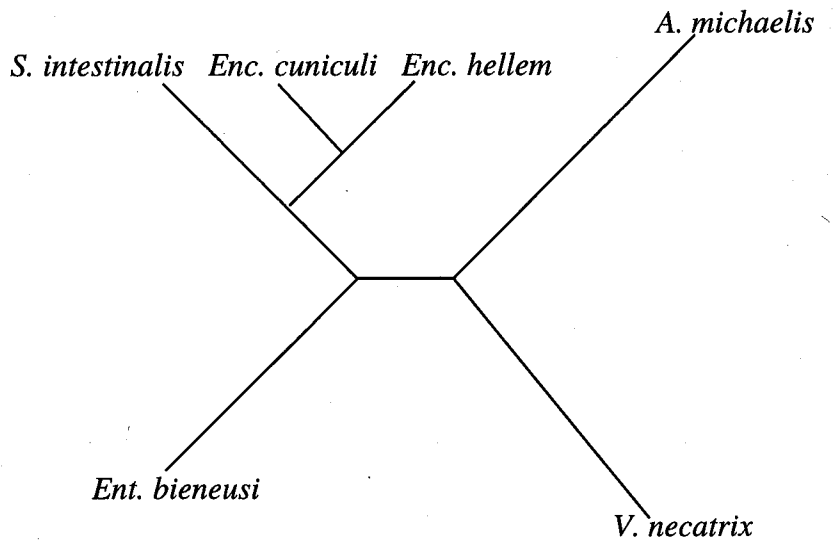

Fig. 4. Unrooted tree based on maximum parsimony analysis (PAUP) of the sequence data from Fig. 1 for the microsporidia *Enterocytozoon bieneusi*, *Septata intestinalis* and *Ameson michaelis*.

and EB450 which is based on *Ent. bieneusi* specific sequences. In our evaluation of other primer sets (data not shown) primer pairs consisting of two *Ent. bieneusi* specific primers only did not reliably amplify infected tissue, although such primers amplified the plasmid containing the cloned *Ent. bieneusi* SSU-rRNA gene. It is possible that the secondary structure of the SSU-rRNA gene present in the organism accounts for this observation.

We were also able to use the sequence data from *S. intestinalis* to define a primer pair V1 and SI500 that could also identify tissue infected with this organism and did not amplify uninfected tissue or tissue from patients with *Ent. bieneusi*. As new microsporidia are identified as opportunistic pathogens in AIDS patients these conserved rRNA primers should prove useful for obtaining rRNA sequence information which can aid in phylogeny or in the development of diagnostic tests. While PCR was used to confirm the utility of the sequence data for diagnosis final epidemiologic studies may require other assays such as *in situ* fluorescence hybridization or the hybridization rRNA assays that have been developed for mycobacteria.

Using the highly conserved primer 580r in the LSU-rRNA together with the conserved SSU-rRNA primer 530r (Vossbrinck et al. 1993) we obtained ribosomal sequence data from several non-cultivatable microsporidian organisms in the presence of non-microsporidian DNA such as in human intestinal biopsy specimens. The primer set 530f-580r appears to recognize highly conserved regions in the phylum Microspora and is a useful tool for further phylogenetic analysis. The highly variable, moderately variable and highly conserved regions seen in *Enc. cuniculi*, *Enc. hellem* and *V. necatrix* are also observed in *Ent. bieneusi*, *S. intestinalis* and *A. michaelis*. Thus, it appears that the region 530f–580r contains sufficient information to be useful for studying the molecular phylogeny of the phylum Microspora. Our analysis confirmed that *Enc. cuniculi*, *Enc. hellem*, and *S. intestinalis* are closely related, most likely at the family level and that each is an independent organism. This method should be especially useful for the study of phylogenetic relationships when material is limited or where culture is not feasible and sufficient morphologic features may not be present for classification. In addition, this method can be applied to archival material to develop a molecular phylogeny of the Microspora. The development of such information may suggest the reservoir hosts for the microsporidia infecting man.

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