

Application of Molecular Techniques to the Diagnosis of Microsporidial Infection

Daniel P. Fedorko, Ph.D., and Yasmine M. Hijazi, M.D.

National Institutes of Health, Bethesda, Maryland, USA

Microsporidia are now recognized as important pathogens of AIDS patients; the ability of these parasites to cause disease in immunocompetent persons is still being elucidated. Improved diagnostic tests for microsporidial infection are continually being sought for establishing diagnosis in order to avoid laborious electron microscopy studies that require invasively acquired biopsy specimens. Modified trichrome or chemofluorescent stains are useful for detecting microsporidia in bodily fluids and stool specimens, but they do not allow for speciation of microsporidia. Polymerase chain reaction with specific primers will allow the detection and speciation of microsporidia in biopsy tissue, bodily fluids, and stool specimens.

Microsporidiosis is truly an emerging infectious disease. Although microsporidia were discovered more than 100 years ago (1), the first well-documented case of human microsporidiosis was not reported until 1959 (2). Human microsporidiosis remained an uncommon infection until the human immunodeficiency virus (HIV) pandemic; the first cases of microsporidial infection in HIV-infected patients were reported in 1985 (3,4). Since then, more than 400 HIV-associated microsporidial infections have been reported, which indicates that microsporidia are common opportunistic pathogens in patients with AIDS (5). Infections caused by three new species, *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, and *E. hellem*, have been described in patients with acquired immunodeficiency syndrome (AIDS). Originally thought to be an opportunistic pathogen of AIDS patients exclusively, *E. bieneusi* was reported in 1994 to be the cause of acute, self-limited traveler's diarrhea in an immunocompetent person who was not infected with HIV (6).

As microsporidia have been increasingly recognized as pathogens of both immunosuppressed and immunocompetent persons, the need for rapid and specific diagnosis of microsporidial infection has arisen. For instance, albendazole therapy can cure *E. intestinalis* infections but is reported to be of little benefit to patients infected with *E. bieneusi* (5); therefore, identifying microsporidia to species level could have important implications in the clinical management of patients. Current diagnostic meth-

ods for microsporidia infection, though continually being improved, have shortcomings that molecular diagnostic techniques may be able to overcome.

Biologic Properties of Microsporidia

Microsporidia are obligate intracellular parasites that infect most invertebrates and all classes of vertebrates. They belong to the phylum *Microspora* in the subkingdom *Protozoa*. They are considered ancient eukaryotes that multiply by binary fission and have a membrane bound nucleus; they lack mitochondria and Golgi membranes (5). In addition to causing disease in humans, these parasites cause disease in insects, mammals, and fish; therefore, they may create economic headaches for industries such as fisheries and silk production. They may also be beneficial to humans as biologic control agents for such pests as grasshoppers and locusts (5).

In host cells, microsporidia replicate either in a parasitophorous vacuole, like the members of the genus *Encephalitozoon*, or directly in the cytoplasm like *E. bieneusi*. Vegetative and spore stages of the organisms can be found in the host cell as the parasite undergoes merogony and sporogony, resulting in the production of the infective spore stage of the parasite (7) (Figure 1). The spores of microsporidia contain the uniquely characteristic coiled polar tubule (Figure 2). Under appropriate conditions within a suitable host, the polar tubule of the spore is extruded (Figures 3 and 4). Contact of the end of the tubule with a host cell membrane allows the spore to transfer its contents (sporoplasm) to initiate infection within the new host cell. An influx of calcium into the spore coincides with the extrusion of the polar tubule, and this mechanism may provide a target for therapy for microsporidiosis since calcium

Address for correspondence: Daniel P. Fedorko, Ph.D., Clinical Pathology Department, Warren G. Magnuson Clinical Center, National Institutes of Health, Building 10, Room 2C-385, Bethesda, MD 20892-1508; fax:301-402-1886; e-mail:dfedorko@nih.gov.

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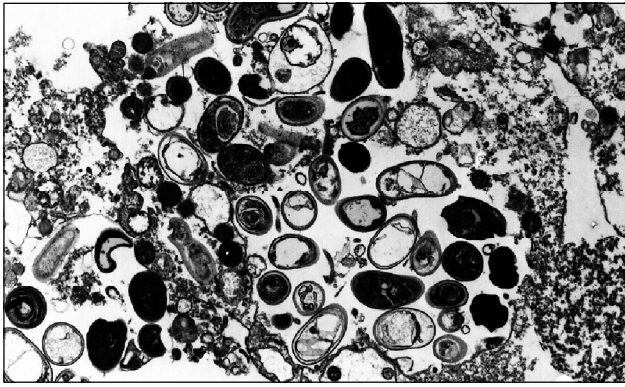


Figure 1. Transmission electron micrograph of a host cell from cell culture parasitized by *Encephalitozoon intestinalis*. Both vegetative forms and spores can be observed inside the parasitophorous vacuole. Original magnification, x5,200.

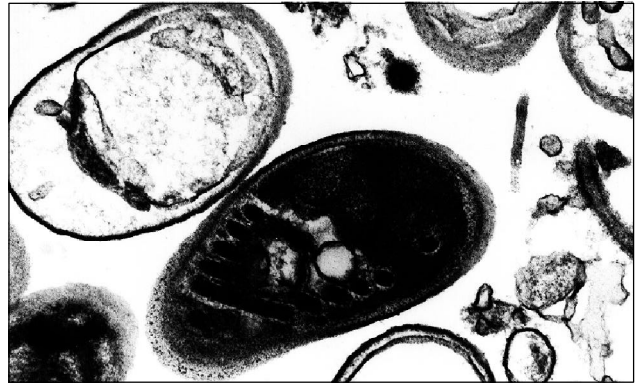


Figure 2. Transmission electron micrograph showing the coiled tubule within an *Encephalitozoon intestinalis* spore from cell culture. This unique structure is diagnostic of microsporidial spores. Original magnification, x28,500.

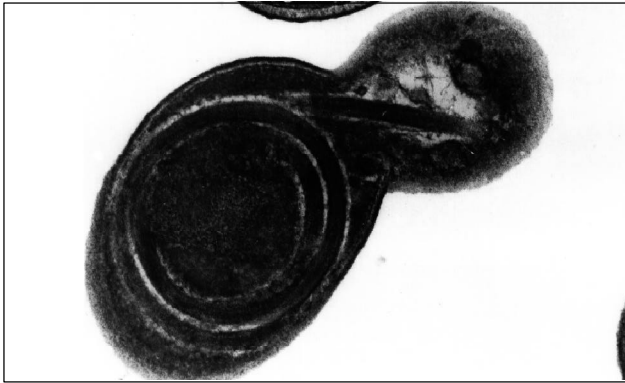


Figure 3. Transmission electron micrograph of a cell culture-derived *Encephalitozoon intestinalis* spore showing the polar tubule in the process of being extruded. The coiled arrangement of the tubule within the spore is clearly demonstrated. Original magnification, x39,000.

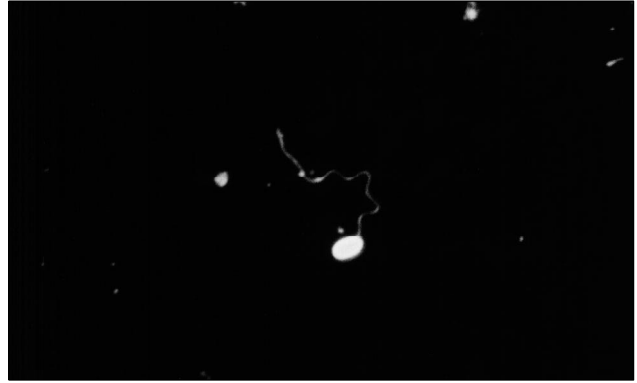


Figure 4. *Encephalitozoon hellem* spore from cell culture showing the extruded polar tubule stained by indirect fluorescence antibody by using rabbit polyclonal antibody and FITC-labeled anti-rabbit antibody. Original magnification, x1,000.

target for therapy for microsporidiosis since calcium channel blockers have been shown to inhibit extrusion and the infection of host cells in vitro (8).

The human microsporidial pathogens and their clinical manifestations are shown in Table 1. The most common microsporidial disease is prolonged diarrhea with wasting caused by *E. bienersi* or *E. intestinalis* in AIDS patients with CD4 T-cell counts below 50 cells/ul. Microsporidia have been reported in up to 39% of AIDS patients with diarrhea (9). Microsporidia may disseminate to cause systemic infection (Table 1); these organisms have been observed in urine, bile, and duodinal aspirates, as well as in ocular, sinus, bronchial, renal, hepatic, and other tissue (5). *E. hellem*, which primarily causes eye infections, has been exclusively found in AIDS patients. Ingestion and inhalation of spores have been suggested as likely modes of transmission for microsporidia (5,10). Biochemical, immunologic, and

molecular studies performed on *E. cuniculi* isolates from mice, rabbits, and dogs indicate that the parasite can be classified into at least three strains (11). An evaluation of the immunologic and molecular characteristics of *E. cuniculi* isolated from humans and rabbits indicated that the isolates were an identical strain, thus suggesting that this microsporidion is a zoonotic parasite (12).

Traditional Diagnostic Methods

Transmission Electron Microscopy

Definitive diagnosis of microsporidial infection relies on observing microsporidia in biopsy tissue, bodily fluid specimens (e.g. urine, sinus aspirates, bile, cerebral spinal fluid), or stool examined by transmission electron microscopy (TEM). Microsporidia can be identified to genus or even species level on the basis of morphologic character-

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Table 1. Named human microsporidial pathogens and their clinical syndromes

Microsporidia genus and species	Clinical syndromes
<i>Pleistophora species</i>	Myositis
<i>Nosema connori</i>	Disseminated infection
<i>N. ocularum</i>	Keratitis
<i>Vittaforma corneae</i>	Keratitis
<i>Encephalitozoon cuniculi</i>	Peritonitis, fulminant hepatitis, seizures, rhinosinusitis
<i>E. hellem</i>	Conjunctivitis, keratoconjunctivitis, bronchiolitis, pneumonia, rhinosinusitis, disseminated infection
<i>E. intestinalis</i>	Diarrhea, disseminated infection
<i>Enterocytozoon bieneusi</i>	Diarrhea, wasting syndrome, cholecystitis, cholangitis, bronchitis, pneumonia

Source: ref. 5.

istics. All stages of the parasite's life cycle can be found in infected tissue, but only the spore stage can be observed in bodily fluids and stool. The size and ultrastructure of spores, particularly the configuration of the coiled tubule, distinguish *E. bieneusi* from the *Encephalitozoon* species (Figures 5 and 6). Limited success was reported when TEM was used to detect spores in bodily fluids and stool specimens to avoid obtaining specimens by invasive procedures (5). Tissue specimens are required for speciation within the *Encephalitozoon* genus because the meronts and sporonts, nuclear configuration, and location of replication in the host cell must be carefully studied (7,10). Problems with the use of TEM include its lack of sensitivity when performed on bodily fluids and stool, the requirement for invasive procedures to acquire biopsy specimens, and the laborious procedures for specimen preparation and examination.

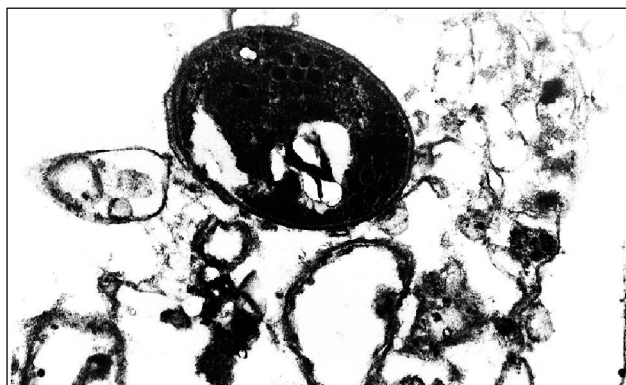


Figure 5. Transmission electron micrograph of an *Enterocytozoon bieneusi* spore in a stool specimen showing the characteristic arrangement of the polar tubule in six turns with two tiers. Original magnification, x28,500.

noticeable tissue inflammatory response make microsporidia detection difficult (5). Once again, the invasive procedure required for obtaining specimens and the length of time needed for processing them are major drawbacks.

Diagnosing microsporidial infections by light microscopy examination of noninvasively acquired specimens has been a challenge for laboratorians. Microsporidial spores are not observed in traditional ova and parasite examinations, and the parasites are generally overlooked in Gram-stained preparations of stool samples because their size, shape, and staining characteristics are similar to those of many enteric bacteria. Weber's modification of the trichrome stain has allowed more definitive identification of microsporidia by light microscopy (15). With this stain, microsporidial spores appear bright pinkish-red, and most have a distinctive diagonal or equatorial line that allows microsporidia and bacteria to be easily differentiated (Figure 7). The

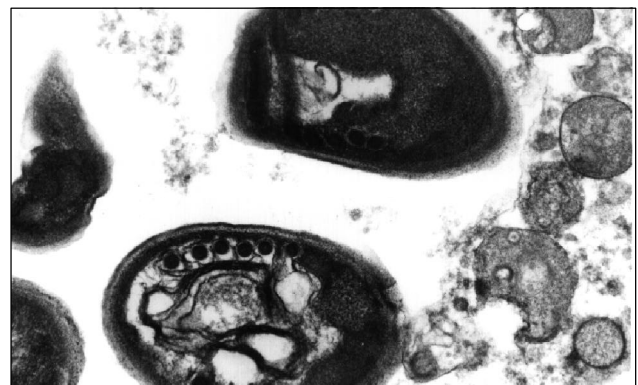


Figure 6. Transmission electron micrograph of an *Encephalitozoon intestinalis* spore from cell culture showing the polar tubule with four to seven coils in single rows, which is typical of the genus *Encephalitozoon*. Original magnification, x28,500.

Light and Fluorescence Microscopy

Histologic examination of biopsy specimens allows diagnosis of microsporidial infection but not genus or species identification of the parasites. Tissue stains used to detect microsporidia include hematoxylin and eosin, Gram, Giemsa, Warthin-Starry, and chromotrope 2R modified trichrome stains (13,14). The small size of the organisms and the lack of a

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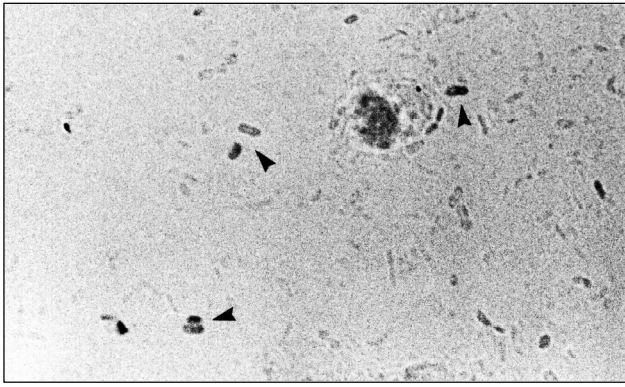


Figure 7. Spores of *Encephalitozoon intestinalis* in a stool specimen stained with modified trichrome stain. Note the characteristic equatorial line in one of the spores. Original magnification, x1,000.

sensitivity and specificity of the modified trichrome stain have not been well established. When results of the modified trichrome stain performed on stool specimens and a TEM examination of duodenal biopsy specimens from HIV-infected patients were compared, *E. bienewsi* spores were detected in stool specimens in 9 of 9 patients with moderate or abundant parasites in tissue and in 8(57%) of 14 stool specimens from patients whose tissue contained few parasites (16). This illustrates that microsporidial spores may not be observed in the stool of some infected patients with the modified trichrome stain.

Various chemofluorescent brighteners (such as calcofluor white and Uvitex 2B) bind to the endospore layer of microsporidia and allow spores to be detected quickly and easily in smears of specimens examined with a fluorescence microscope (5). Fluorescein-labeled polyclonal and monoclonal antibodies are being developed for the detection and speciation of microsporidia (5,17). Didier et al. (17) recently compared modified trichrome, calcofluor white, and a fluorescent polyclonal antibody stain and found that the polyclonal antibody stain was the least sensitive method for detecting microsporidia in stool, urine, and duodenal lavage specimens. They proposed screening specimens with calcofluor white and confirming positive smears by using a modified trichrome stain. However, modified trichrome and calcofluor white stains do not allow for speciation of microsporidia; therefore, species-specific antibodies should be used to provide definitive identifications. *E.intestinalis*-specific monoclonal antibodies have been used in an immunofluorescence assay for detecting spores in urine, stool, bronchial brush biopsy specimens, bronchoalveolar lavage fluid, and samples obtained from nasal swabs (18). *E. bienewsi*-specific antibodies may not be developed until the organism is successfully cultivated in long-term culture.

Cell Culture

Microsporidia have been isolated from a variety of specimen types and in a variety of cell lines (5). *E. hellem*, *E. intestinalis*, and *Vittaforma corneae* have been isolated from human specimens and maintained in continuous culture. Recently, *E. bienewsi* has been cultivated on a short-term (6 months) basis in vitro (19). Detecting microsporidia in infected cell cultures may take 3 to 10 weeks (19,20). Isolating microsporidia in cell culture as a means of diagnosing infection is laborious and lengthy and is prone to failure with specimens from nonsterile sites. Therefore, cell culture is not recommended as a routine laboratory technique for diagnosing microsporidiosis.

Serology

Serologic assays used to detect antibodies to microsporidia in human sera include immunofluorescence, immunoperoxidase, enzyme-linked immunosorbent assay (ELISA), and Western blot (21-23). The sensitivity and specificity of these methods for detecting antimicrosporidial antibody are not known because no comparative evaluations have been published. Studies have demonstrated increased rates of seropositivity to *E. cuniculi* in persons who live in tropical regions and have tropical diseases (21,24). Most notable were *E. cuniculi* seropositivity rates of 4.7% in patients with malaria and 9.1% in patients with schistosomiasis (24). A study of homosexual men in Sweden found that 10 (33%) of 30 were seropositive for antibodies to *E. cuniculi*, and all seropositive patients plus half of the seronegative patients had sometimes visited tropical countries (25). Studies to detect microsporidial antibodies in HIV-infected and non-HIV-infected patients have demonstrated that AIDS patients can mount an immune response to microsporidial infection; however, serologic methods are not useful as diagnostic tools because these studies have found that at least half of the serum specimens from persons without a history of microsporidial infection had positive titers (22,23). Some of the problems with serologic testing include the poor response to antigen challenge in immunosuppressed persons (26), the probability that different (both pathogenic and nonpathogenic) microsporidia contain common cross-reactive antigens (23,26), and the lack of species-specific reagents in part because *E. bienewsi* cannot be grown in continuous culture.

Molecular Techniques

Characterization of the microsporidial genome has focused on the small subunit ribosomal RNA

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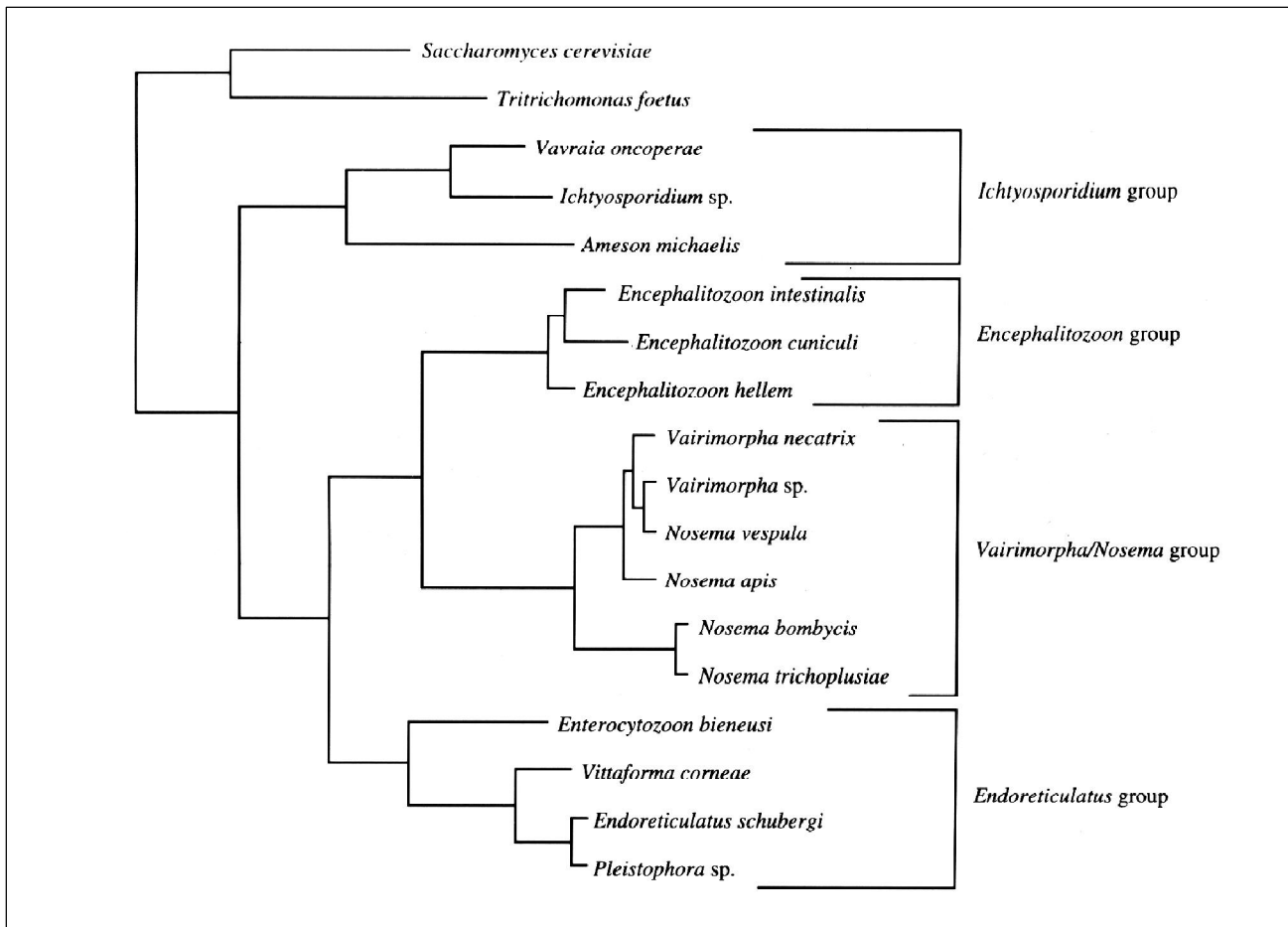


Figure 8. Cladogram representing the phylogenetic relationship of several microsporidial genera as determined by small subunit ribosomal DNA sequence similarity. The human pathogens can be found in the *Encephalitozoon* group and the *Endoreticulatus* group. (Reprinted with permission from ref. 35.)

(SSU-rRNA) gene. The sequence of the SSU-rRNA gene of the microsporidium *Vairimorpha necatrix* was published by Vossbrinck et al. in 1987 (27). The *V. necatrix* SSU-rRNA gene was far shorter than a typical eukaryotic SSU-rRNA gene and lacked several universal (and eukaryotic-specific) sequences. PCR amplification with primers complementary with conserved sequences of the *V. necatrix* SSU-rRNA gene has been used to generate sequence information from microsporidia that infect humans. SSU-rRNA gene sequences have been published for *E. cuniculi* (28,29,30,31), *E. hellem* (30,31), *E. intestinalis* (31,32), and *E. bienersi* (31,33), and an unpublished sequence for *V. corneae* has been deposited into GenBank National Center for Biotechnology Information, National Institutes of Health, accession 0.5 U11046.

Taxonomic decisions concerning the microsporidia have been based historically on morphologic characteristics as established by TEM. These criteria are still valid for organisms with unique

morphology and ultrastructure and were used to justify the reclassification of *Nosema corneum* to *Vittaforma corneae* (34). This reclassification was confirmed by a study of microsporidial phylogeny based on evaluation of SSU-rRNA gene sequences that indicated that *N. corneum* was more closely related to the insect parasite *Endoreticulatus schubergi* than to the other *Nosema* species (35) (Figure 8). Phylogenetic analysis of the sequences of the SSU-rRNA genes of microsporidia is often inconsistent with traditional classifications that are based on morphologic characteristics observed by TEM; this was demonstrated by the use of recent sequence data to determine the correct taxonomic placement of *E. intestinalis* (32,35). This organism was named *Septata intestinalis* because unique septations between spores within the parasitophorous vacuole were observable by TEM (36). Although the various stages of the microorganism throughout its life cycle are indistinguishable from those of *E. cuniculi*, the observation of septations

allowed the organism to be identified. The *E. intestinalis* SSU-rRNA gene contained sufficient unique sequences to establish this organism as an independent species but shared about 90% sequence homology with the other two characterized *Encephalitozoon* species, *E. cuniculi* and *E. hellem*.

Molecular techniques have confirmed the existence of different strains of *E. cuniculi* that have been isolated from mice, rabbits, and dogs (11). Three antigenically different strains were detected by sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and Western blot. Double-stranded DNA heteroduplex mobility, restriction fragment length polymorphism with the restriction endonuclease *Fok* I, and DNA sequencing were performed on the PCR products generated by using a set of primers to amplify the entire microsporidial SSU-rRNA gene and a second set of primers specific for *Encephalitozoon* species. These methods allowed three strains of *E. cuniculi* to be clearly separated. The existence of different strains of *E. bieneusi* was suggested after SSU-rRNA gene sequences of organisms from maxillary sinus mucosa had been compared with those from intestinal enterocytes (37). Sequencing errors in this study were minimized by analyzing multiple recombinant DNA clones. SSU-rRNA sequences from *E. bieneusi* and *Encephalitozoon* species derived from a variety of clinical specimens must be compared to establish the existence of intraspecific genetic variation in these organisms.

Molecular Diagnosis

Primer pairs that amplify the entire microsporidial SSU-rRNA gene sequence produce amplicons of approximately 1,550 base pairs in length from *Encephalitozoon* species and *E. bieneusi* (28,33). Although these primer pairs have proven useful for sequencing and taxonomic studies, the targets are too long to be efficiently amplified from clinical specimens in a diagnostic assay. Targets for diagnostic PCR that can be amplified efficiently usually range from 100 to 400 base pairs for formalin-fixed tissue or up to 700 to 1,000 base pairs in fresh specimens (38). Several primer pairs designed to amplify short regions (250 to 607 base pairs) of the SSU-rRNA gene, and their application in the diagnosis of microsporidial infection by PCR have been published (Table 2). Primers specific for *E. hellem* and *E. cuniculi*-specific primers have been used to identify microsporidia cultured from patient specimens (39,40), but only *E. bieneusi* and *E. intestinalis* SSU-rRNA DNA have been amplified directly from patient specimens (31,33,41,42). A pair of *E. bieneusi*-specific primers amplified cloned *E. bieneusi* SSU-rRNA gene sequences but did not reliably amplify DNA from infected tissue (33). Not unexpectedly, therefore, some primer sets appear to be adequate for amplification from cultured organisms or cloned sequences but may not reliably amplify microsporidial DNA in patient specimens. The primer pair V1 and EB450 (Table 2) amplifies

Table 2. SSU-rRNA PCR primer pairs for the diagnosis of microsporidial infection

Primer pair (no. nucleotides)	Primer designation	Organisms amplified	Amplicon size in base pairs	Source of Target	References
5'-CACCAGGTTGATTCTGCCTGAC-3' (22)	V1	<i>Enterocytozoon bieneusi</i>	348	Biopsied tissue	33, 41
5'-ACTCAGGTGTTATACTACGTC-3' (22)	EB450	<i>Encephalitozoon hellem</i>		Duodenal aspirates	31
5'-GAAACTTGTCCTCCTTACG-3' (21)	EBIEF1	<i>E. bieneusi</i>	607	Bile	43
5'-CCATGCACCACTCCTGCCATT-3' (21)	EBIER1			Duodenal aspirates	
5'-TGAGAAGTAAGATGTTTAGCA-3' (21)		<i>E. hellem</i>	547	Cultured organisms	39
5'-GTAAAAACACTCTCACACTCA-3' (21)					
5'-ATGAGAAGTGATGTGTGTGCG-3' (21)		<i>Encephalitozoon cuniculi</i>	549	Cultured organisms	39, 40
5'-TGCCATGCACTCACAGGCATC-3' (21)					
5'-CACCAGGTTGATTCTGCCTGAC-3' (22)	V1	<i>Encephalitozoon intestinalis</i>	370	Biopsied tissue	31
5'-CTCGCTCCTTACACTCGAA-3' (20)	SI500				
5'-CACCAGGTTGATTCTGCCTGAC-3' (22)	PMP1*	<i>E. bieneusi</i>	250	Stool	42
5'-CCTCTCCGGAACCAACCTG-3' (21)	PMP2	<i>E. cuniculi</i>	268	Cultured organisms	
		<i>E. intestinalis</i>	270	Stool	
		<i>E. hellem</i>	279	Cultured organisms	

*The nucleotide sequence of primer PMP1 is identical to that of primer V1.

E. bienewsi from TEM-confirmed infected tissue and *E. hellem* from cell culture (33). This primer pair has not, however, been evaluated for its ability to amplify *E. hellem* from patient specimens. Primer pair V1 and EB450 was extensively tested by da Silva, et al. (43), who found that the primer pair did amplify *E. bienewsi* DNA from some patient specimens that had evidence of the parasite by electron microscopy. In addition, the primer pair did not amplify DNA of *E. bienewsi* derived from a short-term culture. da Silva et al. (43) described a pair of highly specific PCR primers for amplifying *E. bienewsi*; these are called EBIEF1 and EBIER1 (Table 2), and are based on SSU-rRNA sequences they generated. This primer pair amplified *E. bienewsi* DNA from cultured organisms, cloned regions of the SSU-rRNA, and patient specimens, but did not amplify SSU-rRNA-coding regions of 13 other genera and species of microsporidia. The primer pair V1 and SI500 amplifies *E. intestinalis* from intestinal biopsy material confirmed by TEM as infected, but does not simplify *E. bienewsi*-infected tissue samples or *E. cuniculi* from cell culture (31). This primer pair has not been evaluated for its ability to amplify other microsporidia.

To avoid acquiring specimens by invasive procedures, PCR was used to detect microsporidia in stool specimens (42). Primer pair PMP1 and PMP2 (Table 2) allows *E. bienewsi* and the *Encephalitozoon* species (42) to be amplified and will also amplify *V. corneae* from culture (D.P. Fedorko, unpublished data). DNA is easily extracted from cultured organisms and biopsied tissue specimens, but extracting DNA from spores requires harsh conditions employing both mechanical and chemical disruption. A major problem with performing PCR on stool specimens is the presence of PCR inhibitors. Treating the stool specimens with sodium hypochlorite or 10% formalin before DNA extraction inactivates microorganisms in the stool and has the beneficial effect of inactivating Taq polymerase inhibitors.

Both DNA probes and restriction enzyme digestion have been used to confirm the identity of PCR amplicons (33,41,42). An internal 30 meroligonucleotide EB150 (5'TGTTGCGGTAATTTGTCTCTGTGTGTAAG-3'), complementary to a region of the amplicon produced by primer pair V1 and EB450, has been used to detect *E. bienewsi* by Southern blot (31,41). Probe EB150 has been reported, however, to hybridize with *E. hellem*, amplified by V1 and EB450, albeit at a lower intensity than *E. bienewsi* (31). PCR products amplified

from stool specimens by using primers PMP1 and PMP2 have been digested with restriction endonucleases *Hae*III and *Pst*I to distinguish between infection with *E. bienewsi* and *E. intestinalis* (42). These restriction enzymes do not allow *E. intestinalis* to be differentiated from *E. cuniculi*, thus limiting their use to diagnosing gastrointestinal infection.

An efficient approach for the molecular detection of microsporidia in patient specimens would involve using universal or "pan-microsporidian" primers for amplification. The species of microsporidia detected could be determined by using restriction endonuclease digestion or DNA probe assays of the amplified DNA or repeat PCR with species-specific primers. Negative specimens would require no further evaluation. Primers PMP1 and PMP2 appear to be panmicrosporidian (42), but this primer pair needs to be evaluated for its ability to amplify microsporidian DNA from a wide range of clinical specimens. Didier et al. (44) have used pan-*Encephalitozoon* primers that amplified a product approximately 1,000 base pairs in length, which included a large portion of the SSU-rRNA gene and a small portion of the large subunit rRNA gene. They successfully amplified *E. hellem* DNA from urine and conjunctival specimens from a patient with AIDS. Southern blotting and a species-specific probe were used to identify the organism to species.

The application of molecular diagnostic techniques for microsporidiosis is in its infancy. There have been no published reports of comparisons of PCR to other methods to determine sensitivity and specificity. Careful selection of primers and probes coupled with highly stringent conditions will be required to detect and speciate microsporidia in patient specimens. The potential for PCR to identify species of microsporidia from non-invasively acquired specimens makes this technique an extremely attractive diagnostic option. Although PCR can be used for detection and speciation of microsporidia in patient specimens, screening for microsporidia by using chemofluorescent stains and modified trichrome stains followed by confirmation and speciation with PCR may become the paradigm for the laboratory diagnosis of microsporidiosis.

Dr. Fedorko is a senior staff microbiologist in the Microbiology Service, Clinical Pathology Department, Warren G. Magnuson Clinical Center, National Institutes of Health, Bethesda, Maryland.

Dr. Hijazi is a senior staff pathologist in the Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland.

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