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Diagnosis of Disseminated Microsporidian *Encephalitozoon hellem* Infection by PCR-Southern Analysis and Successful Treatment with Albendazole and Fumagillin

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A 37-year-old AIDS patient presented with foreign body sensation. Microsporidia were detected in smears from a conjunctival swab and urine sediment stained with calcofluor and a modified trichrome blue stain and by indirect fluorescent-antibody staining with murine polyclonal antiserum raised against *Encephalitozoon hellem*. This antiserum cross-reacted with other *Encephalitozoon* species, so PCR was performed to amplify the microsporidian ribosomal DNA (rDNA) with pan-*Encephalitozoon* primers. The PCR DNA products from the urine and conjunctival clinical specimens, along with the tissue culture-derived microsporidian controls, were assayed by Southern analysis with oligonucleotide probes specific for *Encephalitozoon cuniculi*, *E. hellem*, and *Encephalitozoon* (*Septata*) *intestinalis*. The PCR product amplified from the urine specimen hybridized with the *E. hellem* probe only, while insufficient DNA was amplified from the conjunctiva specimen for detection by Southern analysis. For corroboration of the PCR-Southern analysis results, aliquots of the urine and conjunctiva specimens were seeded onto RK-13 cell monolayers. The rDNA extracts of the cultured microsporidia were amplified by PCR with pan-*Encephalitozoon* primers, and the PCR DNA products were subjected to digestion with restriction endonuclease *Fok*I. The amplified rDNA of both the urine and conjunctiva isolates generated digestion patterns that were identical to the *E. hellem* PCR rDNA digestion pattern. In addition, double-stranded heteroduplex mobility shift analysis with these PCR products indicated that the urine and conjunctiva isolates were identical to each other and to *E. hellem*. The patient was treated with albendazole and topical fumagillin and responded rapidly, with no recurrence of ophthalmologic signs. The results of this study demonstrate that PCR-Southern analysis provides a basis for distinguishing *E. cuniculi*, *E. hellem*, and *E. intestinalis* in clinical specimens.

Microsporidia are obligate intracellular protozoan parasites which cause a variety of opportunistic infections in AIDS patients (7, 33, 40, 41, 56). The most common microsporidian associated with AIDS, *Enterocytozoon bieneusi*, primarily infects enterocytes of the small intestine and causes chronic diarrhea (5, 13). *Septata intestinalis* (3), recently identified as *Encephalitozoon intestinalis* (25), and *Encephalitozoon hellem* (14, 19, 38) both cause disseminated infections with clinical signs that include diarrhea, sinusitis, nephritis, pneumonia, and keratitis (32, 40, 56). *Encephalitozoon cuniculi* (12, 27, 28), *Vittaforma corneae* (formerly named *Nosema corneum*) (10, 42, 43), *Nosema ocularum* (4, 6), and *Pleistophora* spp. (9, 30) have been detected less frequently in AIDS patients or individuals seronegative for human immunodeficiency virus.

Microsporidian species infecting humans measure approximately 1.0 to 2.0 by 1.5 to 4.0 μm and are easily overlooked or confused with bacteria and small yeasts (8, 40, 41). Furthermore, species identification often is impossible by the histochemical methods presently available, particularly since the microsporidian species infecting humans are nearly identical to each other in size. The purpose of this study is to describe the use of the PCR in conjunction with Southern analysis with oligonucleotide probes for distinguishing the closely related species *E. hellem*, *E. cuniculi*, and *E. intestinalis*. These results were corroborated with those for organisms cultured in vitro from the urine and conjunctival specimens of this patient and subsequently assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), PCR-restriction fragment length polymorphism (RFLP), and double-stranded DNA heteroduplex mobility shift analysis.

Patient history. This 37-year-old male AIDS patient developed a foreign body sensation in his left eye in April 1994 which did not respond to treatment with ophthalmic lubricants and topical erythromycin. Within 3 months, he progressed from unilateral superficial to bilateral diffuse punctate keratitis, with visual acuity deteriorating from 20/20 to 20/40 (right eye) and 20/25 to 20/60 (left eye). Gram stains of conjunctival fluid and urine sediment revealed multiple intracellular gram-positive oval organisms consistent with microsporidia.

The patient's initial positive human immunodeficiency virus antibody test was in 1986, when he presented with lymphadenopathy and abnormal liver function tests. His opportunistic infections included oral, esophageal, and laryngeal candidiasis (1993), diffuse molluscan contagiosum (1992 to 1994), and herpes simplex virus type 2 perirectal ulcers (1994). He had episodes of recurrent sinusitis and otitis media (1993 and 1994), pneumonitis (*Haemophilus influenzae*, 1992; atypical, 1993), and diarrhea (gastroenteritis with undetermined pathogen, 1991; intestinal amebiasis, 1992; *Campylobacter* enteritis, 1993). He had no urinary symptoms during any stage of his illness. His CD4 cell count dropped from 48 in June 1992 to 1

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in March 1995, the last result before his death. He developed microsporidial keratoconjunctivitis while taking a prophylactic medication regime including acyclovir, trimethoprim-sulfamethoxazole, and clarithromycin.

After showing no response to a trial of oral intraconazole, he began treatment with topical fumagillin (0.03% solution in both eyes every hour) and oral albendazole (200 mg twice a day). He responded rapidly to this regimen, and a follow-up ophthalmologic exam 1 month later revealed no trace of corneal or conjunctival lesions and full recovery of visual acuity to 20/20. Albendazole was discontinued, and fumagillin was tapered to a daily maintenance regimen without any signs or symptoms of recurrence to the time of his death.

MATERIALS AND METHODS

Histochemistry. Smears from the urine sediment and conjunctival swabs were prepared on glass slides, fixed with methanol for 10 min, and stained with calcofluor, modified trichrome blue, and indirect immunofluorescent antibody (IFA) as previously described (16).

Organisms. Microsporidia were grown in RK-13 cells by procedures previously described (14, 17, 42). *E. hellem* was isolated from three AIDS patients with corneal-conjunctival keratitis (14, 23, 61). *E. intestinalis* (3) was isolated from a bronchoalveolar lavage specimen that was provided by Jan Orenstein from an AIDS patient (17) and was found to be identical to the first isolate of *E. intestinalis* described by van Gool et al. (45). *E. cuniculi* I was originally isolated from a rabbit (34, 39). The human isolate of *V. corneae*, originally named *N. corneum* (42), was cultured from the corneal tissue of a human immunodeficiency virus-seronegative individual with stromal keratitis and iritis (10, 43).

The conjunctival scraping and urine sediment from this patient with disseminated microsporidiosis each were suspended in Tris-buffered saline (TBS) containing 0.3% Tween 20 (TBS-Tween), centrifuged for 10 min at 400 × *g* (room temperature), and washed once with TBS. Each pellet was resuspended in tissue culture medium (RPMI 1640 containing 5% heat-inactivated fetal bovine serum, 2 mM L-glutamine, penicillin, and streptomycin), which was then added to a nearly confluent monolayer of RK-13 cells. Medium was replaced every 3 or 4 days, and the supernatants containing parasites were saved until used. Parasitophorous vacuoles containing organisms were observed 2 weeks after the monolayers were seeded, and microsporidia were observed in culture supernatants after approximately 4 weeks.

For assays performed in this study, microsporidia were collected from tissue culture supernatants and washed three times by centrifugation at 400 × *g* for 15 min with TBS-Tween to wash off the bovine serum albumin that adheres to the parasite surface. To remove host cell debris, each pellet was resuspended in TBS, mixed with an equal volume of 100% Percoll (Pharmacia, Piscataway, N.J.), and centrifuged at 400 × *g* for 30 min. The pellets containing the purified microsporidia were washed with TBS, counted with a hemacytometer, and adjusted to the appropriate concentrations.

ELISA. To titrate levels of serum antibodies to microsporidia, an enzyme-linked immunosorbent assay (ELISA) was performed as described by Hollister and Canning (26) and modified as described previously (15).

DNA extraction and PCR methods. DNA was extracted from aliquots of urine sediment and conjunctival tissue as previously described (17, 18, 52). Briefly, the specimens or tissue culture-derived microsporidia were washed with sterile distilled water, pelleted in 1.5-ml microcentrifuge tubes by centrifugation at 15,000 × *g* for 1 min, and resuspended in 200 μl of TE buffer (10 mM Tris-HCl [pH 7.5] and 1.0 mM EDTA) containing 0.1 g of 0.5-mm-diameter sterile glass beads. The tubes were vortexed for 45 s, and this procedure was followed by the addition of 200 μl of lysis buffer (0.2 M NaOH, 2% SDS, 0.5 mg of proteinase K per ml). After an overnight incubation at 56°C, 200 μl of neutralization buffer (2.55 M potassium acetate, pH 4.8) was added to each tube. The tubes were centrifuged at 15,000 × *g* for 5 min, and the DNA was purified with Magic Minipreps (Promega Corporation, Madison, Wis.) as described by the manufacturer.

To amplify the ribosomal genes of the microsporidia, PCR was employed, with a set of primers selected from sequences conserved between *E. cuniculi* and *E. hellem* being used (17, 18, 52). These pan-*Encephalitozoon* primers amplify a product which has a size of approximately 1,000 base pairs and which includes a large portion of the small-subunit rRNA gene, the entire intergenic region, and a small portion of the large-subunit rRNA gene. These primers also amplify ribosomal DNA (rDNA) of *E. intestinalis* but do not amplify rDNA from tissue culture-derived *V. corneae* or intestinal biopsy-derived *E. bieneusi*. The sequences for this set of primers are 5′-TGCAGTTAAAATGTCCGTAGT-3′ (int530f) and 5′-TTTCACTCGCCGCTACTCAG-3′ (int580r).

Gene amplification by PCR was performed in 100-μl reaction mixtures containing each primer at 0.05 μM, each deoxynucleoside triphosphate (Promega Corp.) at 0.2 mM, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 U of *Taq* DNA polymerase (Promega Corp.), approximately 10 ng of genomic DNA, and 100 μl of overlaid mineral oil. The PCR assays were performed with a Perkin-Elmer thermocycler (Norwalk, Conn.), and the profile was 94°C for 1 min, 40°C for 1 min, and 72°C for 2 min. The PCR products were purified with Magic PCR Prep minicolumns as described by the manufacturer (Promega Corp.), and aliquots were examined by electrophoresis in 1.5% agarose gels. The remaining portions of the PCR products were stored at 4°C until used.

Southern analysis. Aliquots of the PCR products (5 and 1 μl) were electrophoresed in 1.2% agarose gels stained with ethidium bromide to visualize the DNA (36) and were blotted onto Magna Graph nylon transfer membranes (Micron Separations, Inc., Westboro, Mass.) with 2× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). DNA was bound to the nylon membrane by UV irradiation (Stratolinker; Stratagene, Menasha, Wis.). The blots were then hybridized and stained with the enhanced chemiluminescence 3′ oligolabelling and detection system as described by the manufacturer (Amersham Life Science, Arlington Heights, Ill.). Briefly, the microsporidian oligonucleotide probes were labelled with fluorescein-11-dUTP and added to the blots at a final concentration of 5 to 10 ng/ml. The oligonucleotide sequences for the *E. cuniculi*, *E. hellem*, and *E. intestinalis* probes are 5′-TAGCGGCTGACGAAGCTGC-3′, 5′-TGAGTGTGAGAGTGTTTTTACAT-3′, and 5′-CGGGCAGGAGAACGA GGACGG-3′, respectively. The blots were incubated at 54°C for 2 h in a shaking water bath when the *E. cuniculi* and *E. hellem* probes were used or at 64°C for 2 h when the *E. intestinalis* probe was used, after which the blots were washed two times in 5× SSC containing 0.1% SDS for 5 min each time and then twice for 15 min at 54°C with 1× SSC containing 0.1% SDS. The blots were then incubated with the blocking solution provided in the enhanced chemiluminescence kit, incubated with horseradish peroxidase-conjugated anti-fluorescein antibody, rinsed with the detection solutions, and exposed on film.

RFLP. PCR products were digested overnight with restriction endonuclease *Fok*I (New England Biolabs, Beverly, Mass.) as described previously (36). The restriction digests were electrophoresed through 2% agarose (FMC Bioproducts, Rockland, Maine) stained with ethidium bromide and photographed under UV illumination.

Double-stranded DNA heteroduplex mobility assay. PCR-amplified products were assayed for the generation of heteroduplexes by a slightly modified version of the method of Soto and Sukumar (44). Briefly, 10-μl aliquots of PCR products from two microsporidians were mixed together, heated to 95°C, and cooled to room temperature to allow random and complete duplex formation. Homoduplexes, which indicate reannealing of homologous strands of DNA, and heteroduplexes, which migrate more slowly because of conformational changes resulting from mismatched DNA sequences, were visualized after electrophoresis of the products in PCR Purity Plus gels (J. T. Baker, Inc., Phillipsburg, N.J.). To control for the fidelity homoduplex formation, products from two different PCR amplifications of the same microsporidian were mixed, denatured, and allowed to rehybridize.

RESULTS

Histochemistry. Smears that were prepared from urine sediment and conjunctival fluid specimens and that were stained with modified trichrome blue, calcofluor, and IFA all demonstrated the presence of organisms consistent for microsporidia (data not shown). These organisms measured approximately 1.5 by 2.5 μm in size, suggesting that they belonged to the *Encephalitozoon*, *Nosema*, or *Vittaforma* genus but not the *Enterocytozoon* genus, the members of which are smaller and measure approximately 1.0 by 1.5 μm. The antiserum used in the IFA was raised against *E. hellem* and cross-reacted with all the *Encephalitozoon* spp. and *E. bieneusi* but only weakly stained *V. corneae*. Therefore, additional studies to determine the identity of this microsporidian species were performed, with emphasis being placed on species in the genus *Encephalitozoon.*

ELISA. Serum obtained from this patient in February 1995 was assayed for antibodies to microsporidia. The ELISA titers were 1:6,400 against *E. cuniculi*, 1:6,400 against *E. hellem*, 1:6,400 against *E. intestinalis*, and 1:3,200 against *V. corneae*. An ELISA titer greater than or equal to 1:800 is considered a positive indication for exposure to or infection with microsporidia (26). In this case, the serum titers of this patient were similar for all four microsporidian species tested, so it was not possible with this method to determine on the basis of serology which species infected this patient.

Southern analysis. PCR was performed on unfixed urine and conjunctival specimens from this patient. Upon examination of the ethidium bromide-stained gels, no PCR products

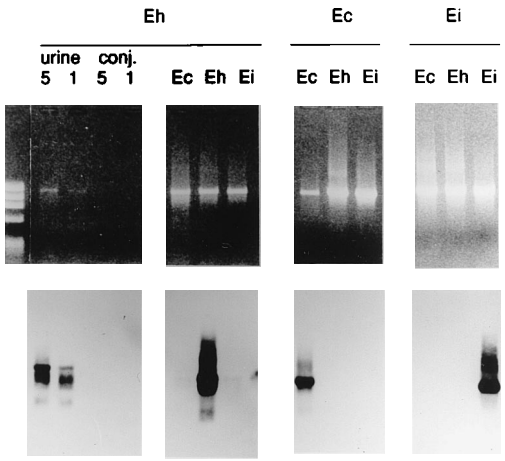

FIG. 1. PCR and Southern analysis of microsporidian rDNA amplified from urine and conjunctival swab (conj.) specimens. Pan-*Encephalitozoon* primers were used to amplify the rDNA, and the products of the PCR are shown in the ethidium bromide-stained gels on the top row. Positive controls were from tissue culture-derived *E. cuniculi* (Ec), *E. hellem* (Eh), and *E. intestinalis* (Ei). Either 5 or 1 μl of PCR product from the specimen assays was added per lane, and 5 μl was added for each of the positive control PCR assays. The lower row of photos shows the results after the blots were probed with oligonucleotides specific for each of the *Encephalitozoon* species as indicated and as described in Materials and Methods.

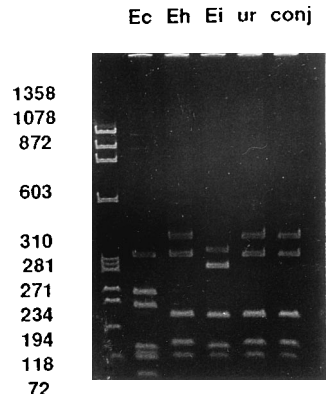

FIG. 2. PCR-RFLP patterns of tissue culture-derived *E. cuniculi* (Ec), *E. hellem* (Eh), and *E. intestinalis* (Ei) as well as the organisms cultured from the urine (ur) and conjunctiva (conj). Pan-*Encephalitozoon* primers were used for PCR, and the PCR-amplified products were digested with restriction endonuclease *Fok*I. The lane at far left shows the molecular weight markers derived from *Hae*III-digested ϕX174. Molecular weights are noted to the left of the lanes.

from the conjunctival specimen could be observed, but a small amount of product from the urine-derived sample could be observed (Fig. 1). The gels were then blotted onto nylon and incubated with species-specific oligonucleotide probes for *E. cuniculi*, *E. hellem*, and *E. intestinalis* (Fig. 1). A band of reactivity was seen on the urine specimen PCR product probed with the *E. hellem*-specific oligonucleotide. The *E. cuniculi* oligonucleotide probe used in this study detects *E. cuniculi* I, II, and III (only strain I is shown) but does not detect *E. hellem* or *E. intestinalis*, and similarly, the *E. intestinalis* oligonucleotide detects only *E. intestinalis.*

Insufficient PCR products were generated from the urine and conjunctival specimens to perform PCR-RFLP or PCR–double-stranded DNA heteroduplex mobility shift analyses to confirm these results. However, aliquots of the original unfixed specimens were placed into culture, and the microsporidia harvested from both cultures were used instead to perform the corroborating experiments.

SDS-PAGE, PCR-RFLP, and double-stranded DNA heteroduplex mobility shift analysis. Percoll-enriched microsporidia were separated by SDS-PAGE, and the two microsporidians cultured from the urine and conjunctival fluids of this patient were identical to each other and to the type species isolate of *E. hellem* (data not shown). PCR products generated from the tissue culture-derived microsporidian isolates were subjected to restriction endonuclease digestion with *Fok*I (Fig. 2). This restriction enzyme was chosen because the RFLP patterns were useful for distinguishing *E. cuniculi*, *E. hellem*, and *E. intestinalis*. The PCR-RFLP patterns of the urine and conjunctival specimens were identical to that for *E. hellem* and differed from those for *E. cuniculi* and *E. intestinalis.*

Double-stranded DNA heteroduplex mobility shift analysis also was performed to demonstrate the identity or lack of identity of two microsporidian PCR products on the basis of the absence or presence of heteroduplexes, respectively (Fig. 3). The combination of the two PCR products from the urine and conjunctival specimens resulted in no formation of heteroduplexes, indicating identity of these two microsporidia (data not shown). Combining the urine PCR product (data shown) or the conjunctival PCR product (data not shown) with the *E. hellem* PCR product generated no heteroduplexes, indicating that the urine and conjunctival microsporidian isolates were identical to *E. hellem*. Heteroduplexes did form for urine (data shown) or conjunctival (data not shown) PCR products that were combined with either *E. cuniculi* or *E. intestinalis* PCR products, indicating a lack of identity (Fig. 3).

DISCUSSION

Microsporidian infections are of clinical concern in immunodeficient individuals, principally in persons with AIDS. Serum antibody ELISA titers in AIDS patients with documented microsporidian infections are highly variable because of progressing immunodeficiency associated with human immunodeficiency virus infections, making serology unreliable as a diag-

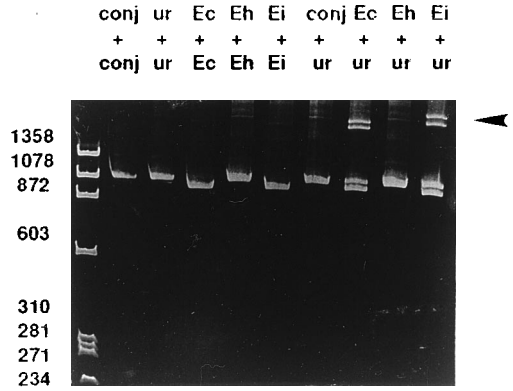

FIG. 3. Double-stranded DNA heteroduplex mobility shift analysis for comparing the PCR rDNA products of the microsporidia cultured from the urine (ur) and conjunctiva (conj) specimens. Pan-*Encephalitozoon* primers were used for PCR amplification, and the PCR products were combined as shown, heated to denature the DNA, and cooled to allow reannealing. Homoduplexes formed when two like PCR products were combined, while heteroduplexes that migrate more slowly formed when strands with regions of dissimilarity reannealed (arrowhead). Molecular markers generated from ϕX174 digested with *Hae*III are shown in the lane to the far left. Molecular weights are noted to the left of the lanes. Ec, *E. cuniculi*; Eh, *E. hellem*; Ei, *E. intestinalis.*

nostic tool (15). In some cases, it is possible to culture microsporidia from affected tissues for growth in tissue culture and then to identify them by SDS-PAGE and Western blot (immunoblot) immunodetection (14, 17, 19, 50, 51). Although useful for species-specific diagnoses and for identifying new microsporidian species, this approach has been too cumbersome and time-consuming for general diagnostics applications. Furthermore, *E. bieneusi*, the most common microsporidian infecting humans, has not been successfully cultured. Instead, diagnosis primarily depends upon detecting microsporidia in patient specimens by electron or light microscopy or, more recently, by molecular biology methods.

Transmission electron microscopy has been the traditional "gold standard" method for diagnosing microsporidia (32). The basis for definitive diagnosis is the observation of the coiled polar tubule, which is the structure found only in microsporidia. For diagnostic purposes, however, transmission electron microscopy is time-consuming and relatively insensitive and requires a great deal of expertise. Histochemical methods using chitin-staining fluorochromes (11, 31, 46–49), modified trichrome staining (29, 35, 55), and IFA (1, 38, 58, 63) were developed and compared for diagnosing microsporidia (16). A resulting diagnostics paradigm was to screen specimens with the calcofluor stain, because it is fast and easy to perform and read. The modified trichrome stain is then used to confirm the presence of microsporidia, because one can distinguish microsporidia from yeast cells by this method. A weakness of the calcofluor and modified trichrome stains, however, is that one cannot distinguish *E. cuniculi*, *E. hellem*, *E. intestinalis* and *V. corneae* from one another. Even though *E. bieneusi* is smaller than the *Encephalitozoon* species and *V. corneae*, it is also often difficult to distinguish this microsporidian from other species in calcofluor- or modified trichrome-stained stool specimens. The IFA stain is less sensitive but may be useful for species identification as reported by Schwartz et al. (38), who described the generation of *E. hellem*-specific polyclonal antiserum for diagnosing seven cases of disseminated microsporidiosis due to *E. hellem*. This approach, however, requires access to tissue culture procedures for generating the high numbers of heterologous microsporidia needed to absorb the antisera and produce species-specific antisera.

As an alternative to histochemical methods, a strong effort is being directed toward development and application of molecular biology methods, and specifically PCR, to increase the levels of reliability, sensitivity, and specificity for diagnosing microsporidiosis. The primary target for PCR amplification is the rRNA genes of microsporidia which were found by Vossbrinck et al. (52–54) to resemble the rRNA of prokaryotes, even though microsporidia are eukaryotes. The rDNA sequences have been used to derive PCR primers that have subsequently been employed in diagnostics (59, 60). Panmicrosporidian primers that amplify the small-subunit rDNA of all microsporidia that infect humans (24, 25, 37, 52), genus-specific primers for the genus *Encephalitozoon* (17, 18), and species-specific primers for *E. bieneusi* (62) and *E. cuniculi* and *E. hellem* (50, 51) have been described. Second-step methods have been employed after PCR to corroborate or specifically identify the PCR products. These methods include DNA sequencing (24, 25, 52, 62), RFLP (17, 18, 25, 52), and heteroduplex analyses (17, 18). Southern analysis also has been used to detect *E. bieneusi* when insufficient PCR-amplified rDNA was generated for visualization on an ethidium bromide-stained gel (62). In the study presented here, the urine-derived PCR product could be seen on the ethidium bromide-stained gel. Only with Southern analysis could the species be identified as *E. hellem*, because insufficient DNA was amplified for PCR-RFLP or heteroduplex mobility shift analysis. Unfortunately, it was not possible to amplify the microsporidian DNA in the conjunctival specimen from this patient by PCR, even though infectious organisms were present, as evidenced by growth in culture. It is possible that PCR inhibitors were present in the conjunctival specimen or that our extraction and amplification methods were not sensitive enough, thereby requiring improvement for use in routine diagnostics with patient specimens.

Species-specific identification of microsporidia in clinical specimens is becoming increasingly important because of various levels of responses to some drugs. For example, albendazole was effective in treating *Encephalitozoon* infections but was less effective against *E. bieneusi* infections (2, 21, 22, 46, 57). Fumagillin was found to be effective against *E. cuniculi* in vitro and against *E. hellem* when used topically on patients with keratitis (20). Both fumagillin and albendazole, when given alone, appeared to be parasitostatic, so continued or repeated treatment is required (20, 21, 56). Therefore, this patient was given both fumagillin topically and albendazole systemically, which appears to have been effective since there was no recurrence. Fumagillin has not been administered systemically for the treatment of microsporidiosis, so far as we are aware, presumably because it is believed to be highly toxic.

The results of this study indicate that PCR-Southern analysis can be used to specifically identify the three *Encephalitozoon* species, namely, *E. cuniculi*, *E. hellem*, and *E. intestinalis*. This method appears to be more sensitive than that involving the detection of PCR products on ethidium bromide-stained gels. In addition, PCR-based diagnostic methods (which could include Southern analysis) may become important for epidemiological studies as a means to define the sources and spread of human microsporidian infections.

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