Microsporidial Keratitis in India: 16S rRNA Gene-Based PCR Assay for Diagnosis and Species Identification of Microsporidia in Clinical Samples

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Purpose. To evaluate 16S rRNA–based polymerase chain reactions for the detection and species identification of the microsporidia that cause keratitis.

Methods. Of the 5892 cases of microbial keratitis seen between September 2002 and December 2005, 31 (0.5%) microscopically diagnosed cases of microsporidial keratitis were included in the test group; 103 patients with nonmicrosporidial keratitis constituted the control group. A 16S rRNA-based pan-microsporidian PCR was chosen for the detection of microsporidian DNA. Species level identification was made using species-specific primer sets of *Encephalitozoon* spp (*E. cuniculi*, *E. hellem*, and *E. intestinalis*). Sequencing and BLAST analysis of amplicons obtained with pan-microsporidian primers were performed for validation.

Results. The corneal scrapings from 26 of 31 cases in the test group and 2 of 103 cases in the control group showed a 250- to 280-bp amplicon in PCR by pan-microsporidian primers (sensitivity of 83% and specificity of 98%). The amplicons of 13 of 26 test group samples were identified by species-specific PCR: *E. cuniculi*, $n = 7$ (549 bp); *E. hellem*; $n = 3$ (549 bp); *E. intestinalis*; $n = 1$ (520 bp). The two cases in the control group were identified to be *E. cuniculi*. The remaining 15 cases (test group) were confirmed to be *Vittaforma corneae* by sequencing and BLAST analysis. All species were confirmed by sequencing and database homology comparison.

Conclusions. This study is the first to validate PCR-based assays for detection of microsporidial DNA in corneal scrapings. Pan microsporidian PCR can be a useful adjunct with smear examination in the diagnosis of microsporidial keratitis. (*Invest Ophthalmol Vis Sci.* 2006;47:4468–4473) DOI:10.1167/iovs.06-0376

Microsporidia are obligate intracellular protozoan parasites that infect both invertebrates and vertebrates.[1] Though not commonly reported, microsporidial keratitis may in fact occur more commonly than believed. A case series of five patients with microsporidial stromal keratitis has been reported recently.[2] The same authors also reported a large case series of microsporidial keratoconjunctivitis.[3] Although these reports have increased awareness about this emerging ocular pathogen, a concerted approach is needed to define the true epidemiologic extent of microsporidial keratitis. Routine diagnosis of microsporidia in clinical laboratories has relied mostly on special staining and microscopic techniques.[1,4] Although it has been reported that the microscopic evaluation of corneal scrapings using different stains for the diagnosis of microsporidial keratitis is sensitive,[5] these often require expertise and the inability to differentiate related species in a specimen may require newer methods to be introduced for the identification of microsporidia causing keratitis. Accurate identification of microsporidia is vital for making a quick diagnosis, and species differentiation of microsporidia may play an important role in treatment assessment and prognosis as well as in understanding the pathogenesis and epidemiology. Although immunofluorescence staining techniques have been developed for species differentiation, they are far from optimal, because of problems of limited availability of antibodies, nonspecific binding, and interference from the sample matrix.[6,7]

The recent development of PCR-based detection methods has greatly enhanced the sensitivity and specificity for identifying microsporidia,[1,4,8,9] and these methods are gradually being introduced in clinical laboratories. Several single and nested primer pairs have been reported to amplify gene sequences of *Encephalitozoon* species and *Enterocytozoon bieneusi*.[1] Mostly, the small subunit rRNA has been used as the target gene, but some authors also used the large subunit rRNA and the intergenic spacer region as a target sequence. However, these studies have been limited to amplification of DNA from biopsy, urine, and stool specimens.[1] Two main approaches have been used in the construction of primers that would amplify all major microsporidian pathogens reported to cause ocular infections, such as *Encephalitozoon* spp (*E. cuniculi*, *E. hellem*, and *E. intestinalis*), as well as *Vittaforma corneae* from ocular specimens.[10] In this study, the design of universal pan-microsporidian primers was chosen for rapid validation in a clinical setting in an ocular microbiology laboratory and the design of species-specific primers was considered for the identification of *E. hellem*, *E. cuniculi*, and *E. intestinalis* DNA from corneal scrapings of patients with microsporidial keratitis. The results were validated by sequencing and BLAST analysis.

Materials and Methods

Reference Strains of Microsporidia

Standard strains of *E. hellem* (ATCC 50504), *E cuniculi* (ATCC 50789), *E. intestinalis* (ATCC 50651), and *Vittaforma corneae* (ATCC 50505) were obtained from the American Type Culture Collection (Manassas, VA).

Cultures of bacteria (*Staphylococcus epidermidis*), fungus (*Aspergillus* spp), virus (herpes simplex virus), and *Acanthamoeba* were

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TABLE 1. Details of the Primer Sequences and PCR Conditions and Amplicon Size for 16S rRNA Gene-Based PCR Assays Used in the Study

Subject	Organism	Primer Sequence	PCR Conditions			Amplicon Size (bp)
1	Pan-microsporidian	F: 5′-CACCAGGTTGATTCTGCCTGAC-3′ R: 5′-CCTCTCCGGAACCAAAACCCTG-3′	Denaturation Annealing Elongation	94°C, 1 min 55°C, 2 min 72°C, 3 min	35 cycles	250–280
2	*E. cuniculi*	F 5′-ATGAGAAGTGATGTGTGTGCG-3′ R 5′-TGCCATGCACTCACAGGCATC-3′	Denaturation Annealing Elongation Extension	94°C, 30 sec 55°C, 1 min 72°C, 2 min 72°C, 5 min	35 cycles	549
3	*E. bellem*	F: 5′-TGAGAAGTAAGATGTTTAGCA-3′ R 5′-GTAAAAACACTCTCACACTCA-3′	Initial denaturation Denaturation Annealing Extension	94°C, 5 min 94°C, 1 min 55°C, 1 min 72°C, 1 min	39 cycles	547
4	*E. intestinalis*	F: 5′-TTTCGAGTGTAAAGGAGTCGA-3′ R: 5′-CCGTCCTCGTTCTCCTGC-3′	Denaturation Annealing Extension	94°C, 1 min 55°C, 1 min 72°C, 90 sec	35 cycles	520

F, forward; R, reverse.

clinical isolates from corneal scrapings processed in our laboratory. Human leukocytes were obtained from blood donated by a volunteer.

Maintenance of Reference ATCC Strains

The reference strains were obtained in dry ice. They had been grown in ATCC CCL-26 (African green monkey) cell line and maintained in ATCC 30-2003 (Eagle's minimum essential medium with 2 mM L-glutamine and Earle's balance salt solution adjusted to contain 1.2 g/L sodium bicarbonate, 0.1 mM nonessential amino acids, and 1.0 mM sodium pyruvate). The cultures were initiated by immediately thawing the reference strains in a water bath set at 35°C for 2 to 3 minutes and inoculating the vero cell line (National Facility for Animal Tissue and Cell Cultures; Pune, Maharashtra, India) that was maintained on Eagle's minimum essential medium (EMEM) fortified with 2 mM glutamine and 10% fetal bovine serum (FBS). The medium also contained 50 μg of gentamicin, 1000 units of penicillin, and 5 μg of amphotericin B per milliliter to prevent bacterial and fungal overgrowth. The culture medium from each flask was removed daily for the first week and twice weekly thereafter and replaced with fresh medium containing the antibiotics. The cultures were incubated at 37°C. After 4 to 6 weeks, when infection of a maximum number of host cells (>70%) was achieved, the cells along with the spores were scraped with a cell scraper, and transferred, and processed for DNA extraction.

Clinical Examination of Patients

The medical records and laboratory records of all patients with microbiologically proven microsporidial keratitis seen at the L. V. Prasad Eye Institute, Hyderabad, India, between September 2002 and December 2005 were retrospectively reviewed. In addition, 103 patients with suspected microbial keratitis who had a corneal scraping smear negative for microsporidia were included as the control group. After relevant history was recorded and careful slit lamp examination performed, the patients were subjected to microbiologic analysis.

Microbiologic Investigation of Clinical Samples

The microbiologic work-up included collection of corneal scrapings by an ophthalmologist using a sterile blade number 15 on a Bard Parker handle, under a slit lamp biomicroscope. In all cases, the cornea was topically anesthetized (1% proparacaine hydrochloride; Sunways, India Pvt. Ltd., Mumbai, India) before scraping. Multiple scrapings were taken from the lesions and smeared on presterilized slides for staining. These procedures have been described in detail in an earlier publication.[11] In general, smears of corneal scrapings were stained with (1) potassium hydroxide+calcofluor white (KOH+CFW; Sigma-Aldrich, St. Louis, MO), (2) Gram's stain (HiMedia, Mumbai, India), (3) Giemsa stain (Diff Quick, an equivalent of the Wright/Giemsa stain; Bacto Laboratories Pty. Ltd., Liverpool, NSW, Australia) and (4) modified Ziehl Neelsen stain (1% H_2SO_4; HiMedia). Material was also inoculated onto blood agar, Sabouraud dextrose agar, and brain–heart infusion broth (HiMedia). Scrapings were also collected in 0.5 mL of phosphate-buffered saline (pH 7.2) and stored at −20°C until tested by PCR.

Additional scrapings were taken from cases with suspected viral keratitis and smeared on a glass slide for immunofluorescence assay (IFA), which was performed as described earlier.[12] The primer details and PCR procedure were as per our earlier publication.[13]

DNA Extraction

The harvested vero cells infected with reference strains of microsporidia, as well as the clinical samples were centrifuged at 1500*g* for 3 minutes. The pellet was washed twice using 5 mL of PBS buffer and resuspended in 0.5 mL of lysis buffer (8 M urea, 2% SDS, 0.15 M NaCl, 0.001 M EDTA, 0.1 M Tris-HCl; Unset; Sigma-Aldrich) for DNA isolation. The aqueous lysate was extracted twice with 0.5 mL of phenol-chloroform-isoamyl alcohol (25:24:1). The DNA was finally precipitated using 0.1 mL volume of 3 M NaCl and 2 volumes of ethanol and resuspended in 50 μL of double-autoclaved water (Milli Q; Millipore, Bedford, MA).

The DNA was extracted from *Mycobacterium tuberculosis*, *Aspergillus* species, herpes simplex virus, *Acanthamoeba*, and human leukocytes, according to procedures described elsewhere.[14–17]

Standardization of Polymerase Chain Reaction

The pan-microsporidian primers were designed according to sequences published by Muller et al.,[10] to amplify a conserved region of the small-subunit (SSU)-rRNA of *V. corneae*, *E. cuniculi*, *E. bellem*, and *E. intestinalis*. These primers generate amplicons of 268 bp for E. *cuniculi*, 270 bp for *E. intestinalis*, 279 bp for E. *bellem*, and 250 bp for *V. corneae.* For species identification, the primers used in our study were based on the published sequences of *E. bellem, E. cuniculi*, and *E. intestinalis* SSU-rRNA.[18–20]

The primer pairs were tested initially for amplification of the reference strains obtained from culture, before using them on clinical samples. The PCR conditions and the amplicon size for all the PCR assays are outlined in Table 1.

Each set of reactions included a negative reagent control and 1 μL of extracted DNA positive control from the reference strains. All PCR reactions were performed in a laminar flow hood after 30 minutes of UV irradiation, to decontaminate surfaces and all supplies within the hood. Presterilized PCR tubes and double autoclaved water were used to reduce the possibility of contamination. The products of amplification were electrophoretically resolved on a 1.5% agarose gel and visualized for analysis after being stained with ethidium bromide.

Analytical Specificity of Primers

All the primer pairs were tested against clinical isolates of *Mycobacterium tuberculosis*, *Aspergillus* species, herpes simplex virus, *Acanthamoeba* and human leukocytes DNA along with a negative reagent control and positive control of DNA obtained from reference strains.

Analytical Sensitivity of PCR

The DNA isolated from the standard ATCC strains was serially diluted (100 ng/mL to 100 fg/mL) and amplified using specific primers along with a negative reagent control and neat undiluted DNA of all strains as positive controls.

PCR-Based Diagnosis of Clinical Samples

After standardization of the PCRs the ability of the primers to detect microsporidian DNA in clinical specimens from test and control groups was tested. PCR was performed on the DNA extracted from corneal scrapings (3 μL template) using the pan-microsporidian primers. All samples that tested positive using pan-microsporidian primers were tested with species-specific primer pairs for species identification of *Encephalitozoon* spp. The DNA extract of respective reference strains was used a positive control against each primer and a suitable negative reagent control was used.

Test for PCR Inhibitors in Clinical Samples

To test the presence of PCR inhibitors, the samples that tested negative with the pan-microsporidian primers were spiked with 1 pg DNA of *E. bellem* and subjected to PCR.

Sequencing

The PCR products of all samples that tested positive using pan-microsporidian and species-specific primers were purified (QIA-quick PCR purification kit; Qiagen, Hilden, Germany) and were resuspended in sterile water. The corresponding forward PCR primer was then used for dye termination PCR sequencing, which was performed at the GENEI Sequencing Facility, Bangalore, India.

16S rRNA Sequence Analysis

Database homology searching was performed with BLAST 2.0 on the National Center for Biotechnology Information's Web Site (http://www.ncbi.nlm.nih.gov).

Results

Of the 5892 cases of microbial keratitis seen during the study period, 2960 were culture-positive for bacteria, fungus, *Acanthamoeba*, or mixed infection. In 31 patients (0.5%) microsporidial keratitis/keratoconjunctivitis was diagnosed, based on demonstration of typical spores of microsporidia in any one or more of the smear examination methods of corneal scrapings. The demographic details and predisposing factors are shown in Table 2. Twenty-one of 31 patients, were male and 10 were female. The mean age of the patients was 35.87 ± 16.47 years (range, 2–73). Of the 31 patients with microsporidial keratitis, the clinical diagnosis in 27 was superficial keratoconjunctivitis, and in 4 it was stromal keratitis. In 11 patients, a predisposing factor related to trauma was identified. Three patients gave a history of bathing in unclean river water, and three had a history of use of topical corticosteroids. One patient reported applying honey to his eyes. In the remaining 12, the predisposing factor could not be ascertained. None of the patients wore contact lenses.

Also shown in Table 2 are the microbiologic tests and PCR results of all 31 patients in the test group. Microsporidial spores were detected by KOH+CFW in 29 of 31 samples (93.5%; 95% CI 84.9–100), by Gram's stain in 28 of 31 samples (90.3%; 95% CI 79.9–100), by Giemsa stain in 20 of 31 samples (64.52%; 95% CI 47.67–81.37), and by modified Ziehl Neelsen stain in 27 of 31 samples (87%; 95% CI 75.29–98.89). Direct examination of the corneal scrapings did not reveal any other bacteria or fungi in any of the cases (Table 2). One of 11 patients tested by viral investigations was positive for herpes simplex virus (HSV) antigen by IFA and HSV DNA by PCR.

Of the 103 patients included in the control group, 75 were male and 28 female. The mean age of the patients was 41.5 ± 19.1 years (range, 3–85). Although culture from 40 cases grew bacteria, 13 grew fungus and 9 showed the presence of *Acanthamoeba* (Table 2). Culture was not collected in eight cases that were submitted for viral investigations. The corneal scrapings were sterile in culture in 33 cases.

Analytical Specificity and Sensitivity of the Primers Used for PCR

With pan-microsporidian primers, an expected diagnostic amplicon of approximately 250 to 280 bp was amplified from DNA of all reference strains. All three primer pairs of *Encephalitozoon* spp amplified, an expected, amplicon of approximately 520 to 549 bp for each reference strain. No amplicons were obtained with *Mycobacterium tuberculosis*, *Aspergillus* spp, *Acanthamoeba*, herpes simplex virus, and human leukocyte DNA. DNA up to ~10 pg for all the strains using pan-microsporidia primers could be detected. Using species-specific primers, *E. bellem* and *E. cuniculi* of DNA up to ~10 pg was detected, and for *E. intestinalis* DNA up to ~1 pg was detected.

Validation of PCR on Clinical Samples

Using pan-microsporidian primers, the corneal scrapings from 26 of the 31 cases showed a 250- to 280-bp amplicon (Fig. 1) in PCR. The results of PCR in correlation with microscopy in all the cases are shown in Table 2 Of the 103 samples in the control group 101 tested negative, and two samples showed an amplification of ~250 bp with pan-microsporidian primers.

Using microscopic detection of microsporidia in corneal scrapings as the gold standard, we had 26 true positives, 2 false positives, 5 false negatives, and 101 true negatives. Thus, our PCR using pan-microsporidian primers was estimated to have a sensitivity of at least 83% and a specificity of 98%.

Of the 26 samples in the study group that were PCR positive with the pan-microsporidian primers, 7 samples showed an amplification of 549 bp (Fig. 2a) with primers specific for *E. cuniculi*, 3 showed an amplification of 547 bp (Fig. 2b) with primers specific for *E. bellem*, and 1 showed an amplification of 520 bp with primers specific for *E. intestinalis* (Fig. 2c). Fifteen samples positive by pan-microsporidian primers did not show any amplification with all three primers. The two samples in the control group that were positive using pan-microsporidian primers, showed an amplification of 549 bp with primers specific for *E. cuniculi.*

Test for PCR Inhibitors in Clinical Samples

An expected amplification corresponding to ~270 bp specific for *E. bellem* was obtained with all samples in both the test and control groups after spiking, which ruled out the presence of PCR inhibitors in the clinical samples.

Sequencing and Database Homology Comparison

Using DNA sequence analysis for amplicons with generic (pan-microsporidian) primers for unknown microsporidia species, the sequence of 15 samples exhibited 96% to 99% homology with the *V. corneae* 16S-rRNA sequences available in GenBank (http://www.ncbi.nlm.nih.gov/Genbank; provided in the pub-

TABLE 2. Demographic, Direct Smear Examination and PCR Results of Corneal Scrapings from Patients with Microsporidial Keratitis/Keratoconjunctivitis

						Direct Smear Examination					PCR	
Patient No.	Lab No.	Age	Sex	Predisposing Factor	Clinical Diagnosis	KOH/CFW	Gram	Glemsa	1% Acid-fast	Test Results for Bacterial/Fungus/Virus	Pan-microsporidian Primers	Species-Specific Primers
1	L490/02	2	M	—	Stromal	+	+	+	+	Negative	+	*V. corneae*
2	L538/02	23	M	—	Stromal	+	+	−	+	Negative	−	NI
3	L229/02	70	F	Injury-grass	Stromal	+	+	+	+	Negative	+	*V. corneae*
4	L1870/02	40	F	Injury-fields	Epithelial	+	+	+	+	Negative	+	*V. corneae*
5	L1296/03	24	M	Trauma-insect	Epithelial	+	+	+	+	Negative	+	*V. corneae*
6	L1820/03	73	M	Water	Epithelial	+	+	+	+	*Corynebacterium* spp.	+	*V. corneae*
7	L1841/03	30	M	Water	Epithelial	+	+	+	+	*Corynebacterium* spp.	+	*V. corneae*
8	L2460/03	41	M	—	Epithelial	+	−	−	−	Negative	+	*E. bellem*
9	L2631/03	33	F	Dust	Epithelial	+	+	+	+	Negative	−	NI
10	L2599/03	60	M	Trauma-wood	Epithelial	−	+	−	+*	Negative	+	*E. bellem*
11	L1359/04	39	M	—	Epithelial	−	+	+	+	Negative	−	NI
12	L1686/04	26	M	Trauma-dust	Epithelial	+	+	+	+	Negative	+	*V. corneae*
13	L1763/04	30	F	Dust	Epithelial	+	+	−	+	Negative	+	*V. corneae*
14	L1770/04	28	M	Insect/dust	Epithelial	+	+	+	+	Negative	+	*E. cuniculi*
15	L1864/04	23	M	Water	Epithelial	+	−	−	+	Negative	+	*V. corneae*
16	L2013/04	60	M	PK	Epithelial	+	+	−	+	*Proteus mirobilis*	+	*E. cuniculi*
17	L2055/04	30	F	—	Epithelial	+	+	+	+	Negative	+	*E. cuniculi*
18	L2288/04	51	F	—	Epithelial	+	−	−	+	Negative	+	*V. corneae*
19	L350/05	38	M	Dust	Epithelial	+	+	+	+	Negative	+	*E. cuniculi*
20	L494/05	17	M	—	Epithelial	+	+	+	+	Negative	+	*E. cuniculi*
21	L991/05	23	F	—	Epithelial	+	+	+	+	Negative	−	NI
22	L1355/05	37	F	—	Epithelial	+	+	+	+	*Pseudomonas aeruglnosa*	+	*V. corneae*
23	L1786/05 L1828/05	59	M	—	Epithelial	+	+	+	+	*Staphylococcus epidermidis*	+	*V. corneae*
24	V360/05	56	M	Injury-iron rod	Epithelial	+	+	+	+	Negative	+	*E. cuniculi*
25	L1835/05	20	M	PK	Epithelial	+	+	+	+*	Negative	+	*E. cuniculi*
26	L1851/05	25	F	—	Epithelial	+	+	+	+	Negative	+	*E. intestinalis*
27	L2096/05	29	M	Injury	Epithelial	+	+	−	−	Negative	+	*V. corneae*
28	L2237/05 L2457/05	43	M	—	Epithelial	+	+	−	−	Negative	−	NI
29	V367/05	35	M	—	Stromal	−	+	+	+	*Herpes Simplex Virus*	+	*V. corneae*
30	L2631/05	26	F	Topical honey	Epithelial	+	+	−	+	Negative	+	*V. corneae*
31	L2828/05	21	M	Topical steroids	Epithelial	+	+	−	+	Negative	+	*F. bellem*

+, presence of microsporidial spores; −, absence of microsporidial spores; +*, non-acid-fast spores present; NI, not identified.

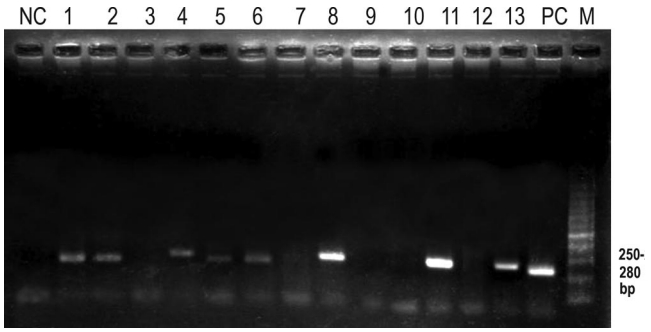

FIGURE 1. Image of a gel, showing results of PCR with pan-microsporidian primers used on 13 corneal scrapings. *Lanes 3*, *7*, *9*, *10*, and *12*: negative, showing no amplicon; *lanes 1*, *2*, *4–6*, *8*, *11*, and *13*: show positivity for microsporidian DNA. NC, negative control; PC, positive control (*V. corneae*), *lane M*: 100-bp marker.

lic domain by the National Center for Biotechnology Information, Bethesda, MD) and hence a positive identification of this species was made for these 15 samples. The species identification made by PCR using the species-specific primers for the remaining 11 isolates in the study group as well as the two isolates in the control group were also confirmed after sequencing the amplicons obtained by PCRs with both generic and species-specific primers. The sequence homology was 96% to 99% after BLAST analysis in all cases. The details are given in Table 2.

DISCUSSION

Routinely used microbiologic staining techniques have been shown to have a high sensitivity and specificity in the detection of microsporidia in ocular samples.[5] However, a practical and simple confirmatory test to support direct smear examination findings is not available. Apart from being subjective, detection of microsporidial spores in smears requires expertise and training In addition, the species of microsporidia cannot be determined by these staining techniques. A technique that is rapid, sensitive, scaleable, and capable of being used for the identification of human pathogenic microsporidia to the species level, in both clinical and environmental sample matrices, has been lacking to date. Culture of microsporidia requires access to tissue culture facilities which, apart from being expensive, are unable to identify species. Consequently, a strong effort is being directed toward development and application of molecular biology methods, particularly PCR, to increase the levels of reliability, for detection and species identification of microsporidia. This test can be easily added to the armamentarium of diagnostic methods in a microbiology laboratory.

To enhance our diagnostic capability, we decided to include PCR for detection of four species of microsporidia in corneal scrapings, in addition to smear examination. Confirmation of smear results using PCR with pan-microsporidian primers can increase confidence in making the diagnosis of microsporidial keratitis. Although PCR-based assays for the detection of microsporidia in stools, urine, and sputum have been described,[1,8–10] none has been tested on corneal scrapings. To validate the PCR method on clinical samples, in this study, 31 samples from the test group and 103 from the control subjects were examined using pan-microsporidial PCR. The results showed that 26 of the test samples were PCR positive. Five smear-positive samples were not positive by these primers probably due to lack of DNA in the sample, since invariably the last scraping was used for PCR. Another possibility could be the presence of other species of microsporidia that are not detectable by the pan-microsporidia primers. Possibility of PCR inhibitors was ruled out in these samples.

Two samples in the control group were positive with pan-microsporidian primers. These were identified as *E. cuniculi* with species-specific primers and confirmed by sequencing and database homology comparison. A review of clinical records of these two patients showed that they had had microsporidial keratoconjunctivitis clinically diagnosed and that they had a few, minute, coarse, punctate, superficial epithelial lesions on the cornea, with associated conjunctivitis. These cases were probably missed on direct examination of corneal scrapings. One patient was seen in the emergency clinic whose scraping was collected after working hours of the laboratory, and the smears were examined on the following day. This delay may account for quenching of the fluorescence from the calcofluor white-stained smear and may have caused an error in interpretation. The cause of missing the diagnosis in the second case, however, could not be established. Because the corneal lesions were very minimal, it is possible that the occasional spores in the smears missed detection. This study has shown PCR using pan-microsporidian primers to have a sensitivity of at least 83% and specificity of 98%. Considering the low prevalence of the disease, a very large sample size of negative control subjects would be necessary to be tested to determine predictive values of the PCR assays. Owing to this limitation in this study, the positive predictive value was 21.9% after adjustment with Bayes Theorem. The negative predictive value of the assay was 95.3%, however. The sensitivity may improve by further optimizing PCR reaction conditions; nevertheless, the results of this study indicate that pan-microsporidia PCR can be used in routine diagnosis for a quick confirmation of the clinical diagnosis. This PCR can also be used for epidemiologic studies as a means to define the source and spread of human microsporidian infections. The limitation of this PCR is that the species-representative amplicons are too close in size to be distinguished from each other.

Species-specific identification of microsporidia in clinical specimens is becoming increasingly important because of various levels of responses to some drugs.[1] Although albendazole and fumagillin have been reported to be useful against many species of *Encephalitozoon*, no drug has been shown to be effective against *E. bieneusi*.[1] Though most species of microsporidia respond favorably to fumagillin in the United States,[1] these may be only anecdotal observations and blinded, placebo-controlled, comparative trials are lacking to date. Therefore, these PCR assays may also find utility as a means of monitoring the effectiveness of antimicrosporidial therapy.

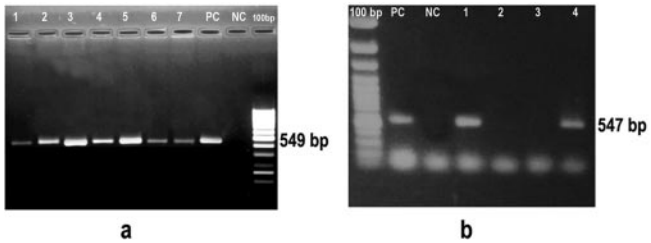

FIGURE 2. Gel photograph of ocular samples showing amplification with primers specific for (**a**) *E. cuniculi* in *lanes 1* to *7* (patients 14, 16, 17, 19, 20, 24, 25); (**b**) *E. hellem* in *lanes 1*, *4* (patients 8, 10, 31); (**c**) *E. intestinalis* in *lane S* (patient 25). NC, negative control; PC, positive control.

The ability of PCR sequencing and database searching to distinguish between very closely related species has already been shown[1] and allows human and nonhuman microsporidia to be distinguished. This study considered a homology of 96% or above to be definitive. Amplicons showing less than 100% homology to the database sequences in our cases were probably due to the computer sequencing software's inability to identify bases during sequencing, especially when the sequencing software incorporated an "N" in place of an actual base due to a weak or covered signal from the actual base. Taking this and the fidelity of *Taq* polymerase into account, we felt thought that at least 96% homology was necessary for definitive species determination of microsporidia. Examination of sequences deposited in GenBank for the gene encoding the SSU rRNA of *E. bellem*, *E. cuniculi*, *E. intestinalis*, and *V. corneae* reveals considerable disagreement about the degree of sequence conservation in the 5′ region (base pairs 1–300) of this gene. Because DNA from all four parasites was efficiently amplified in the assay using the pan-microsporidian primers, it is concluded that this region covered by the primer is conserved in these organisms and therefore a single PCR using these primers can be a useful adjunct to smear examination of corneal scrapings by routine staining methods. In epidemiologic studies that require species identification, one may use sequencing after pan-microsporidian PCR or resort to species-specific PCR.

To the best of our knowledge, this is the first study in which PCR was used for detection and identification of species of microsporidia from ocular specimens. The short time and relatively lower cost and expertise required for PCR testing is a distinct advantage over electron microscopy. We believe that our work will increase the awareness of this rare disease and open avenues for further research.

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