PCR for the diagnosis and species identification of microsporidia in patients with keratitis

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Abstract

Corneal scrapings from 30 patients with microbial keratitis were subjected to microsporidial PCR. PCR was positive for microsporidia in ten of 30 patients. The species was identified as *Vittaforma corneae* by sequencing in all ten patients. The remaining 20 patients were negative for microsporidia and showed the growth of other organisms (*Acanthamoeba*, fungi or bacteria).

Keywords: Corneal scrapings, keratitis, microsporidia, PCR, *Vittaforma corneae*

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Microsporidia cause a variety of human infections, including intestinal, ocular, sinus, pulmonary, muscular and renal, in both immunocompetent and immunocompromised patients [1]. Ocular manifestations include superficial punctate keratoconjunctivitis and stromal keratitis [2]. Ocular microsporidiosis is diagnosed in the laboratory by identification of microspordial spores using different staining techniques, or by electron microscopy of corneal scrapings, corneal biopsy samples and corneal buttons [2]. In this study, we evaluated PCR as a tool for the diagnosis and species identification of microsporidia in patients with keratitis.

Thirty patients diagnosed with microbial keratitis between June 2008 and December 2008 were included in the study. A detailed history was taken in all cases and a slit lamp bimicroscopic examination was performed. Corneal scrapings were taken from patients using a sterile no. 15 surgical blade on a Bard-Parker handle under 4% lignocaine topical anaesthesia. The material obtained was smeared on clean, presterilized glass slides for microscopic examination using Gram stain, Giemsa stain, potassium hydroxide with calcofluor white preparation and 1% acid-fast stain (Kinyoun's stain). The scraped material was also inoculated onto sheep blood agar, chocolate agar, Sabouraud's dextrose agar, potato dextrose agar, thioglycollate broth, brain–heart infusion broth and non-nutrient agar with *Escherichia coli* overlay for *Acanthamoeba*. The corneal scraping was also collected for microsporidial PCR in 300 μL of phosphate-buffered saline.

DNA was extracted from corneal scrapings and from American Type Culture Collection (ATCC) strains of microsporidia using a Qiamp DNA kit (Qiagen, GmbH, Hilden, Germany). Primers for PCR were designed from a small-subunit rRNA of microsporidia as described by Raynaud *et al.* [3] (forward, 5′-CACCAGGTTGATTCTGCC-3′; and reverse, 5′-GTGACGGGCGGTGTGTAC-3′). Microsporidial PCR was standardized using ATCC strains of *Encephalitozoon hellem* (ATCC 50504), *Encephalitozoon cuniculi* (ATCC 50789), *Encephalitozoon intestinalis* (ATCC 50651) and *Vittaforma corneae* (ATCC 50505). The microsporidial primers were tested for specificity using cultures of bacteria (*Staphylococcus aureus*), fungi (*Fusarium* spp.) and *Acanthamoeba* isolated from corneal scrapings.

The PCR cycles consisted of an initial denaturation step at 94°C for 3 min, 35 cycles of denaturation at 94°C for 45 s and annealing at 57°C for 45 s with an extension at 72°C for 1 min, and a final extension at this same temperature for another 7 min. The products of amplification were electrophoretically resolved on a 1.5% agarose gel and visualized for analysis after being stained with ethidium bromide. The expected positive PCR product size was 1200 bp in length.

Sequencing was performed with fluorescently labelled dideoxynucleotide terminators, using an ABI 3130 XI automated sequencer, following the manufacturer's instructions (PE Applied Biosystems, Foster City, CA, USA). Each sequence was manually aligned and analysed to ensure high-quality sequence data. The sequences were analysed and identified using the MEGABLAST search program of GenBank databases. An isolate was assigned to a particular species if the small-subunit rRNA sequence of the isolate was >98% similar to that of the type strain of the most closely related species indicated by the NCBI BLAST. The percentage similarity was determined using alignment of the sequence of the isolate in question with the GenBank sequence of the type strain, using the BLAST program.

All four ATCC strains of microsporidia were amplified in the PCR. No amplicons were obtained with *S. aureus*, *Fusarium* spp. or *Acanthamoeba* isolated from clinical corneal scrapings. PCR was positive for microsporidia in ten of 30 patients, and culture was negative for bacteria, fungi and *Acanthamoeba* in all of these ten cases. Microsporidial spores were detected by potassium hydroxide with calcofluor white preparation in all ten patients (100%), by Gram stain in five of seven (71.4%), by Giemsa stain in one of two (50%) and by 1% acid-fast stain in seven of eight (87.5%) patients. Of the remaining 20 patients, ten showed fungal growth, eight showed bacterial growth and two showed *Acanthamoeba* growth; all of these 20 patients were negative for microsporidial PCR. PCR products of all ten patients with microsporidial keratitis on sequencing showed a high similarity (98–99%) with the *V. corneae* deposit sequence in the GenBank database. The species of microsporidia identified in the study and the degree of similarity between the study isolate and the reference isolate sequence deposited in GenBank are shown in Table 1.

Ocular microsporidiosis was considered to be a rare disease, but recently there was an outbreak of epidemic keratoconjunctivitis caused by microsporidia in India [4,5]. We noticed an increase in the number of microsporidia cases in the previous year (Reddy AK, unpublished data) at our institute. Microsporidial keratitis may mimic herpes simplex virus keratitis [2] and epidemic keratoconjunctivitis, which is usually caused by adenovirus. Laboratory diagnosis plays an important role in differentiating microsporidial infection from other forms of keratitis.

The various methods available for the diagnosis of microsporidial infection include direct microscopic examination of microsporidial spores in corneal scrapings, using various staining techniques, culture and PCR [2]. Direct microscopic examinations of microsporidial spores in corneal scrapings, using different staining techniques, have been shown to have high sensitivity [2]. However, detection of microsporidial spores with the use of different stains requires expertise and immediate screening of the sample. Any delay in screening of slides, especially those stained with potassium hydroxide with calcofluor white (a highly sensitive method for the detection of microsporidial spores), leads to the likelihood of the smear becoming dry and quenching the fluorescence from the calcofluor white-stained smear [6]. The detection also depends on the quantity size of the sample. Occasional microsporidia can be overlooked when the sample quantity is very small. On the other hand, microsporidial culture requires tissue culture facilities, is expensive and is also time-consuming [2].

TABLE 1. The species of microsporidia identified in the study and the degree of similarity between the study isolate and reference isolate sequence deposited in GenBank

Study isolate (GenBank accession number)	Identified species	Reference sequence[a] accession number	Percentage similarity with reference sequence
AKR 1 (GQ924593)	*Vittaforma corneae*	UI1046	99
AKR 2 (GQ924594)	*V. corneae*	UI1046	99
AKR 3 (GQ924595)	*V. corneae*	UI1046	98
AKR 4 (GQ924596)	*V. corneae*	UI1046	98
AKR 5 (GQ924597)	*V. corneae*	UI1046	98
AKR 6 (GQ924598)	*V. corneae*	UI1046	99
AKR 7 (GQ924599)	*V. corneae*	UI1046	98
AKR 8 (GQ924600)	*V. corneae*	UI1046	99
AKR 9 (GQ924601)	*V. corneae*	UI1046	99
AKR 10 (GQ924602)	*V. corneae*	UI1046	99

[a]GenBank sequence to which similarity was observed.

Several published studies describe PCR-based methods to amplify different regions of the small and large subunits of the rRNA gene and the intergenic spacer region for diagnosis and species differentiation of microsporidia infecting humans [7]. In this study, we have evaluated small-subunit rRNA primers for the diagnosis of keratitis caused by microsporidia. The primers used in this study amplified all four species of microsporidia (*E. hellem*, *E. cuniculi*, *E. intestinalis* and *V. corneae*) reported to cause ocular infections [7]. Joseph *et al.* [6] evaluated the 16S rRNA-based pan-microsporidial PCR for the diagnosis and species identification of microsporidia in ocular samples, and they found that the sensitivity of the assay was 83% and the specificity was 98%. In this study, of 30 patients with microbial keratitis, microsporidial PCR was positive in ten, and all of these cultures were negative for bacteria, fungi and *Acanthamoeba*. Of the remaining 20 patients, ten showed fungal growth, eight showed bacterial growth and two showed *Acanthamoeba* growth; all of these 20 patients were negative for microsporidial PCR. There were no false positives and false negatives with the microsporidial PCR in our study. Although potassium hydroxide with calcofluor white preparation (direct microscopic examination) detected all cases of microsporidia that were positive in PCR, the advantage of PCR is that it can be performed on stored samples and samples with occasional microsporidia. Species identification is not possible with direct smear examination methods, whereas this is possible using PCR. Although species identification is possible with tissue cultures, it is very expensive and also time-consuming (approximately 2–3 weeks).

PCR is a rapid technique. The detection and species identification of microsporidia can be done within 72 h using PCR. The most common species of microsporidia isolated from ocular infections include *V. corneae*, *E. hellem*, *E. cuniculi* and *E. intestinalis* [7]. In this study, we identified *V. corneae* in all ten patients infected with microsporidia.

PCR is highly sensitive and specific for the detection and identification of microsporidia in patients with keratitis, and

can be used for the confirmation of diagnosis. The major limitation of this study was the small sample size, and PCR as a diagnostic tool needs to be evaluated with large numbers of ocular samples.

Transparency Declaration

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