Microsporidia are obligate intracellular parasites that infect eukaryotic cells and have emerged as major opportunistic human pathogens. Due to the difficulties in definitive laboratory diagnosis and insufficient knowledge, ocular microsporidiosis is infrequently reported in India. To improve diagnostic facilities, we have developed a novel duplex PCR (dPCR) for the simultaneous identification of both genera and species of isolates with microsporidian aetiology that cause keratitis. The material scraped from the corneas of 12 clinically diagnosed microsporidial keratitis patients was subjected to routine microbiological examinations and molecular diagnosis using a novel dPCR that targeted the small-subunit rRNA gene (SSU-rRNA) of microsporidia and *Vittaforma corneae* using genus- and species-specific primers. Of the 12 corneal scrapes, 6 showed positive results in smears, while dPCR provided positive amplification with both pan-microsporidial and *V. corneae* species-specific primers for 9 corneal scrapes. The results were validated by sequencing and BLAST analysis. The sensitivity of this novel dPCR method was higher than that of conventional microscopy in the diagnosis of corneal microsporidial infection. dPCR with specific primers is potentially more sensitive, specific and depends less on more complicated methods for exact identification of the aetiology of microsporidial keratitis.
of microsporidia; however, the large-subunit rRNA gene and the intergenic spacer region have also been used as the target DNA (Joseph et al., 2006c; Franzén & Müller, 1999). An efficient approach for the molecular detection of microsporidia is the construction of universal or pan-microsporidial primers that amplify all major microsporidian pathogens, as well as species-specific primers for microsporidial species reported to cause ocular infections. In this context, we have developed a novel methodology, duplex PCR (dPCR), for rapid simultaneous detection of both genus and species of microsporidia using genus- and species-specific primers. The results have been validated by sequencing and through BLAST analysis.

METHODS

Ocular specimens and inclusion of study subjects. This prospective and consecutive analysis included 1270 corneal scrapes that were submitted for microbiological evaluation from patients clinically diagnosed as having infectious keratitis by a group of ophthalmologists. It was based on typical clinical features, such as eyes with a loss of corneal epithelium, with underlying stromal infiltration and suppurature associated with signs of inflammation, with or without hypopyon, seen at the Cornea Clinic of Aravind Eye Hospital & Postgraduate Institute of Ophthalmology, Tirunelveli, Tamil Nadu, India, between October 2010 and September 2011. Ulcers with typical features of viral infection and healing ulcers were excluded, as were Mooren’s ulcers, interstitial keratitis, sterile neurotropic ulcers and any other ulcers associated with autoimmune conditions.

Among the 1270 patients clinically diagnosed with infectious keratitis, a provisional diagnosis of microsporidial keratitis was made in 12 patients based on their typical clinical features, such as progressive loss of vision, with multiple diffuse, minute, dot or punctate epithelial lesions, sometimes with multiple target-like epithelial lesion evolving into a nummular pattern or mixed follicularpapillary conjunctival reaction. Sometimes a dense greyish stromal infiltrate with or without surrounding oedema was observed (Fig. 1a), mostly presenting as an atypical viral keratoconjunctivitis or mimicking herpes simplex virus keratitis, and sometimes associated with iritis. Immunocompromised patients typically develop conjunctivitis and an epithelial keratitis accompanied by pain, photophobia, blurred vision, conjunctival hyperaemia and foreign body sensation (Das et al., 2012; Friedberg & Ritterband, 1999). After detailed ocular examination with a slit-lamp bio-microscope, using standard techniques, corneal scrapes were performed under aseptic conditions for each ulcer by an ophthalmologist using a sterile Bard–Parker (no. 15) blade after instillation of...
0.5 % proxymetacaine hydrochloride. The scraped material of the 12 patients who were clinically diagnosed as having microsporidian keratitis was included in the study. Part of the scraped material was subjected to microbiological evaluation such as smear analysis [10 % potassium hydroxide wet mounting, Gram-staining, acid-fast staining, modified Ziehl–Neelsen acid-fast staining (1 % H2SO4 techniques)] and culture analysis (bacterial culture on blood agar supplemented with 5 % sheep’s blood, chocolate agar, brain heart infusion broth and thioglycollate medium, fungal cultures on potato dextrose agar and Sabouraud’s dextrose broth, and acanthamoebic cultures on non-nutrient agar with *Escherichia coli* overlay) (Bharathi et al., 2007). Part of the corneal scrapes from the 12 study subjects were collected in 1.5 ml vials containing 250 μl Tris–EDTA (TE) [1×TE – 10 mM Tris (pH 8.0 with HCl), 1 mM EDTA] buffer for performing dPCR analysis. The protocols mentioned in the manuscript are the routine diagnostic procedures at our institute, and hence Institutional Review Board approval was not necessary.

**DNA extraction.** Extraction of DNA from the 12 samples from the study subjects and from 4 American Type Culture Collection (ATCC) control strains of microsporidia (*Encephalitozoon hellem* (ATCC 50504), *Encephalitozoon cuniculi* (ATCC 50789), *Encephalitozoon intestinalis* (ATCC 50651) and *Vittaforma corneae* (ATCC 50505)), were carried out using a QIAamp DNA mini kit (Qiagen) in accordance with the manufacturer’s recommendations.

**Oligonucleotide primers.** Oligonucleotide primers as described by Raynaud et al. (1998) (forward 5′-CACCAGTTGATCTGC-3′ and reverse 5′-GTGACGGCCGTTGTCGACT-3′) targeting the SSU-rRNA gene were used for the amplification of the pan-microsporidial genome, and species-specific primers targeting the SSU-rRNA gene of *V. corneae* were designed using sequences in GenBank with an MEGALIGN search program of the GenBank database. Finally, organism identification was determined by comparing our sequences with existing sequences in the GenBank database using the BLAST program available at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). A sequence similarity of <99 % for the SSU-rRNA gene was the criterion used to define a new bacterial strain.

**Optimization of dPCR and amplification of microsporidial genomes.** Reactions (50 μl) for dPCR were optimized in an Eppendorf Mastercycler ProS thermalcycler with PCR reagent mixture consisting of 10× buffer [10× buffer – 10 mM Tris-HCl pH 8.3 (at room temperature), 50 mM KCl, 1.5 mM MgCl2], 200 μM each dNTPs, oligonucleotide primer sets (10 pmole each primer), 1 unit Taq DNA polymerase, with or without MgCl2 (2 mM), 10 ng template DNA from corneal scrapes and/or from positive control strains and sterile water. The dPCR programs consisted of an initial denaturation at 94 °C for 5 min, followed by 30 cycles of DNA denaturation at 94 °C for 45 s, primer annealing at 58 °C for 60 s, and primer extension at 72 °C for 90 s. After the last cycle, a final extension step at 72 °C for 8 min was added and the products were stored at 4 °C. The annealing temperature of both the pan-microsporidial and *V. corneae* primers was optimized as 58 °C for 60 s. All PCRs were carried out in a 50 μl reaction volume contained in a 0.2 ml thin-walled polypropylene microfuge tube (Eppendorf), using 10 μl DNA from each corneal scrape, in addition, there were three negative controls (DNA extraction control, reagent mixture control and non-infectious vitreous flush) and one positive control (*V. corneae* ATCC 50505), after determining the specificity and sensitivity of all the primer sets. The amplicons were resolved in 1.5 % Tris/borate–EDTA agarose gel with ethidium bromide (0.5 μg ml−1), visualized with a UV transilluminator and results recorded with a gel documenting system (Vilber Lourmat). The expected PCR amplicon size was 1200 and 472 bp for pan-microsporidial and *V. corneae* specific-primers, respectively. A 20 kb DNA ladder (GeNei) was used for comparison.

**Sequencing of PCR products.** For further confirmation, the PCR products of six study subjects were subjected to sequencing analysis at MWG AG Biotech, Bangalore, India, using an ABI 3730 XL automated sequencer (PE Applied Biosystems) following the manufacturer’s instructions. The sequences were analysed and identified using the MEGABLAST search program of the GenBank database. Finally, organism identification was determined by comparing our sequences with existing sequences in the GenBank database using the BLAST program available at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). A sequence similarity of <99 % for the SSU-rRNA gene was the criterion used to define a new bacterial strain.

**RESULTS**

Of the 12 study subjects, 6 showed positive results for microsporidia in smears (Figs 1b, c, d), while cultures exhibited negative results for any microbial growth. Smears and cultures of the remaining six study subjects were found to be negative for micro-organisms. DNA amplified with pan-microsporidial primers from all four ATCC strains of microsporidia produced 1200 bp amplicons, while DNA amplified with *V. corneae* species-specific primers from an ATCC strain of *V. corneae* alone produced a 472 bp amplicon (Fig. 2a). None of the primers produced amplicons with bacterial, fungal and acanthamoebic DNA (Fig. 2a). The lowest concentration at which pan-microsporidial primers amplified DNA from the four ATCC control strains was 10 pg, while *V. corneae* species-specific primers with DNA of the *V. corneae* control strain required 1 pg (Fig. 2b). Of the 12 study subjects, the DNA of 9 (75 %) (of which 6 showed positive results and 3 showed negative results in smears) was amplified positively with both pan-microsporidial primers and *V. corneae* species-specific primers producing 1200 and 472 bp amplicons, respectively (Fig. 2c). None of the DNA from the remaining three subjects showed any positive amplification. Similarly, neither of the two negative controls showed any positive amplification. DNA sequencing of the six amplicons, followed by a DNA database comparison analysis with a partial sequence of the SSU-rRNA gene of *V. corneae* identified the isolates as six new strains of *V. corneae* and the sequences have been deposited in the NCBI GenBank database (accession numbers JQ087416–JQ087421). A sequence similarity of <99 % for the SSU-rRNA gene was the criterion used for defining a new strain.

**DISCUSSION**

Microsporidia are a diverse group of intracellular eukaryotic parasites that are highly derived relatives of fungi and harbour very small genomes, the most extreme being the 2.3 Mbp genome of *E. intestinalis* (Peyretaillade et al., 1998).
In these genomes, the genes are flanked characteristically by short intergenic regions along with few repetitive sequences. Since microsporidia are obligate and intracellular in nature, the numbers of genes present in the genome are severely reduced and are packed into a smaller space with a significant reduction of intergenic space (Katinka et al., 2001; Slamovits et al., 2004; Vivarès & Méténier, 2000). Although microsporidia are true eukaryotic organisms, the features of the rRNA-encoding DNA resemble prokaryotic sequences (Franzen & Müller, 1999), and are composed of a 16S SSU-rRNA gene and a 23S LSU-rRNA gene, which are separated by an intergenic non-transcribed spacer. Whereas, true eukaryotic organisms possess a 5.8S rRNA gene, and this is a unique feature of true eukaryotes. In addition to this, the SSU-rRNA and LSU-rRNA genes of microsporidia are shorter than typical eukaryotic SSU-rRNA and LSU-rRNA genes, and lack several universal sequences (Franzen & Müller, 1999). The discovery of a gene for a mitochondrion-type chaperone (Peyretaillade et al., 1998) combined with molecular phylogenetic data, led to microsporidia being reclassified as fungi that have lost mitochondria during evolution (Van de Peer et al., 2000).

Several PCR-based methods have been reported for amplifying different regions of the SSU and LSU rRNA genes, as well as the intergenic spacer region, for diagnosis and species differentiation of microsporidia infecting humans (Fedorko & Hijazi, 1996). SSU-rRNA sequences are used for species-specific detection, as well as for the analysis of the phylogenetic relationship of an organism (Franzen & Müller, 1999). Hence, to enhance our diagnostic capability, conserved, repetitive and highly variable sequences within the rRNA genes need to be identified that amplify microsporidian genomes specifically. The highly variable sequences of the SSU-rRNA genes provide a unique signature for any microsporidian and useful information about possible relationships (Woese, 1987), because SSU-rRNA genes accumulate mutations at a low and constant rate over time. The first such report on the use of conserved rRNA primers was for the SSU-rRNA gene of Vairimorpha nectatrix, a pathogen of agricultural pests, which indicated that evolutionary development path leading to microsporidia branched very early from that leading to other eukaryotes (Vossbrinck et al., 1987; Ghosh & Weiss, 2009). Primers complementary to conserved sequences within this gene were used to amplify and subsequently obtain sequence data on the rRNA gene of several human-infecting microsporidia (Zhu et al., 1994; Vossbrinck et al., 1993; Raynaud et al., 1998; Franzen & Müller, 1999; Reddy et al., 2011). Therefore, such pan-microsporidial primers are very useful in the
screening of clinical specimens for microsporidia. Two sets of primer were used in our novel dPCR for targeting both the genus of microsporidia and the species of *V. corneae*. The genus-specific or pan-microsporidial primers amplified the region between sequence positions 1 and ~1205 of the SSU-rRNA gene of microsporidial species, producing 1200 bp products from the DNA of nine study subjects, whereas, the species-specific primers for *V. corneae*, which were novel and primers designed by us, amplified the region between sequence positions 191 and 662 of the SSU-rRNA gene of *V. corneae*, [which was complementary with the reference sequence of *V. corneae* (GenBank accession no. L39112)] and produced 472 bp amplicons from the DNA of six study subjects, as well as showing a positive amplification with genus-specific (pan-microsporidial) primer sets.

Diagnoses of microsporidial infections are primarily based on the identification of microsporidial spores in clinical samples using light microscopy, but genus and species differentiation are uncertain (Sharma et al., 2011). In the present analysis, of the 12 clinically suspected microsporidial keratitis samples, corneal scrapes from 6 cases showed positive results for microsporidia by smear-based light microscopy examinations revealing a uniform oval shape, and a non-budding sporulant nature, which helped to differentiate them from bacteria and yeasts. The spores stained strongly with Gram-stain and modified Ziehl–Neelsen acid-fast staining and were highly remarkable. Spores are the only easily recognizable stage in microsporidia and which can survive outside their host cells (Vavra & Larsson, 1999). Spores range in size from 1 to 40 μm, and can be spherical, ovoid, rod-shaped or crescent shaped, although most are ovoid. The classification of microsporidia in humans has depended on transmission electron microscopy techniques, and this remains the gold standard for the identification and speciation of microsporidia; however, it is not suitable for use in routine diagnosis. Apart from being subjective, detection of microsporidial spores in smears requires expertise and training, and in addition, the species of microsporidia cannot be determined by these staining techniques. Furthermore, culturing of microsporidia in mammalian cell lines is costly incorporating time-consuming procedures, which are prone to failure with specimens from unsterile sites (Franzen & Müller, 2001). Therefore, the molecular biology of microsporidia has drawn attention for the accurate detection and speciation of isolates from clinical specimens. The data presented here clearly show that pan-microsporidial primers and species-specific primers of *V. corneae* can be used for the determination of both the genus and species of *V. corneae* simultaneously by utilizing a novel dPCR for corneal specimens. In addition, the analysis presented also underlines the superiority of dPCR by providing a higher diagnostic rate (75%) over microscopy diagnosis (50%). Also, considering the reagent consumption, test cost, duration of time and requirement of thermalcyclers for the amplification of two genomes, dPCR has a distinct advantage over conventional PCR in rapid, simultaneous identification of the genus of microsporidia and of *V. corneae*.

For the taxonomic system of microsporidia, both rRNA genes and protein-coding DNA sequences have been used. By using sequencing analysis, it has been shown that *Nosema trichopusia* is a synonym for *Nosema bombycis* based on the sequences of the SSU-rRNA encoding region (Pieniazek et al., 1996). Nucleotide sequences of the SSU-rRNA of microsporidial species have been published in NCBI databases and are accessible via the GenBank database. These are helpful in designing new primers for the identification of unknown microsporidial species. Here, by using the GenBank database, we have designed species-specific primers for the amplification of the *V. corneae* genome and for the sequencing of the PCR products. The sequencing analysis revealed six novel strains of *V. corneae* and the sequences of their SSU-rRNA genes are accessible via the GenBank database (accession numbers JQ087416–JQ087421), and will be helpful for developing newer identification tools. Sequence-based identification relies on limited, yet phylogenetically informative, SSU-rRNA sequence variation between related bacterial taxa. The entire SSU-rRNA gene of microsporidia is ~1550 bp in length; however, partial sequencing of SSU-rRNA generally provides sufficient taxonomic information while limiting costs. Partial SSU-rRNA sequences can be compared with sequences of *V. corneae* available in GenBank to determine the species with maximum similarity. For most taxa, sequence identity >99% with a valid reference sequence is required for species-level identification.

The phylogeny of various species of microsporidia has been reported based on the sequence data of the whole SSU-rRNA gene obtained by PCR with primers amplifying the region between sequence positions 18 and 1537, complementary to the conserved region of SSU-rRNA gene at the 3’ and 5’ ends (Baker et al., 1995). Results of bio-informatics analysis describe the sequence difference between *V. corneae* (formerly *Nosema corneum*) and *N. bombycis*, and it seems clear that *V. corneae* is unrelated to other *Nosema* spp. Baker et al. (1995) placed *V. corneae* in the *Endoreticulatus* group because the parasite is most closely related to *Endoreticulatus schubergi* (7.3% distance). This supports the reclassification of *N. corneum* to a new genus as *V. corneae*, which was carried out based on the ultrastructure of developmental stages in the livers of experimentally infected athymic mice (the diplokaryotic arrangement of the nuclei was the only characteristic that conformed to the description of the genus *Nosema*) (Silveira & Canning, 1995). Similarly, the degree of sequence homology of the SSU-rRNA gene from *E. intestinalis*, *E. cuniculi*, *E. hellem* and *Enterocytozoon bieneusi* analysed by pairwise comparison with the BESTFIT program, after *HphI* and *BsmI* restriction enzyme digestion of the amplified DNA, have been depicted by Hartskeerl et al. (1993, 1995) who concluded that *E. cuniculi*, *E. hellem*, and *E. intestinalis* belong to the same genus and proposed the reclassification of *Septata intestinalis* to *E. intestinalis*. The
taxonomy of microsporidia has undergone several changes during the last few years and will continue to change significantly in the near future when new DNA-based data are incorporated into new classification systems. Therefore, new molecular techniques have led not only to the discovery of new organisms but also to the elimination of an old organism.

Accurate identification up to the species level is possible through sequencing, which requires the use of long sequencing runs and time-consuming processes, and is becoming mandatory for the specific management of ocular microsporidiosis. Therefore, the utilization of species-specific primers is an elegant alternative for a sequencing methodology. This report is believed to be the first of its kind that validates a novel dPCR, a more sensitive and specific method, which depends less on more complicated methods, which will help in the reliable and quick diagnosis of microsporidiosis, including species determination. This systematic approach for the recognition of novel species with potential diagnostic or therapeutic relevance provides a basis for epidemiological surveys and improvement of sequence databases that may lead to identification of new clinical entities.

ACKNOWLEDGEMENTS

The financial support of the Aravind Eye Care System, India, is acknowledged.

REFERENCES


