Use of PCR Targeting of Internal Transcribed Spacer Regions and Single-Stranded Conformation Polymorphism Analysis of Sequence Variation in Different Regions of rRNA Genes in Fungi for Rapid Diagnosis of Mycotic Keratitis†

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The increased incidence of fungal infections in the recent past has been attributed to the increase in the number of human immunodeficiency virus-positive and AIDS patients. Early diagnosis of mycoses in patients is crucial for prompt antifungal therapy. Immunological methods of diagnosis have not been found to be satisfactory, and recent research has been diverted to the use of PCR for the sensitive and early diagnosis at the molecular level. In the present study we targeted different regions of the rRNA gene to diagnose cases of mycotic keratitis and identify the causal agents. Six fungus-specific primers (primers ITS1, ITS2, ITS3, ITS4, invSR1R, and LR12R) were used, and the amplified products were analyzed by single-stranded conformation polymorphism (SSCP) analysis. Dendrograms of these SSCP patterns, prepared on the basis of Jaccard’s coefficient, indicated that the PCR products obtained with primer pair ITS1 and ITS2 were the best for the identification of fungi. The results were confirmed by sequencing of the PCR products, and the approach was successfully tested experimentally for the detection of mycotic keratitis caused by Aspergillus fumigatus and was used for the diagnosis of fungal corneal ulcers in patients.

Mycotic keratitis or fungal corneal infections have a worldwide distribution, and the incidence is higher in tropical and subtropical countries (8). More than 10 species of fungi belonging to 56 genera have been reported to cause ocularmycosis. However, species of Fusarium, Aspergillus, Candida, and other hyaline and dematiaceous hyphomycetes are the usual isolates from patients with mycotic keratitis (20). The management of keratomycosis depends on rapid identification of the causal agent. The diagnosis is often delayed because of the poor availability of infected material from the cornea and the slow growth of a large number of fungi in routinely used culture media, and therefore, early intervention is not always possible and the patient’s vision is often lost. In general, the diagnosis of fungal corneal ulcer is dependent on Gram and Giemsa staining, which have low sensitivities of about 50 to 80% (4). Recent advances in molecular biology techniques have opened the door for culture-independent diagnostic methods. Immunological detection (13) and identification by use of distinctive metabolites (2) and nucleic acid probes (7, 25) are the tools most often used for diagnosis. One such technique is PCR, which has been shown to be useful for the culture-independent diagnosis of various microbial infections, including mycoses (10, 11, 22). To date, a few cases of mycotic keratitis have successfully been diagnosed by PCR (5, 6).

Ribosomal DNA is the most conserved region in the genome, with capabilities of phylogenetic divergence (14). The whole rRNA gene contains a small subunit (SSU) 18S rRNA, 5.8S rRNA, and a large subunit (LSU) 28S rRNA. Internal transcribed spacer (ITS) region I (ITSI) and ITSII are more variable than the rest of the ribosomal gene subunits and are found between SSU rRNA and 5.8S rRNA and between 5.8S rRNA and LSU rRNA, respectively. Besides this, intergenic spacer (IGS) region I (IGSI) and IGSII are found between the end of the LSU and start of the next SSU sequence (24). Many workers (9, 15–17) have used the single-stranded conformation polymorphism (SSCP) technique to identify sequence variations in a single strand of DNA due to its adoption to a unique conformation under nondenaturing conditions (12). Here we report on the experimental proof and the clinical laboratory diagnosis of three cases of corneal ulcer by PCR by the ITS SSCP technique, in which useful vision could be restored due to prompt diagnosis and specific antifungal therapy.

MATERIALS AND METHODS

Cultures. Aspergillus fumigatus, Aspergillus flavus, Candida albicans, Candida krusei, Candida parapsilosis, Cryptococcus neoformans, Fusarium spp., Sporothrix schenckii, Trichophyton mentagrophytes, and three patient isolates (responsible for mycotic keratitis) were maintained on Sabouraud dextrose agar (SDA). Pseudomonas aeruginosa was maintained on nutrient agar.

Extraction of fungal DNA. All the fungal strains were inoculated in 100 ml of Sabouraud dextrose broth under shaking conditions at 37°C to obtain log-phase cultures. Microscopic examination was done to test the purity of the cultures, and the cells were harvested by centrifugation at 6,000 × g for 15 min at 4°C. The pellets were washed twice with 0.8% physiological saline and transferred to 200 μl of extraction buffer (0.2 M Tris-HCl [pH 7.6], 0.5 M NaCl, 0.1% sodium dodecyl sulfate, 0.01 M EDTA). Glass beads were added to this mixture at a 1:1 ratio and vortexed vigorously in a bead beater (HamiltonBeach/Proctor-Silex, Inc., Southern Pines, N.C.) to achieve 60% lysis of the cell mass. Fungal DNA from this lysate was recovered with a DNeasy mini kit (Qiagen, Hilden, Germany).
Germany), electrophoresed on a 1% agarose gel with 1× TBE buffer (8.9 mM Tris-borate, 0.2 mM EDTA), and analyzed after staining with ethidium bromide. The purity of the extracted DNA was checked at 260 and 280 nm (UV/VIS 911A; GBC Scientific Equipment, Dandenong, Australia) and stored at −20°C for further analysis.

**Sample collection.** (i) Experimental mycotic keratitis. Experimental A. fumigatus keratitis was produced in bred albino New Zealand rabbits by the method of Agrawal et al. (1). A spore suspension of *A. fumigatus* keratitis was produced in bred albino New Zealand rabbits by the further analysis.

The amplified products were denatured at 95°C for 10 min and snap-cooled on ice before they were mixed with denaturing buffer (80% [wt/vol] deionized formamide, 10 mM EDTA [pH 8.0], 1 mg of xylene cyanol per ml, 1 mg of bromophenol blue per ml). The samples were then electrophoresed on a 5% acrylamide gel. The gels were then silver stained and analyzed (3).

**Similarity-dissimilarity analysis.** SSCP profiles were generated by using all of the samples from the PCRs and the four sets of primers. These profiles were analyzed by the presence or the absence (which were given values of 0 and 1, respectively) of bands to prepare a dendrogram for these fungal strains on the basis of Jaccard's coefficient.

**Results** The 260 nm/280 nm ratios for the DNAs extracted from all the fungal strains were found to be between 1.7 and 1.8. These DNA samples gave positive results in all sets of reactions. Amplification of DNA samples with primer pairs ITS1-ITS2, ITS3-ITS4, ITS1-ITS4, and invS1R-LR12R resulted in fragments of approximately 200 bp, 350 bp, 550 bp, and 2.0 kb, respectively. None of these primers amplified bacterial DNA.

Experimental mycotic keratitis caused by *A. fumigatus* was successfully produced in the right eyes of the albino rabbits (Fig. 1a). The progression of the corneal infection was monitored daily for up to 7 days, and the scrapings from the infection sites were found to be positive by PCRs with all the primer pairs described above. Also, *A. fumigatus* was isolated on SDA at 2, 3, 4, and 5 days postinoculation. We could achieve amplification of fungal DNA from the corneal scrapings by PCR as early as 48 h postinoculation (Fig. 2). These primer pairs did not amplify fragments from the corneal tissue from the control eye of any of the rabbits. Scrapings from three of the four patients suspected of having mycotic keratitis produced amplification of fungal DNA from the corneal scrapings by PCR.
be 22°C and 5 V/cm, respectively. Overall, four SSCP patterns specific for different regions of the ribosomal gene were obtained from a total of 15 DNA samples (9 from standard cultures, 3 from isolates from the patients with mycotic keratitis, and 3 from the corneal scrapings from these patients). Variations in the sequences of the different regions of the ribosomal gene were clearly evident in the SSCP patterns (Fig. 4a to c).

Agarose gel electrophoresis of the PCR products showed that ITSII, amplified by primer pair ITS3 and ITS4, was almost of the same size in all the fungi tested, whereas the PCR products of ITSI, which was amplified with primers ITS1 and ITS2, varied in size, thereby indicating that ITSII is the more conserved region in the 18S and 28S rRNA genes (Fig. 3a and b). However, a considerable difference in the band patterns of the amplified products of both the ITSI and the ITSII regions was observed by SSCP analysis (Fig. 4a and b). Similarly, the products amplified from the ITSI and the ITSII regions, including the 5.8S rRNA region (amplified with primer pair ITS1 and ITS4), showed little variation in size, as detected by aga-
rose gel electrophoresis (Fig. 3c), whereas a significant difference in the band patterns of these amplified products (from all 15 DNA samples) was obtained by SSCP analysis (Fig. 4c). All the PCR products obtained with primer pair ITS1 and ITS4 were sequenced with primer ITS1 and were identified by using the BLASTn program. These sequences were found to be 90 to 100% similar to the sequences of the ITS1, 5.8S rRNA gene, and ITSII regions of the respective fungi. On the basis of the results of these studies, the three patient isolates were identified as the Colletotrichum state of Glomerella cingulata, Curvularia inaequalis, and Epidermophyton floccosum by PCR of the corneal scrapings as well as by conventional identification methods. The multiple-sequence alignment obtained with the Clustal W program is presented in Fig. 5. The fourth patient had a case of bacterial keratitis and did not give positive results for fungi by PCR analysis.

Primers invSR1R and LR12R, used in the present study for amplification of the IGS region of the fungi, were inferior, as they failed to amplify the DNA of C. albicans and T. mentagrophytes under normal conditions. These primers also did not give positive results with DNA from the corneal scrapings. However, variation of the MgCl2 concentration in the reaction mixtures resulted in positive results. SSCP analysis of the amplified products obtained with this primer pair was not satisfactory, as the product size was found to be about 2.0 kb (Fig. 2) for all the fungal isolates; therefore, almost similar patterns were obtained (data not shown).

The SSCP patterns of the ITS regions were found to differentiate the fungal strains tested to the species level. The SSCP patterns of the Candida and Aspergillus strains differed significantly (Fig. 4a to c, lanes 1, 2, 3, 5, and 6), but no such difference was evident in the products amplified from the DNA from the corneal scrapings or in those from their subsequent cultures (Fig. 4a to c, lanes 10, 11, 12, 13, 14, and 15). Dendrograms were prepared from the three SSCP patterns described above on the basis of Jaccard's coefficient. Analysis of these dendrograms also confirmed the findings indicated above (Fig. 6).

DISCUSSION

The identities of the fungi determined by PCR in the present study matched 100% the conventional identities of the respective strains. Three of four patients with mycotic keratitis were positive for fungi by this technique, while the fourth patient, who was negative for a fungus in this study, was found to be negative for fungus in culture as well, thereby indicating the specificity of the ITS PCR-SSCP technique.

The high purity of the fungal DNA isolated in the present study was indicated by the 260 nm/280 nm ratios, which ranged from 1.7 to 1.8 for all fungi. We achieved the same high grade of purity of DNA obtained by the extraction methods used earlier, including those with commercial kits, by use of the QIAamp tissue mini kit (Qiagen). We also used the DNeasy plant mini kit (Qiagen) for DNA extraction, but this kit was not found to be suitable for use with corneal scrapings, in which it is supposed that only a few fungal elements may be present. The added advantage to our extraction method was that we could isolate the pure form of DNA in significantly less time (<1 h) than is required for other methods (11, 15). In addition, the kit that we used apparently had a capacity of removing all PCR-inhibiting substances (5) from DNA extracts of the corneal scrapings.

We were interested in the section of the genome that in-
includes the 18S, 5.8S, and 28S genes, which code for rRNA and whose nucleotide sequence is also relatively conserved among fungi. This section also includes the intervening ITS regions, called ITSI and ITSII, whose DNA sequences vary. Although the ITS-coding regions are not translated into proteins, they have a critical role in the development of functional rRNA; and because of the sequence variations of these regions among species, these regions show promise for use as signatures for molecular biology-based assays (25). A number of probes have been designed by many workers for the identification of fungal DNA by the hybridization procedure, but PCR is the most sensitive and widely used technique for the identification of fungi and is also best suited for use with clinical samples in which DNA is poorly available. Ferrer et al. (5) used primers ITS1, ITS4, and ITS86 to identify fungal strains by nested PCR, and Kumeda and Asao (15) also used PCR with primers ITS1 and ITS4 followed by SSCP analysis to identify fungi pathogenic for plants (15). Besides these, other primer pairs have been designed for the identification of fungi (21). On the basis of these facts, the primers described by White et al. (24) and Vilgalys et al. (23), which anneal to different regions of the ribosomal gene, were selected and the sequence variations in each segment were determined simultaneously. An IGS region-specific primer pair was also used to compare the sequence variations within the ITS region. To analyze the variations in nucleotide sequences, direct sequencing of the amplified product, restriction fragment length polymorphism analysis (21), temperature gradient gel electrophoresis, denaturing gradient gel electrophoresis, amplified rRNA gene restriction analysis for the ribosomal gene, and SSCP analysis (9, 15–17) are being used by various workers. Among all these techniques, SSCP analysis is one of the most accurate and is often used for mutational studies and the detection of single nucleotide polymorphisms (12). In the present study the ITS PCR-SSCP technique was used for the successful identification of fungi.

We tried to assess these techniques for their abilities to diagnose experimental keratomycosis in rabbits. The prognosis of the disease depends on an early and a prompt diagnosis. In the case of mycotic keratitis, the delay in diagnosis is due to the lack of sensitive techniques and the continued dependence on classical methods, such as direct microscopic examination, culture of material obtained from deep corneal scrapings, corneal biopsy, and anterior chamber paracentesis. The availability of less clinical material and the slow growth of fungi often lead to delays in therapy and ultimately result in corneal damage. Using all four primer pairs, we succeeded in diagnosing experimental fungal keratitis due to *A. fumigatus* within 8 h from the time of collection of the corneal scrapings from the rabbits (Fig. 2), which was faster than the time to the recovery of fungi by conventional culture on SDA. Encouraged by this we applied our technique to four patients who were suspected of having fungal corneal ulcers and who consulted a private ophthalmic practitioner (N. K. Mishra).

The fungi isolated from the human cornea were identified in

![FIG. 6. Dendrograms of all the standard strains and patient isolates (the Colletotrichum state of *G. cingulata*, *C. inaequalis*, and *E. floccosum*) prepared with the NTSYS program (Exter Publishing, Ltd., Setauket, N.Y.). (a) Dendrogram generated by using the SSCP pattern shown in Fig. 4a; (b) dendrogram generated by using the SSCP pattern shown in Fig. 4b; (c) dendrogram generated by using the SSCP pattern shown in Fig. 4c.](http://jcm.asm.org/)

![FIG. 5. Multiple-sequence alignment of the sequences of ITS1, 5.8S rRNA, and ITSII of patient isolates with the sequences of standard fungi by use of the Clustal W program. The sequence of the isolate from patient 1 matches 100% the sequence of the *G. cingulata* anamorph *Colletotrichum floccosporoides* (GenBank accession no. AY177329), followed by matches of 99% with *Colletotrichum fragariae* (GenBank accession no. AY536223) and 99% with *G. cingulata* strain CMUBE1851 (GenBank accession no. AY266391), the sequence of the isolate from patient 2 matches 98% the sequence of *C. inaequalis* strain CBS 185.47 (GenBank accession no. AF120261), with the next best matches being to the sequences of *Bipolaris papendorfii* (96% similarity; GenBank accession no. AF163075) and *Cochliobolus hawaiiensis* (80% similarity; GenBank accession no. AF071324); the sequence of the isolate from patient 3 matches 96% the sequence of *E. floccosum* (GenBank accession no. AJ000629), with the next best matches being to the sequences of *Arthroderma benhamiae* (90% similarity; GenBank accession no. AF506036) and *T. mentagrophytes* (88% similarity; GenBank accession no. 297999).](http://jcm.asm.org/)
the laboratory on the basis of micro- and macromorphological characteristics. One of the isolates was identified as the Colletotrichum state of *G. cingulata*. Interestingly, this is the second case of mycotic keratitis caused by this fungus (Fig. 1b) in the vicinity of our city (Lucknow, India) and has been encountered after a gap of more than 20 years (18). The other two isolates were identified as a *Curvularia* sp. and an *Epidermophyton* sp. Species of the genus *Curvularia* are frequently isolated, while an *Epidermophyton* sp. has rarely been reported as a cause of mycotic keratitis (19, 26). The PCR product sequencing studies resulted in the identification of these isolates as the Colletotrichum state of *G. cingulata* (100% similarity), *C. inaequalis* (99% similarity), and *E. floccosum* (98% similarity). This indicates the authenticity and superiority of PCR-based identification of fungi (Fig. 5).

The amplified products obtained with primer pair invSR1R and LR12R were almost similar; therefore, the SSCP patterns derived from this IGS region did not differentiate all the fungi, whereas the ITS region-specific primer pair ITS1 and ITS4 resulted in amplified products of various sizes that could be differentiated on SSCP gels due to sequence variations (Fig. 4c). ITS1 has a more variable segment than ITSII, and ITS1 was amplified by primers ITS1 and ITS2 (Fig. 4a and b). This was also confirmed by the use of Jaccard’s coefficient, as represented in the dendrogram in Fig. 6. The dendrogram prepared with the ITSII sequences showed no difference between *A. flavus* and *Fusarium*; similarly, the dendrogram prepared with the complete sequences of ITS1, 5.8S, and ITSII failed to differentiate *S. schenckii* and the Colletotrichum state of *G. cingulata*. On the other hand, the dendrogram prepared with the ITS sequences differentiated all the fungal strains to the species level (Fig. 6). However, the patient isolates of the Colletotrichum state of *G. cingulata*, *C. inaequalis*, and *E. floccosum* were found to be closely related to *A. fumigatus*, *A. flavus*, and *T. mentagrophytes*, respectively. This satisfies our sequencing results for the DNA sample amplified with primers ITS1 and ITS4. In conclusion, the successful diagnosis of three sequencing results for the DNA sample amplified with primers

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