Optimization and Application of Molecular Techniques for Rapid Detection of Fungal Genome from Ocular Specimens: Our Experience in a Tertiary Care Centre in India

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Ocular Mycoses
Ocular fungal infections are an important cause of morbidity and blindness, especially in tropical countries like India. Mycotic keratitis (corneal fungal infection) is the most frequent presentation, but the intraocular structures (fungal endophthalmitis) orbit, sclera, and rarely the adnexal organs like lacrimal apparatus may also be involved. An important problem is in assessing the accuracy of the genus or species identification of a fungal strain isolated in culture. The criteria to associate a particular fungus with ocular mycoses were as follows: when an adequate clinical history was presented that suggested a mycotic infection; when the fungus was seen in the clinical specimens; and when the morphology of the fungus was consistent with the reported etiologic agent.1

Corneal blindness is a major public health problem in India. Fungal keratitis is one of the most important infections leading to such grave complications. This can be prevented by making an early diagnosis and instituting appropriate antifungal therapy. The use of nested polymerase chain reaction (PCR) in detection of fungi in ocular specimens would help in administration of antifungal drugs and patient management. Since the revolutionary molecular biology technique of PCR involves enzymatic amplification of even minute quantities of a specific sequence of DNA, it is of great benefit in rapidly detecting the presence of organisms which are difficult to culture. Ocular samples that can be submitted for PCR include intraocular fluid (aqueous or vitreous), any fresh ocular tissue, formalin-fixed or paraffin-embedded tissue, and even stained or unstained cytology slides or tissue. The results of all these studies suggest that PCR is more sensitive than culture as a diagnostic aid in ophthalmic mycoses. However, concern persists regarding the specificity of this technique and the problems that may arise from the production of false-positive results. In most of these studies, insufficient detail has been provided to permit an independent assessment of the adequacy of the techniques used for culture. In the diagnosis of ophthalmic mycoses, PCR would probably be most valuable in providing a positive result in a shorter period than that required for culture and in identification of a fungal isolate which does not sporulate.2

Present Status of Ocular Mycoses in India
Fungal keratitis is more common in tropical countries. The detection and management of fungal keratitis are mainly by direct smear study and culture. Mycotic keratitis can easily be detected and/or diagnosed using conventional methods. Fungi were identified as the principle aetiological agent in 44% of corneal ulceration in India.3 There are a series of reports on the spectrum of fungi causing keratitis from Southern India.4 The management of fungal keratitis poses a problem when the conventional methods yield a negative result. Development of rapid and accurate tests for laboratory diagnosis of fungal infections could improve outcome of affected patients.

In India, a specific PCR targeting the small subunit ribosomal RNA has been developed and applied on corneal specimens for detection of fungal genome and proved to be extremely sensitive technique. However, a routine mycology laboratory in India is not in a position to implement the technique for rapid detection. Only a few well-equipped mycology laboratories in India can adapt this technique for application. Identification of fungi to species level is essential to direct the antifungal treatment. In about 75% cases, fungal identification to species level is possible by conventional methods. In the remaining, the identification is not possible because of atypical characters of certain fungi, ability to grow in different morphological forms and emergence of new species. The only way to identify these fungi is by using rapid molecular techniques – PCR-based RFLP and DNA sequencing technique.

The conventional methods involved in the detection of fungal infections are less sensitive due to lesser amount of available clinical specimens and low microbial threshold. The techniques are laborious and time consuming. Rapid diagnosis by molecular methods aids in the institution of specific antifungal drug and management. The advantages of molecular methods are it is rapid, extremely sensitive and specific. The explosion in the rates of opportunistic fungal infections has propelled interest in clinically relevant methods for rapid detection and identification of fungi from ocular specimens.

Fungal endophthalmitis ranges 4–11% in tropical countries. The laboratory diagnosis of fungal
endophthalmitis is mainly by direct smear study and culture. Unlike fungal keratitis, the conventional methods are often negative in fungal endophthalmitis. There are a series of case reports on fungi causing endophthalmitis and the methods involved in detection. In India, a specific PCR targeting the large subunit ribosomal RNA has been developed and applied on intraocular specimens to detect fungal aetiology.\(^5\) The technique targeting the 28S ribosomal RNA gene was optimized and applied on ocular specimens to detect panfungal genome. PCR detected fungal genome in 45% in comparison with the conventional technique positive in 20.23% by smear examination and 25% by culture. This technique proved to be extremely sensitive to detect the fungal aetiology.

Aspergillus endophthalmitis is the commonest type of vision-threatening fungal endophthalmitis encountered in India. Since conventional methods lack sensitivity, we evaluated PCR against the conventional mycological methods in the diagnosis of Aspergillus endophthalmitis. In this study, 27 intraocular specimens from 22 patients with suspected fungal endophthalmitis (proved as non-bacterial origin) were included. The intraocular specimens from these patients were subjected to the conventional methods, viz. microscopy and culture for growth of fungi, as well as PCR for the detection and differentiation of species of Aspergillus. Among the 27 test samples, 4 samples that were positive by culture were also positive by PCR for Aspergillus species.

In addition, PCR detected and identified Aspergillus species in 2 culture-negative specimens. The average time required for PCR was only 24 h against a week’s time by conventional mycological methods. Thus, the study indicated that PCR was not only a more sensitive but also a rapid diagnostic tool compared to the conventional mycological methods in the diagnosis of Aspergillus endophthalmitis.\(^6\)

**Development and Application of a Novel Multiplex PCR Targeting 18S rRNA, ITS region and 28S rRNA for Detection of Panfungal Genome in Ocular Specimens**

Fungi have a ribosomal DNA (rDNA) complex region including a sequence coding for 18S rRNA, ITS1, 5.8 S, ITS 2, 28S rRNA gene. All three genes within the rRNA complex have been used in studies on the molecular evaluation of fungi. The 18S gene region, which is about 1800 bp in size with both conserved and variable domain sequences, has been used to assess the taxonomic relationships of the major groups of living organisms and to separate genera and species based on sequence polymorphisms. However, the drawback of using this region with the identification of species is the relative sequence homology among fungal species and the need to sequence a large number of bases to do complementary analysis. The 5.8S region on the other hand is only about 160 bp long and conserved with the major organism groups. Owing to its small size and conserved nature, it is not appropriate for phylogenetic studies to classify fungal species. However, this conserved region has been useful as an attachment site for universal primers to amplify flanking spacer regions within the eukaryotic genome. The 28S rRNA, which is around 340 bp in size, also contains both conserved and variable nucleotide sequence regions. The variable domains of this large ribosomal subunit have also been used to allow comparisons from high taxonomic level to the species level. Much of the 28S rRNA gene, however, is conserved among organism groups limiting the usefulness of this region for species identification. mPCR optimized to detect fungal DNA in corneal scrapings and intraocular fluids from patients clinically suspected to have fungal keratitis and endophthalmitis; and develop a multiplex PCR targeting the ribosomal unit consisting of 18S rRNA region, the ITS region, and 28S rRNA region.

Our findings suggest that mPCR is a potentially valuable tool for detecting keratomycosis. A variety of modifications in the optimization will
also require ongoing evaluations in multiple clinical settings with more rigorous control specimens for comparison. Eventually, PCR might solidly complement the current “gold standard” diagnostic techniques for guiding management or supporting research studies of fungal keratitis and endophthalmitis. The novel multiplex PCRs were optimized with three sets of primers to detect panfungal genome in ocular specimens. Another multiplex PCR was also developed to detect eu-bacterial, Propionibacterium acnes and panfungal genomes simultaneously in intraocular specimens. The application of mPCR proved to be cost effective and aided in rapid detection of infectious aetiology.

ITS noncoding regions flanked by the structural rRNA genes. Approximate binding sites of the ITS3 and ITS4 PCR primers are shown by arrows. Most molecular diagnostic methods are able to screen patients in the initial stages of fungal infection, but not all protocols can identify the source of the DNA to the genus or species level. For example, 18 different species of fungi were detected by a PCR method employing a universal primer that amplified a highly conserved region in the 18S rDNA, but this method cannot differentiate among these species.

The nucleic acid-based amplification techniques are helpful in management. To evaluate semi-nested polymerase chain reaction (snPCR) targeting internal transcribed spacer (ITS) region for detection of panfungal genome in ocular specimens, standardization of PCR targeting ITS primers was carried out by determining analytical sensitivity and specificity.

**Application of a Semi-nested Polymerase Chain Reaction Targeting the Internal Transcribed Spacer Region**

PCR amplification may facilitate the identification of ITS region DNA sequences with sufficient polymorphism to be useful for identifying fungal species. Coding regions of the 18S, 5.8S, and 28S nuclear rRNA genes which evolve slowly are relatively conserved among fungi and provide a molecular basis for establishing phylogenetic relationships between coding regions ITS 1 and 2 (ITS1 and ITS2, respectively), which evolve more rapidly and may therefore vary among different species within a genus.

SnPCR was applied onto a total of 168 ocular specimens with clinically suspected fungal aetiology. PCR was specific and sensitive and detected fungal genome in 90 (53.57%) ocular specimens.
in comparison with the conventional technique, positive in 34 (20.23%) by smear examination and in 42 (25%) by culture. The increase in clinical sensitivity by 28.57% using PCR was found to be statistically significant \( P < 0.001 \) using Z-test for two proportion. The accuracy of the test was found to be 70.85%. PCR proved to be a rapid diagnostic technique for detection of panfungal genome directly from clinical specimens.\(^8\)

**Strain Variations Encountered in Aspergillus Species**

The increasing popularity of molecular approaches for the identification of fungal pathogens reflects significant improvements in DNA analysis in recent years. A number of targets for molecular identification of *Aspergillus* species have been investigated including the mitochondrial cytochrome \( b \) gene, DNA topoisomerase gene, betaglobulin gene and various ribosomal RNA genes. The most promising target to date has been the 5' end of the large-subunit rRNA gene (D1–D2 region) and the ITS region. Primary structural analysis of *Aspergillus* species has revealed negligible intraspecies variability and recognizable interspecies divergence within the D2 region. DNA sequencing technique has been standardized to analyse the genotypic differences existing among *Aspergillus* strains causing ocular infections using nucleic acid-based amplification techniques targeting the ITS region by PCR, PCR-based RFLP, and DNA sequencing of ITS amplicons. The ITS region has been used as target for phylogenetic analysis because it generally displays sequence variation between species but only minor variation within the strains of same species. In our study, all *A. flavus* isolates showed similar pattern of digestion with Hae-III. The intraspecies variation among *Aspergillus* species is 2.3% alignment of contiguous fungal sequences, which demonstrated that both single-nucleotide differences and short lengths of sequence diversity due to insertion or deletion existed in the ITS regions among the pathogenic *A. flavus* strains. *A. flavus* isolates in our study had a BLAST score of 97.7% identity with the standard strain of *A. flavus* (ATCC 16883) – GenBank Accession No. AB008415. The inspection of BLAST alignments generated with *A. flavus* ITS1 and ITS 2 data from GenBank revealed that many *A. flavus* sequences in the database had truncated ends and/or heterogeneities at positions found to be conserved at the subgeneric level among reference sequence of type and authenticated culture collection strains. In accordance with this in our study, *A. flavus* isolates revealed a variation of 2.3% when compared with the standard strain of *A. flavus*. The sequences were deposited in GenBank, and Accession Nos DQ683118, DQ683119, DQ683120, DQ683121, DQ683122, DQ683123, and DQ683124 were assigned for the isolates.

The AH and VF isolates of *A. flavus* obtained from two different patients showed single-nucleotide polymorphisms pertaining to substitution, insertion, inversion, and deletion. *A. flavus* isolated from eviscerated material revealed distinct single- and multiple-nucleotide polymorphisms. The pattern of dissimilarity among *A. flavus* isolates varied from single- or double-nucleotide polymorphisms in intraocular specimen isolates, and multiple-nucleotide polymorphisms were seen in *A. flavus* isolated from corneal scraping and eviscerated material. Ribosomal sequence analysis for *A. flavus* group still requires important improvements in GenBank database, including submission of additional sequence information for ITS 1 and ITS 2 regions, implementation of quality control measures for sequence entries with nomenclatural updates. Upon major improvement of present sequence databases, identification to the species level should be feasible through ITS sequence analysis. The association of this particular pattern of nucleotide polymorphism contributing to the pathogenicity of *A. flavus* strain making it different from the environmental strain needs to be investigated further. Also, the pattern of polymorphism exhibited by external and intraocular isolates of *A. flavus* strains proves to be a potential area of research in near future. The nucleotide polymorphisms existing among *A. flavus* strains seems to be novel and the first to be reported in the literature to the best of our knowledge.\(^9\)

**Application of PCR-based DNA Sequencing for Identification of Non-sporulating Fungi Using Primers Targeting ITS Region**

Non-sporulating moulds (NSMs) results from unfavourable growth conditions due to which no fruiting structures are produced. The other common names include hyaline mycelia and mycelia sterilia. Mycelia sterilia is a form order that contains the filamentous fungi that remain sterile despite attempts to induce the formation of conidia or spores. Sterile isolates represent species of fungi that simply are not producing conidia, spores, pycnidia, ascoscarps or basidiocarps because of compatibility systems, the lack of appropriate environmental and nutritional needs, or both. In rare instances, these fungi are opportunistic pathogens of humans. If an isolate is suspected of causing disease, it is important to try to induce the formation of conidia, spores or fruiting bodies, so it can be identified. There is no universal medium or set of environmental conditions that will stimulate conidiogenesis or sporogenesis. Various media and techniques must be tried until the correct combination of variables is found. Since most mycelia sterilia are not significant isolates, it is not practical to expend much time and material attempting to
induce sporulation. These isolates should be tested for resistance to cycloheximide and the ability to grow at 35–37°C. Production of reproductive structures or expression of specific biochemical phenotypes by fungi grown in culture is required for phenotypic analyses.

The identification of filamentous fungi in the clinical laboratory can be challenging as some of the fungal isolates do not sporulate (mycelia sterilis), making identification by microscopic morphology not possible. The integration of molecular analyses targeting ITS1, ITS2 and 28S DNA sequences with traditional phenotypic methods of fungal identification can significantly increase the specificity and decrease the turnaround time. ITS sequences may provide accurate identification of closely related isolates and species which cannot readily be distinguished using 26S or 28S rRNA gene sequences. The phenotypically validated ITS DNA sequence database is useful for identification of routinely isolated moulds, previously unidentified moulds, and moulds that do not show specific or expected morphological and biochemical phenotypes. We have optimized nested PCRs for early detection of fungal genome directly from intraocular specimens and nested PCR targeting ITS region to identify NSMs. In addition, a novel multiplex PCR for simultaneous detection of pan-fungal genome targeting three different genes was optimized and proved to be a rapid and reliable tool in rapid detection of fungal genome from intraocular fluids. The development of sensitive and rapid diagnostic molecular techniques would not only allow confident verification of the diagnosis but also allow early commencement of specific and appropriate treatment.

Traditionally, the recovery of NSM has been dismissed in the laboratory as insignificant environmental organisms without further testing. Additionally, for those laboratories who attempt to augment sporulation specifically for identification, the process can require up to 3 weeks of incubation and often without success. A small proportion of NSM (12% – Statistics of L & T Microbiology Research Centre) were found to be associated with fungal keratitis. These fungi fail to produce the characteristic identification structures which are a prerequisite to identify the fungal genera. DNA-based identification has been utilized successfully to identify pathogenic fungi. In order to identify the fungi by molecular methods, it is imperative that phenotypically well-characterized mould isolates from clinical samples are used for developing the method and building the sequence database. In L & T Microbiology Research Centre, in order to identify the NSM, a study was undertaken by targeting the ITS region of these fungi by designing a PCR-based DNA sequencing technique. A representative subset of 50 isolates reported as NSM was randomly selected for gene sequencing. PCR-based DNA sequencing identified 11 (22%) to genus and 39 (78%) to species. The isolates were identified by sequencing the ITS 1 region identifying 15 different genera, and these genera were confirmed by sequencing the ITS2 region. Thirteen isolates had reference sequences that shared 100% identity to species level involving 8 genera, and 27 isolates had sequences that shared 99% identity to species level and 10 isolates had sequences that shared 95–98% identity to genus level. Of the 50 fungal isolates sequenced, 27 were found to be emerging pathogens involving seven genera (Botryosphaeria species, Lasiodiplodia species, Thielaviopsis, Glomerella singulata, Macrophomina phaseolina, Rhizoctina bataticola, Podospora species) and 23 as established pathogens involving 8 genera (Aspergillus, Fusarium, Bipolaris, Pythium, Cochliobolus, Exserohilum, Pseudoallescheria and Scedosporium species) and 12 were found to be emerging pathogens (plant/soil) involving 5 different genera spectrum of mycotic disease continues to expand well beyond the familiar entities of candidiasis and aspergillosis.

The last 20 years or so have seen a growing number of fungal infections coincident with a dramatic increase in the population of severely immunocompromised patients. Another difficulty for microbiologists experienced in mycology is that fungi are mostly classified on the basis of their appearance rather than on the nutritional and biochemical differences that are of such importance in bacterial classification. The integration of molecular analyses with traditional phenotypic methods of fungal identification can significantly increase the specificity and decrease the turnaround time for the identification of clinically important moulds. The use of PCR-based DNA sequencing has several advantages over the conventional methods like rapidity, accuracy and definite identification. On the other hand, the conventional methods are time consuming, laborious and no possibility of definitive identification. Future research in ophthalmic mycoses needs to focus on improvement in diagnostic techniques, development of new antifungal compounds to overcome antifungal drug resistance. Nucleic acid-based amplification techniques involving real-time PCR and reverse-transcriptase PCR need to be optimized to assess the transcripts produced by the pathogenic fungi. Further studies on the growth kinetics of NSMs, its susceptibility patterns
to the in use and emerging antifungal agents are a potential area of research in the near future. Development of commercial kits at cost affordable rates for rapid detection and identification of fungi from any clinical specimen will aid in better management of fungal infections prevalent in a developing country like India.

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References

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