Role of Nucleic Acid Based Amplification Techniques in Detection, Characterization and Species Level Identification of Fungi in Clinical Specimens and a Study on *In Vitro* Sensitivity Pattern of Ocular Fungal Isolates

THESIS

Submitted in partial fulfilment
of the requirements for the degree of
DOCTOR OF PHILOSOPHY

By

R.BAGYALAKSHMI

2003PHXF029

Under the Supervision of

Dr. K. LILY THERESE

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE

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CERTIFICATE

This is to certify that the thesis entitled, "**Role of Nucleic Acid Based Amplification Techniques in Detection, Characterization and Species Level Identification of Fungi in Clinical Specimens and a Study on *In Vitro* Sensitivity Pattern of Ocular Fungal Isolates"** and submitted by R. Bagyalakshmi ID No 2003 PHXF029 for award of Ph. D. Degree of the Institute embodies original work done by her under my supervision.

Signature in full of the supervisor:

Name in Block letters : **Dr. K. Lily Therese**

Designation : **Reader in Larsen & Toubro**
Microbiology Research Centre
Vision Research Foundation
Sankara Nethralaya, Chennai

Date :

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ABSTRACT

The present study was undertaken to design and develop nucleic acid based amplification methods for rapid detection and identification of fungi from clinical specimens. The study design is categorized into two groups, the first method included nucleic acid based amplification methods for detection and identification of fungi causing ocular mycoses and the second involved the optimization of nucleic acid based techniques to detect and identify fungi causing dermatomycoses.

Polymerase Chain Reaction (PCR) targeting the Internal Transcribed Spacer region (ITS) was optimized and applied on ocular specimens to detect panfungal genome. PCR detected fungal genome in 76 (45.2%) out of 168 specimens in comparison with the conventional technique positive in 34 (20.23%) by smear examination and 42 (25%) by culture. The next approach was to design rapid methods for identification of *Fusarium* species since it is the second common isolate (38%) in L&T Microbiology Research centre, Sankara Nethralaya, Chennai. A total of 50 ocular isolates of *Fusarium* species were identified to species level by genus and species specific PCR, PCR based Restriction Fragment Polymorphism (RFLP) and DNA sequencing.

Two novel multiplex Polymerase Chain reactions (mPCR) were optimized with 3 sets of primers to detect panfungal genome in ocular specimens and to detect eubacterial, *P. 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.

The identification of fungi to species level is very important in directing the treatment strategy due to the high incidence of antifungal drug resistance and with regard to newly emerging fungal pathogens. Twelve percentage of fungal isolates (12%- Larsen & Toubro Microbiology Research Centre data) remained unidentified by conventional methods and it becomes important to identify these group of fungi. This was facilitated by applying nucleic acid based amplification techniques. PCR based DNA sequencing applied on the ITS region to identify these non sporulating fungi . Of the 50 fungal isolates sequenced 27 were found to be emerging pathogens

involving seven genera *Botryosphaeria, Lasidiplodia* species, *Thielavia tortuosa*, *Glomerulla singulata*, *Macrophomina phaseolina*, *Rhizoctonia bataticola*, *Podospora* species). 26 as established pathogens involving 8 genera (*Aspergillus*, *Fusarium*, *Bipolaris*, *Pythium*, *Cochilobolus*, *Exserohilum*, *Pseudoallescheria* and *Scedosporium* species)

The recent explosion in the rates of opportunistic fungal infections, combined with the increasing number of reports of resistance to the available antifungal agents has propelled interest in clinically relevant methods for antifungal sensitivity testing. So a phenotypic study was designed to study the sensitivity pattern of ocular fungal isolates to amphotericin B, fluconazole and ketoconazole. All the 50 Candida species tested were susceptible to amphotericin B, 2(4%) and 5 (10%) were resistant to fluconazole and ketoconazole respectively . Among the 130 filamentous fungi tested, 6 (4.61%) were resistant to amphotericin B, 49 (37.7%) and 10 (7.6%) were resistant to fluconazole and ketoconazole respectively.

The second part of the study focused on nucleic acid based amplification methods to detect and identify fungi in clinical specimens obtained from cases of dermatomycoses. A dermatophyte specific PCR was optimized targeting 18SrRNA specific for detection of dermatophytes and subsequently PCR based RFLP was carried out on the amplicons to reveal the identity of dermatophytes. Apart from that, PCR targeting ITS region was also performed to confirm detection of dermatophyte as fungus. The results clearly demonstrated not only the specificity, but also an increased clinical sensitivity by 31.67 % by this PCR method over the conventional methods of smear and culture.

The application of nucleic acid based amplification techniques were found to be rapid and reliable method for detection and identification of fungi in ocular and other clinical specimens.

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ABBREVIATIONS	DESCRIPTIONS
ABC	ATP –binding casette
AIDS	Acquired immuno deficiency syndrome
AH	Aqueous humor
ATCC	American type culture collection
AFLP	Amplified Fragment Length polymorphism
AP-PCR	Arbitrarily primed polymerase chain reaction
ATP	Adenosine tri phosphate
BAL	Broncho alveolar lavage
BBA	Brucella blood agar
BHIB	Brain Heart Infusion Broth
Bp	Base pair
BPB	Bromophenol blue
CHS	Chitin synthase
$CHCl_3$	Chloroform
CLSI	Clinical laboratory Standards Institute
CMA	Corn meal agar
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetra acetic acid
EIA	Enzyme immuno assay
EK	Electrophoretic karyptype
EVS	Endophthalmitis vitrectomy study
FC	Flucytosine
FRET	Fluorescence resonance energy transfer
FU	Fluorouracil
F dUMP	Fluoro dUMP
Fg	femtogram
G	Grams
GMS	Gomori's methanamine silver staining
HIV	Human Immuno deficiency virus
HLA	Human leukocyte antigen
IPA	Invasive pulmonary aspergillosis
IGS	Intergenic Spacer region
ITS	Internal Transcribed Spacer region
ITZ	Itraconazole
KOH	Potassium hydroxide
L1A1	Lanosterol alpha demethylase
LPCB	Lactophenol Cotton blue
LSU	Large subunit
mPCR	Multiplex PCR

ABBREVIATIONS	DESCRIPTIONS
MLEE	Multilocus enzyme electrophoresis
mtDNA	Mitochondrial DNA
$MgCl_2$	Magnesium chloride
MQW	Milli Q water
μg	Microgram
μl	Microlitre
mg	Milligram
ml	Millilitre
MIC	Minimum Inhibitory Concentration
MFC	Minimum Fungicidal Concentration
MW	Molecular weight marker
nPCR	Nested Polymerase chain reaction
NaOH	Sodium hydroxide
NSM	Non sporulating moulds
PCR	Polymerase Chain Reaction
PCR-RFLP	PCR – Restriction Fragment Length Polymorphism
PDA	Potato dextrose agar
Pg	picogram
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
28SrRNA	28S ribosomal ribonucleic acid
18SrRNA	28S ribosomal ribonucleic acid
RAPD	Randomly amplified polymorphic DNA
Rep PCR	Repetitive sequence based PCR
RR mix	Ready reaction mix
RSCP	Reference strand mediated cofirmational analysis
RT-PCR	Reverse transcriptase PCR
SDS	Sodium dodecyl sulfate
snPCR	Semi nested Polymerase Chain reaction
SCAR	Sequence confirmed amplified region analysis
SSCP	Single strand confirmation polymorphism
SDA	Sabouraud’s dextrose agar
SSU	Small sub unit
SWAPP	Sequencing with arbitrary primer pairs
uPCR	Uniplex polymerase chain reaction
YEA	Yeast extract agar

CHAPTER - 1

An Overview of Ophthalmic Mycoses and Laboratory Methods for Detection and Identification of Fungi

1.1 Introduction

Fungal infections of the eye, are being increasingly recognized as an important cause of morbidity and blindness; certain types of ophthalmic mycoses may even be life-threatening. The study of rapid detection methods for ocular mycoses is an immediate need. So far the studies about ophthalmic fungal infections and its detection is hampered by several factors. The first is that there are few controlled or comparative studies on this subject, and much of the material is in the form of single case reports, reports of small numbers of patients, or papers dealing with a retrospective review of patient records. The second is that many fungal genera and species have been implicated in ocular infections, and it is difficult to give appropriate weight to the significance of these organisms. A third problem is in assessing the accuracy of the genus or species identification of a fungal strain isolated in culture. The present study was undertaken to design and develop methods for rapid detection and identification of fungi from clinical specimens.

Standard approaches to the laboratory diagnosis of ocular fungal infections include (i) direct microscopic visualization for the presence of organisms in freshly obtained specimens, (ii) histopathologic demonstration of fungi within tissue sections, and (iii) cultivation of the causative fungus and its subsequent identification. However, these approaches often are not sufficiently sensitive and/or specific to detect fungal infections, and they sometimes require invasive procedures to obtain the necessary specimens.

Early and accurate detection of fungal infections is important for several reasons, including timely institution of antifungal therapy and to decrease the unnecessary use of toxic antifungal agents. In addition, the availability of accurate and timely diagnoses could reduce the use of empirical antifungal therapy, thereby reducing

antifungal selection pressure and the emergence of antifungal resistance. Unfortunately, a major obstacle to the successful treatment of fungal infections is the lack of sensitive and specific methods for early detection.

The conventional methods involved in the diagnosis of fungal infections are less sensitive due to lesser amount of available clinical specimen and low microbial threshold. The techniques are laborious and time consuming. Rapid detection 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. Target genes used in molecular detection of fungal infections includes single and multicopy nuclear and mitochondrial genes.

Numerous targets within the fungal genome have been evaluated with much of the current work using sequence areas within the ribosomal DNA (rDNA) gene complex. Multiple applications using this as target includes direct detection from clinical specimens, culture identification, phylogenetic research and molecular typing for epidemiological purposes. The breadth of applications of rDNA complex gene have great potential in characterization and identification of fungi. Development of rapid and accurate assays to detect fungal infections could potentially impact care and improve outcome of affected patients.

Twelve percentage of fungi (L & T Microbiology Research Centre, Sankara Nethralaya) were unidentifiable by the conventional method and are termed as mycelia sterilia, i.e these organisms fail to produce the spores even after prolonged incubation in sporulating media. A definite identification of such fungi becomes possible only when nucleic acid based amplification methods are applied.

The present study is undertaken to design and optimize nucleic acid based amplification methods to detect the presence of fungi in ocular specimens using Polymerase Chain Reaction (PCR), and multiplex polymerase chain reaction (mPCR) and to identify them by using Polymerase Chain Reaction based Restriction Fragment Length Polymorphism (PCR-RFLP) and DNA sequencing. The application of these techniques would favour earlier and specific detection of fungal etiology improving the patient care and management of ocular mycoses.

1.2 Hypothesis:

Nucleic acid based laboratory techniques are more useful in rapid detection and identification of fungi particularly the non sporulating fungi in clinical specimens as compared to conventional mycological methods

1.3 Objectives:

Objective 1

- To optimize a semi nested Polymerase Chain Reaction (snPCR) targeting Internal Transcribed Spacer (ITS) region for detection of panfungal genome
- To apply snPCR targeting ITS region on ocular specimens for detection of ocular mycoses and evaluate ITS PCR against conventional methods.

Objective 2

- To standardize a multiplex Polymerase chain reaction (mPCR) targeting three regions the small subunit (18SrRNA), ITS and the large subunit region 28SrRNA for detection of panfungal genome
- To apply mPCR for detection of panfungal genome in ocular specimens
- To standardize a nested PCR (nPCR) targeting 18SrRNA for detection of *C. albicans* in ocular specimens
- To standardize a nPCR targeting 18SrRNA for detection of *A. fumigatus* in ocular specimens
- To standardize a nPCR targeting 18SrRNA for detection of *F.solani* in ocular specimens
- Application of PCR targeting the 18SrRNA, ITS region and 28S rRNA region on ocular specimens for detection of panfungal genome

Objective 3

- To develop a novel mPCR targeting eubacterial, *P. acnes* and panfungal genome for rapid aetiological diagnosis of infectious endophthalmitis
- To apply mPCR on intraocular specimens for rapid and simultaneous detection of eubacterial, *P. acnes* and panfungal genome

Objective 4

- To analyze the sequence similarity in ITS region of ocular *A. flavu*s isolates by PCR based DNA sequencing.

Objective 5

- To identify non sporulating moulds by PCR based DNA sequencing on ITS region

Objective 6

- To identify *Fusarium* species by phenotypic methods – colony morphology and microscopic characters and evaluate it against genotypic methods – PCR based RFLP and DNA sequencing.
- To optimize a genus specific *Fusarium* PCR targeting 18S and 5.8S region for detection of *Fusarium* species.
- To optimize a species specific *Fusarium* PCR targeting 18S and 5.8S region for detection *of F. solani, F. oxysporum*, *F. moniliforme*

Objective 7

- To standardize agar dilution method using standard ATCC strains of *Candida* species to determine MIC of amphotericin B, ketoconazole and fluconazole
- To determine MIC of amphotericin B, ketoconazole and fluconazole on ocular fungal isolates by agar dilution method.

Objective 8

- To standardize and apply a dermatophyte specific PCR targeting 18S rRNA for detection of dermatophytes in specimens collected from patients with dermatomycoses
- To apply PCR based RFLP on 18S rRNA for identification of dermatophytes
- To apply PCR on ITS region for detection of fungi in dermatological specimens including dermatophytes
- To apply PCR based RFLP on ITS region for identification of fungi causing dermatomycoses

1.4 Ocular mycoses

Ocular fungal infections, or ophthalmic mycoses, are being increasingly recognized as an important cause of morbidity and blindness; certain types of ophthalmic mycoses may even be life-threatening (Levin et al, 1996). Keratitis (corneal infection) is the most frequent presentation (Srinivasan et al, 1991), but the orbit, lids, lacrimal apparatus, conjunctiva, sclera, and intraocular structures may also be involved. An important publication by Wilson et al in 1998 listed 105 species in 35 genera of fungi as causes of keratitis and other ophthalmic mycoses . However, the criteria by which these fungi were considered to be genuine ophthalmic pathogens and not simply contaminants inadvertently introduced into specimens during or after collection (D'Mellow et al, 1991), were not clearly delineated. An evaluation made in 1980 (McGinnis et al, 1980) of more than 300 reports pertaining to human fungal infections published in the literature from the late 1940s to the beginning of 1979 encountered similar difficulties. That assessment included reports on 30 genera (60 species) of fungi isolated from ophthalmic infections, principally keratitis; only reports pertaining to 32 species in 19 genera of fungi satisfied strict criteria of acceptability (McGinnis et al, 1980).

An important problem is in assessing the accuracy of the genus or species identification of a fungal strain isolated in culture. For example, a fungal strain isolated from a patient with keratitis was initially identified as *Arthrobotrys oligospora* but later reidentified as *Cephaliophora irregularis* (Guarro et al, 1999 a). Similarly, a filamentous fungus isolated from an intraocular lesion arising out of a retained contact lens was identified as *Scedosporium prolificans* (Arthur et al, 2001); it now appears that this identification may have been erroneous (Guarro et al, 2002). Hence the criteria to associate a particular fungi with ocular mycoses were 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 in the clinical specimens was consistent with the reported etiologic agent (Thomas, 2003 a).

1.5 Mycotic Keratitis (Keratomycosis)

Mycotic keratitis presents as a suppurative, usually ulcerative, corneal infection. This entity may account for more than 50% of all cases of culture-proven microbial keratitis and of ophthalmic mycoses (Hagan et al, 1995), especially in tropical and subtropical areas. The fungi most frequently implicated appear to vary depending on the geographical location and the period for which the infection is observed. In the first half of a 9-year study of microbial keratitis in south Florida, nine strains of *C. albicans* were isolated, but only one strain was isolated in the second half of the study (Liesegang et al, 1980). Although *F. solani* has been reported as the most common cause of mycotic keratitis in many parts of the world (Srinvasan et al, 1997), species of *Aspergillus* have predominated in some authentic, carefully documented studies from the Indian subcontinent (Upadhyay et al, 1991) and other countries (Cuero et al, 1980, Clinch et al 1992,Vajpayee et al, 1993,Garg et al, 2000). *C. albicans* was reported to be the most common cause or one of the most common causes of mycotic keratitis in the United States and Nepal, but it has been infrequently reported in several other major studies. The salient observations of 14 major studies of mycotic keratitis reported in the literature since 1991; 6 of these are studies done in the Indian subcontinent, that is, India, Nepal, Bangladesh and Sri Lanka (McClellan et al, 1994, Srinivasan et al, 1997, Gopinathan et al, 2002); 3 are studies done in the United States (Tanure et al, 2000, Wilhelmus et al, 2001); 4 are studies done in Paraguay (Kaspar et al, 1991, Ghana (Hagan et al, 1995), Singapore (Wong et al, 1997), and the People's Republic of China (Xie et al, 2001); and 1 published study was performed simultaneously in Ghana and southern India (Leck et al, 2002). Mycotic keratitis apparently occurs much more frequently in developing countries such as India than in developed countries such as the United States. Srinivasan et al,1997 reported on 139 patients with culture-proven mycotic keratitis seen over a 3-month period in Madurai, India; in contrast, 125 patients with culture-proven mycotic keratitis were seen over a 10-year period in south Florida (Rosa et al, 1994), while 24 patients with mycotic keratitis were treated over a 9-year period in Philadelphia (Tanure et al,2000).

1.6 Endophthalmitis

Fungal pathogens in endogenous endophthalmitis and posttraumatic endophthalmitis are similar to those causing fungal keratitis. The reports include *Fusarium moniliforme* , *Exophiala jeanselmei* , *P. boydii* , *A. niger* , *Scytalidium dimidiatum* , *Helminthosporium spp*. , *S. schenckii* , *Penicillium chrysogenum* , and *L. theobromae* (Klotz et al, 2000) and *Aspergillus* species (Callanan et al, 2006)

1.7 Present status of ocular mycoses in India

Fungal keratitis is more common in tropical countries. The detection and management of fungal keatitis is mainly by direct smear study and culture. Mycotic keratitis can easily be detected and /or diagnosed using conventional methods. There are a series of reports on the spectrum of fungi causing keratitis from Southern India (Gopinathan et al, 2002, Thomas 2003b). The management of fungal keratitis poses a problem when the conventional methods yield a negative result. The need for a sensitive and specific technique arises to detect the presence of a etiological agent. In India, a specific PCR targeting the small subunit ribosomal RNA has been developed and applied on corneal specimens to detect fungal aetiology (Gaudio et al, 2002). This technique proved to be extremely sensitive to detect the fungal aetiology. 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 PCR based DNA sequencing.

Another area of research which still requires expertise is antifungal susceptibility testing on ocular isolates. Though the Clinical Laboratory Standards Institute (CLSI)

standards are set to determine susceptibility patterns, none of the laboratories in India carry out antifungal susceptibility testing on a routine basis. The reasons for the same are CLSI method is laborious and involves the cost factor. This could be easily overcome by developing the basic infrastructure facilities in the routine mycology laboratories in India.

Fungal endophthalmitis ranges 4 to 11% in tropical countries. (Jaeger et al, 2000) The detection and management 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 (Anand et al, 2001a, Tarai et al, 2006). This technique proved to be extremely sensitive to detect the fungal aetiology.

Thus the development of sensitive and specific nucleic acid based methods is essential for detection and identification to improve the management of mycotic keratitis.

1.8 Need for molecular diagnosis

The conventional methods involved in the detection of fungal infections are less sensitive due to lesser amount of available clinical specimen and low microbial threshold (White et al, 1990). 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.

Target genes used in molecular diagnosis of fungal infections includes single and multicopy nuclear and mitochondrial genes. Multicopy genes provide better detection threshold than single copy genes and are employed in different molecular assays. Ribosomal DNA of fungi includes the conserved sequence (common to all fungi) helping in detection of fungi from clinical specimens. The Internal Transcribed Spacer (ITS region) has variable domain used for species identification .The mitochondrial DNA is used for detection of *C. albicans* and *Aspergillus* species.

The ability to determine the nucleic acid sequence of the genomic DNA has revolutionized most areas of contemporary biomedical research. Numerous targets within the fungal genome have been evaluated, with much of the current work using sequence areas within the ribosomal DNA (rDNA) gene complex. Multiple applications using this as target includes direct detection from clinical specimens, culture identification, phylogenetic research and molecular typing for epidemiological purposes. The breadth of applications of rDNA complex gene has great potential in characterization and identification of fungi. Development of rapid and accurate assays to diagnose fungal infections could potentially impact care and improve outcome of affected patients. (Iwen et al, 2001, Lau et al, 2007)

1.9 Organization of fungal genome:

DNA and RNA have been used in clinical microbiology applications for the classification and identification of bacteria and fungi. The ribosomal DNA (rDNA) genes are found in all microorganisms and are known to accumulate mutations at a slow constant rate over time. Nucleotide sequence heterogeneity within this region can be used to phylogenetically classify the microorganisms. Interspaced among the highly conserved regions of the DNA genes are regions of variable sequences called spacer regions. Since mutations within the spacer regions are known to occur in a greater frequency than the rDNA genes the sequence heterogeneity within the area has been useful for the separation of both genera and species.

Fungi have a rDNA gene complex with comparable characteristics. The organization of this complex in fungi includes a sequence coding for the 18S rDNA gene, an internal transcribed spacer region 1(ITS1), the 5.8 S rDNA gene coding region, another ITS region called ITS2 and the sequence coding for 28S rDNA gene. The 28S rDNA gene evolved slowly and are relatively conserved among fungi providing a molecular basis of establishing phylogenetic relationships. Early work in molecular testing using rDNA complex as target concentrated in the region of 18S rDNA also referred as the small subunit rDNA gene. Other genes within the molecular complex has also been used for the

molecular evaluation of fungi include the 5S rDNA, 5.8S rDNA,28S like rDNA. The comparison of nucleotide sequences within this region has been successful for the separation of genera and species.

The 18S gene of fungi is about 1800 bp in size with both conserved and variable domain sequences. Sequence variation within this region has been used to assess the taxonomic relationships and to separate genera and species based on sequence polymorphisms. However, the drawback in using this region for the identification of the species is the relative sequence homology among fungal species and the need to sequence a large number of bases in order to do comparative analysis. The 5.8S region on the other hand is only about 160 bp long and highly conserved. Owing to its small size and conserved nature, it is not used for phylogenetic studies. However this sequence is used as an attachment site for primers to amplify flanking spacer regions. The 28S DNA which is around 3400 bp in size and the variable domains of this subunit is have also been used to allow comparisons from higher taxonomic levels to the species level. Much of the 28S rDNA gene is conserved limiting the usefulness of this region for species identification. (Iwen et al, 2001)

1.10 Application of molecular methods for detection of fungi

The incidence of fungal infections has dramatically increased in recent years due in part to the onset of AIDS and improved medical techniques including the use of antibiotics, immunosuppressive therapy and organ transplantation. In many instances fungi such as *C. albicans, A. fumigatus* are normally benign or even endogenous organisms but they can lead to serious and indeed fatal infections in immunocompromised individuals. The application of PCR technology to molecular diagnostics holds great promise for the early identification of medically important pathogens. PCR has been shown to be useful for the detection of the presence of fungal DNA in clinical specimens. Considerable interest has been focused on the utility of selecting universal primers those that recognize constant regions among most, if not all, medically important pathogens.The different molecular techniques which are used for detection of fungi include PCR, RFLP, Hybridization, Single strand

confirmation Polymorphism (SSCP) (Orita et al, 1989, Lyons et al, 2000). The SSCP technique facilitates species level identification of fungi.

The application of PCR based DNA sequencing is used to determine the species identity of fungi and study on the mutations in genetic regions leading to antifungal drug resistance. *Fusarium* species have emerged as major opportunistic fungal agents. Since new antifungal agents exhibit variable activity against *Fusarium* isolates depending on the species, rapid identification at the species level is required. Conventional culture methods are difficult and sometimes inconclusive. By DNA sequencing of 28SrRNA *Fusarium* can be identified to species level within 48 hours (Walsh et al, 1995)

1.11 Conclusion

Molecular based methods are rapid, sensitive and specific than conventional methods in detection and identification of fungi from clinical specimens. These techniques would be of great importance in an ophthalmic setting to detect and identify fungi causing ocular mycoses.

CHAPTER - 2

Development and Application of a Seminested Polymerase Chain Reaction (snPCR) for Detection of Panfungal Genome From Ocular Specimens

2.1 Review of literature

The laboratory detection and identification of fungi in clinical specimens remains a significant problem in medical mycology. The clinical presentation is difficult to interpret, and the findings of noninvasive methods (computed tomographic scanning and X ray) are not specific . Culture results are available at the earliest in 2 to 3 days, and blood and deep-tissue sample cultures from infections with focal lesions are frequently negative (Vincent et al, 1998). Direct microscopy and histopathological examination are rapid, but they do not always allow identification of the infecting agent to the species level (Vincent et al, 1998) and serological methods lack sensitivity . Identification of this increasing diversity of pathogens by conventional methods is often difficult and sometimes inconclusive (Reiss et al, 1999). Morphological features and reproductive structures useful for identifying isolated fungi may need days to weeks to develop in culture, and evaluation of these characteristics requires expertise in mycology. At least 150 fungal species have been identified as human pathogens and have been isolated from virtually all body sites (Fromtling et al, 1995). Thus, rapid methods that are sensitive and specific are needed, and PCR has been applied to fulfill these requirements.

A variety of PCR protocols for human samples have been published, including panfungal PCR assays (Van Buirk et al, 1998) and methods that detect one species (Reddy et al, 1993,Spreadbury et al 1993, Chryssanthou et al, 1994), members of a fungal family (Yamakami et al, 1996), or several species (Sandhu et al 1995, Einsele et al, 1997). The incidence of individual fungal species in various diseases is relatively low, e.g., *Candida* spp., 10%; *Aspergillus spp*., 5 to 15%; and *Fusarium* spp., 2% (Gamis et al, 1991). Identification of the infecting agent to the species level

is required to guide appropriate treatment. Therefore, the most efficient and economic approach may probably be a single protocol that is able to detect and identify many species. However, the protocols for detecting several species (Sandhu et al, 1995) use labor-intensive blotting procedures and sequential hybridizations with various radiolabelled probes for differentiation of species, which make these approaches impractical for routine laboratory use.

Molecular techniques utilizing amplification of target DNA provide alternative methods for detection and identification (Kurtzman et al, 1997).PCR based detection of fungal DNA sequences can be rapid, sensitive, and specific (Makimura et al, 1999). 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 of establishing phylogenetic relationships (White et al, 1990). Between coding regions are the ITS 1 and 2 regions (ITS1 and ITS2, respectively) which evolve more rapidly and may therefore vary among different species within a genus (Figure 2a). Thus, PCR amplification may facilitate the identification of ITS region DNA sequences with sufficient polymorphism to be useful for identifying fungal species.

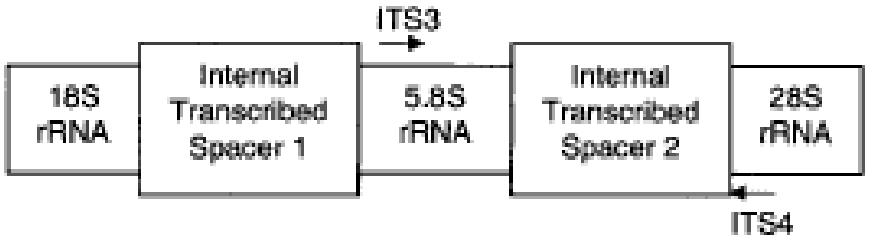


Figure 2a. Schematic representation of ribosomal unit of fungi

ITS noncoding regions flanked by the structural rRNA genes. Approximate binding sites of the ITS3 and ITS4 PCR primers are shown by arrows.

2.1.1 Detection of specific nucleic acids

The use of molecular diagnostic tools to detect fungal specific nucleic acid sequences has been reviewed (Vazquez et al 1994, Walsh 1997), and many researchers have reported the usefulness of DNA-based methods for the diagnosis of invasive fungal infections. However, most of the studies were performed in limited

numbers of patients, and no large prospective clinical trials have yet been reported. Furthermore, several questions need to be addressed before the DNA-based method which can be adapted to daily clinical routine; these include questions of (i) which DNA targets are best for commercial kits used in routine diagnostic laboratories, (ii) what are the optimal methods for extracting fungal DNA from clinical specimens obtained from various sites, and (iii) which detection methods are best for routine clinical laboratory use.

2.1.2 Sample Preparation

Specimen preparation can have a significant impact on the sensitivity and reproducibility of a molecular diagnostic test. In general, the sample preparation method should release intracellular DNA from the fungal cell wall and/or thick capsule; it must concentrate DNA targets that may be present in very small amounts; and it must eliminate protein debris, contaminants, potential inhibitors, and other extraneous materials without degrading the target DNA. At present, there are many protocols for sample preparation, but no universal method for optimally extracting, purifying, and concentrating fungal DNA from clinical specimens is available. Nonetheless, fungal DNA has been extracted and purified from different clinical samples, including whole blood (Buchman et al, 1990), bronchoalveolar lavage fluid (Tang et al, 1993) ,CSF (Ralph et al, 1996). serum and plasma (Yamakami et al 1998, Loeffler et al, 2000) However, the efficiencies of the molecular diagnostic methods applied to different types of clinical samples might not be equivalent, as DNAs present in different clinical samples are probably different in origin. For example, one study showed that PCR was more often positive when serum was used for testing than when whole blood was used for testing of specimens from patients with invasive candidiasis (Bougnoux et al, 1999).

2.1.3 Target Selection

Targets that have been used in molecular diagnostic tests for fungal infections include single and multicopy nuclear and mitochondrial genes and RNA. In general, molecular diagnostic methods targeting multicopy genes have better detection

thresholds than those targeting single-copy genes. Among multicopy genes, mitochondrial DNA (mtDNA) has been used in the PCR-based detection of *C. albicans* (Miyakawa et al, 1992) and *Aspergillus* species (Jones et al, 1998). However, the variability of mtDNA among different strains may be a limiting factor. Others (Ferrer et al 2001, Jaeger et al 2000), have targeted the multicopy ribosomal genes in order to maximize sensitivity and specificity. The ribosomal genes contain conserved sequences that are common to all fungi and also variable domains and highly variable ITS regions. The conserved sequences can be used to screen for fungal infection, while the variable sequences can be exploited for species identification. Indeed, favorable results targeting this ribosomal gene as a tool for fungal detection and identification have been obtained.

2.1.4 Amplification and Detection Methods

Nucleic acid hybridization and amplification methods are fundamental to molecular diagnosis. Hybridization techniques employ a DNA probe to determine whether a particular organism is present. The probe is a single strand of DNA synthesized such that it corresponds with a recognized sequence in the DNA or RNA of the suspected infectious agent. *In situ* hybridization assays has been used effectively to localize the DNA and RNA of infectious agents in routinely processed tissues, and no DNA extraction is required (Reiss et al, 1999).This technique has been reported for the identification of *Candida* spp. (Lischewski, 1996), and *Aspergillus* spp. (Park et al, 1997). Although the entire procedure is rapid and easy to perform, the sensitivity of the assay is often lower than those of other molecular biological assays, especially those including nucleic acid amplification (Reiss et al, 1999).

PCR is the most frequently used amplification procedure because it can readily adapt to many applications. However, when a single-copy gene is used as the target for PCR, special amplification steps such as nested PCR (nPCR) are often necessary to achieve the necessary degree of sensitivity. For example, the sensitivity with npCR was 1,000 times higher than that of the single PCR for the detection of fungal infections (Wong et al 1990, Kairuz, 1994, Chryssanthou et al, 1994). The PCR-generated product is usually analyzed by ethidium bromide-stained gel electrophoresis. Although gel electrophoresis is simple and inexpensive, it is

much less sensitive than Southern blotting (Tang et al, 1993).Detection of PCR products by ethidium bromide staining and by Southern blotting is time-consuming, and the interpretation of results may be subjective. Hence, PCR-Enzyme immuno assay (EIA) —a three part method which included PCR amplification, hybridization with the complementary labeled probe, and detection of reaction products in an EIA that provides either colorimetric or fluorescence readout was developed by Loeffler et al, 2000). In addition, the PCR-EIA format provided further amplification without losing the species-specific binding associated with Southern blotting and ultiple samples can be assayed in parallel and semiquantitation of DNA is possible ((Fujita et al, 1995). A quantitative PCR assay with the LightCycler (Roche Diagnostics, Mannheim, Germany) amplification and detection system was described by Loeffler et al, 2000 which proved to be very useful in rapid detection within 45 minutes. Another technique, Reference Strand mediated Conformational Analysis (RSCA) is a heteroduplex-based typing technique which differs from other sequence-based typing methodologies in that the type is assigned on the basis of differences in DNA conformation (due to mismatches) that occur when two different sequences are hybridized to each other (heteroduplex), retarding the migration of the heteroduplex in a non-denaturing polyacrylamide gel (Arguello et al, 1998). This technique had high sensitivity, specificity and reproducibility.

2.1.5 Species Identification

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 fungus were detected by a PCR method employing a universal primer that amplified a highly conserved region in the 18S ribosomal DNA (rDNA) (Polanco et al, 1995), but this method cannot differentiate among these species. However, the subsequent probing of the amplicons with species-specific probes discriminated among individual fungal pathogens to species level (Sandhu et al, 1995). Although these methods appear promising in the field of diagnostics, the use of species-specific probes is not always an efficient approach in mycology, given the large number of potentially pathogenic fungi. Others have reported the use of RFLP by enzyme digestion of PCR product (Ferrer et al 2001). This approach was able to classify five broad groups of fungi:

Candida spp., *Cryptococcus*, *Aspergillus* and clinically related septate molds, zygomyces, and dimorphic fungi. Another approach is the use of single-strand conformational polymorphism to delineate the difference between fungal species and/or genera (Walsh et al, 1995). This technique included PCR amplification of a conserved region of the 18SrRNA with further separation of genus and species on the basis of exploiting small but phylogenetically important base pair differences among medically important fungi. Minor sequence variations in highly conserved DNA segment will cause subtle changes in conformation that forms in short-strand DNA changes after they are denatured. This conformationally different fragment can then be separated electrophoretically under nondenaturing conditions.

2.1.6 Diagnostic Considerations

The detection and identification of fungal pathogens by DNA-based methods can yield results sooner than cultivation (Prariyachatigul et al,1996). Furthermore, in experimentally infected animals and patients, the sensitivity can be higher than those of cultures and serologic tests for diagnosing invasive fungal infections (Wong et al, 1990). DNA results have correlated with clinical improvement and effect of treatment, and these have been demonstrated in patients with invasive candidiasis (Einsle et al, 1997), invasive aspergillosis (Yamakami et al, 1998) and cryptococcal meningitis. Although the disappearance of fungal DNA from the blood of patients correlated with successful therapy, no experimental data are available to explain these aspects of therapy. Further evaluation is necessary for the DNA detection in monitoring of invasive fungal infections with animal models. Molecular diagnostic methods may not distinguish individuals who are colonized from those who are infected . For example, false-positive results of 31% and ranging from 8 to 38% have been reported in the diagnosis of candidiasis and aspergillosis . The most likely explanations of false-positive results are the colonization of the patients' airway by conidia and/or hyphae and the contamination of sample and/or reaction buffers by environmental fungi. Adoption of rigorous working practices and appropriate decontamination procedures may help to control the risk of contamination. In addition, avoiding specimens that are more susceptible to aero contamination (e.g., bronchoalveolar lavage fluid) may reduce the chance of false positivity. False-negative results could be due to the low sensitivity of a method designed to detect a single-copy gene (Yeo et al, 2002)

2.1.7 Mycotic keratitis

Mycotic keratitis or fungal corneal infections have a worldwide distribution, and the incidence is higher in tropical and subtropical countries (Garg et al, 2000). More than 105 species of fungi belonging to 56 genera have been reported to cause oculomycosis. However, species of *Fusarium, Aspergillus, Candida*, and other hyaline and dematiaceous hyphomycetes are the usual isolates from patients with mycotic keratitis (Thomas, 2003 b). *C. albicans* is the most common aetiological agent causing keratitis in Melbourne, Australia (Bhartiya et al, 2007). Clinical suspicion of fungal keratitis is essential to diagnosis, but once the diagnosis is made, the treatment remains difficult.(Nitulescu, 2006). Therapeutic keratoplasty may treat severe, refractory infectious keratitis effectively. High cure rates are achievable, although infection recurrence despite prolonged treatment remains a significant problem in fungal keratitis (Ti et al, 2007). 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% (Clellan et al, 1994). Recent advances in molecular biology techniques have opened the door for culture-independent diagnostic methods. Immunological detection (Iwastu et al, 1982) and identification by use of distinctive metabolites (Ahmad et al, 2002) and nucleic acid probes (Wu et al, 1996) 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 (Tullari et al, 1998). To date, a few cases of mycotic keratitis have successfully been diagnosed by PCR (Gaudio et al, 2002).

2.1.8 Fungal endophthalmitis

The microbiological spectrum of infectious endophthalmitis shows that the percentage of isolates that are fungi is 8 to18.5% (Okhravi et al, 1998).However, standard microbiological tests are positive in only 54 to 69% of endophthalmitis cases by culture (Kunimoto, et al, 1999). In fungal infections,even when positive, results usually take longer than a week because these organisms are difficult to identify and/or are slow-growing,early diagnosis and rapid intervention is a critical element for an effective treatment of ocular infections.This has led to the development of culture-independent diagnostic tests such as PCR. PCR-based detection methods with universal primers for bacterial DNA in ocular samples (Okhravi et al, 1997, Kunimoto, et al, 1999,Carroll et al, 2000, Chen et al, 2000,Lohman et al, 2000) have been developed. For detection of fungal pathogens, multicopy gene targets have been evaluated for increasing the sensitivity (Tang et al, 1993) and universal fungal PCR primers have been developed for broadening the range of detectable fungi (Jordan et al, 1994 Haynes et al,1995 Einsele et al, 1997). Studies on fungal DNA detection in ocular samples have been performed (Alexanderakis 1998, Hidalgo et al, 2000, Jaeger et al, 2000); the small number of conidia in the samples, the difficulty of DNA extraction Loeffler et al, 2000, Vanburik et al, 1998 as some filamentous fungi have a sturdy cell wall which is resistant to standard DNA extraction procedures for yeast and bacteria, and the presence of PCR inhibitors in human specimens (Wiedbrauk, et al,1995) are some of the difficulties with fungal detection in ocular samples. The ideal marker to detect a fungal infection should be present in all fungal genera (but should contain enough internal variation in its sequence to define a given species) and should be a multicopy gene to maximize the sensitivity of the detection method. The rRNA genes are good candidates, and the sensitivity of their detection may be dramatically increased by the use of npCR. The ITS region fits in perfectly to be used as a detection target and the region is conserved among all medically important fungi enabling the design and use of panfungal primers. Hence in this study, ITS region was used as a target to detect fungal genome in ocular specimens.

2.2 Objectives

- To optimize a snPCR targeting ITS region for detection of panfungal genome
- To apply snPCR targeting ITS region on ocular specimens for detection of panfungal genome and evaluate ITS PCR against conventional methods.

2.3 Materials and Methods:

2.3.1 Standard strains of *Candida* species

C. albicans ATCC 24433, *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 90028, *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258, *C. tropicalis* ATCC 750, *A . fumigatus* ATCC 10894

2.3.2 Clinical specimens

A total of 168 specimens consisting of 145 intraocular specimens and 23 corneal scrapings were processed for detection of fungal etiology from the period between 2003- 2005. A total of 20 specimens consisting of 12 aqueous humor (AH) collected from patients undergoing uncomplicated cataract surgery and 8 vitreous fluid (VF) from patients having vitreous hemorrhage, proliferative diabetic retinopathy, inflammation of the eye due to non-infectious etiology were used as controls. However, the break up of endophthalmitis could be obtained only for 133 as against 145. This was due to the 12 specimens received from other referral hospitals located in various parts of India. The clinical details with regard to endophthalmitis and keratitis is provided in Table 2a.Informed consent was obtained from the patients from whom the ocular specimens have been collected. The methodology of collection and processing of ocular specimens is given in Appendix I

Table 2a: Categorization of ophthalmic presentation of patients included in the study

Clinical diagnosis **Total number :** **168***	**Aqueous humor** **83**	**Vitreous fluid** **56**	**Corneal scrapings and other ocular specimens** **29**
Classified endoph 133			
P.O.E 68	38	28	Fibrin material from aqueous chamber 2
Chronic endoph 42	25	14	Fibrin material from aqueous chamber 2 Orbital pus 1
Low grade endoph 8	4	4	-
Endogenous endoph 8[#]	6	1	Blood 1
Post traumatic endoph 7	-	7	-
Unclassified endoph 12	10	2	**-**
Keratitis 23	-	-	**23**

NOTE: Endoph : Endophthalmitis, **P.O.E** : Post operative endophthalmitis

***** There were a total of 156 cases (133 out of 145 cases of endophthalmitis and 23 keratitis) in which the clinical details could be obtained. It was not possible to obtain the clinical details in 12 cases of endophthalmitis as these patients specimens were sent from other referral hospitals in various parts of India.

Paired sample of vitreous fluid (1) and blood (1) were included in the study

2.3.3 DNA extraction

DNA extraction from intraocular fluids and corneal scrapings were carried out using Biogene kit (Biogene, USA), for tissue and purulent specimens, Qiamp DNA minikit (Qiagen, Cat. No. 51304 Germany) was used. DNA extraction was carried out on fresh specimens according to manufacturer's instructions.

2.3.4 Semi nested PCR (snPCR)

The snPCR targeting ITS region was optimized as per the method of Ferrer et al, 2000. The protocol for carrying out amplification and agarose gel electrophoresis is given in Appendix II

2.3.5 Sensitivity of snPCR

The sensitivity of snPCR was determined using serial ten fold dilutions of standard strain of *C. albicans* ATCC 24433. Ten μg of *C. albicans* ,*A.fumigatus* and were used as the template.

2.3.6 Specificity of snPCR

The specificity of snPCR was carried out with the DNA extracts of micro –organisms as given in Appendix II.

2.3.7 DNA sequencing

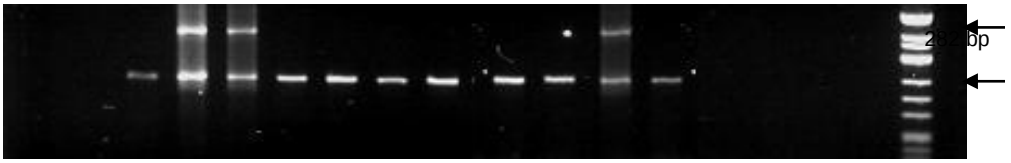
PCR was performed using primers targeting the D2 region of the large subunit of the ribosomal RNA gene using the Micro Seq^{R} D2LSUr DNA PCR Kit (Applied Biosystems, Foster City ,CA). PCR amplicons were sequenced with MicroseqD2 Large Sub Unit ribosomal DNA Fungal Sequencing Kit^{R} (Applied Biosystems, Foster City,CA) and that of ITS PCR amplicons as described in Appendix II

2.4 Results:

2.4.1 Standardization of PCR - Sensitivity of snPCR targeting ITS region

The results of snPCR after two rounds of amplification is shown in Figure 2b (*C.albicans*), Figure 2c (*A. fumigatus*). ITS primers had sensitivity of 1 femtogram of *C. albicans* DNA (Single cell of *C. albicans* cell). The ITS primers had a sensitivity to detect 10 fg of *A. fumigatus* and *F. lichenicola* DNA

Figure 2b: Agarose gel electrophoretogram showing the sensitivity of snPCR targeting ITS region using ATCC *C. albicans* 24433



Lane 1 : Negative control I **Lane 2 :** Negative control II round **Lane 3:** *C. albicans* neat DNA (10µg/ µl) **Lane 4 – Lane 13 :** Serial ten fold dilutions of *C.albicans* DNA showing positivity -282 bp of *C. albicans* after two rounds of amplification **Lane 14-Lane 17:** Ten fold dilutions of *C.albicans* DNA showing no amplification after two rounds of amplification **MW:** Molecular weight marker *Hinf* –I digest of *Phi X* 174 Bacteriophage DNA **Sensitivity: 1 fg**

Figure 2c: Agarose gel electrophoretogram showing the sensitivity of snPCR targeting ITS region using ATCC *A. fumigatus* 10894

Lane 1 : Negative control II round **Lane 2 :** Negative control I round

Lane 3: *A.fumigatus* ATCC 10894 DNA positive control **Lane 4 – Lane 13 :** Serial ten fold dilutions of *A. fumigatus* neat DNA showing positivity -298 bp of *A. fumigatus* after two rounds of amplification **Lane 14-Lane 17:** Ten fold dilutions of *A. fumigatus* DNA showing no amplification after two rounds **MW:** Molecular weight marker *Hinf* I digest of Phi X 174 bacteriophage DNA

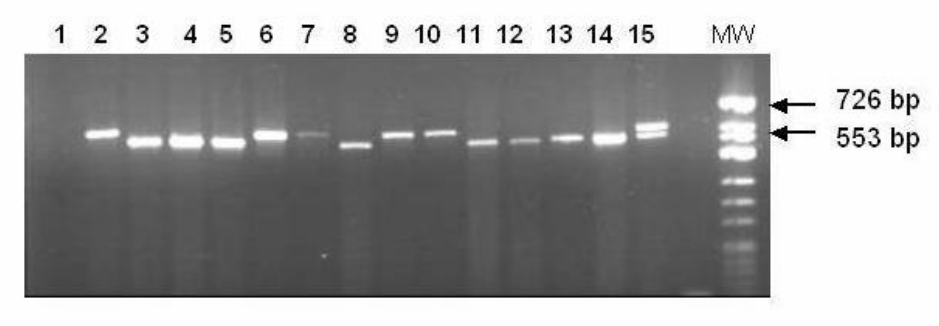
Sensitivity *A. fumigatus* : 10 fg

NOTE: 596 bp I round product, 298 bp II round product

2.4.2 Specificity of PCR

The specificity of ITS PCR is shown in Figure 2d. The primers were specific amplifying all the fungal DNA .No amplification was obtained with bacterial, viral and human DNA

Figure 2d : Agarose gel electrophoretogram showing specificity of snPCR targeting ITS region of panfungal genome

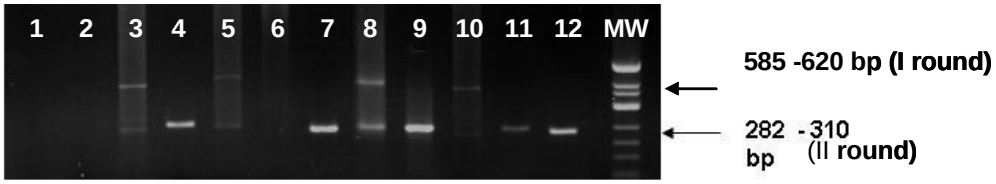


Lane 1 : Negative control **Lane 2** : A. *flavus* 595 bp **Lane 3:** *C. albicans* (ATCC 24433) 536 bp **Lane 4:** *C .parapsilosis* (ATCC 22019) 520 bp **Lane 5:** *C. krusei* (ATCC 6258) 510 bp **Lane 6 :** *A .niger* 599 bp **Lane 7:** *A.fumigatus* 596 bp Lane 8: *Penicillium* species 518 bp **Lane 8:** *Paecilomyces* species 506 bp **Lane 9**: *A.terreus* 594 bp **Lane 10** : *F. lichenicola* 592 bp **Lane 11:***Rhizopus species* 522 bp **Lane 12 :** *Curvularia* species 524 bp **Lane 13:** *C. lipolytica* 520 bp **Lane 14:** *T. rubrum* 532 bp **Lane 15:** *F. falciforme* 592 bp **MW:** Molecular weight marker *Hinf* I digest of Phi X 174 bacteriophage DNA

Note: The base pair size of amplicons were indicated as per the method of Ferrer et al, 2001

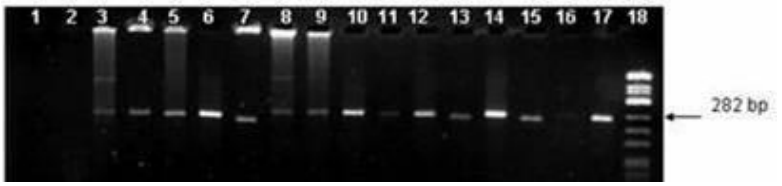
The results of PCR using ITS primers applied on 168 clinical specimens for detection of fungal genome is given in Table 2c. Fungal genome was not detected in all the 20 control specimens. The results of PCR applied on clinical specimens for fungal genome detection is shown in Figure 2e, and 2f.

Figure 2e: Agarose gel electrophoretogam showing application of ITS PCR on ocular specimens



Lane 1: Negative control II round **Lane 2:** Negative control I round **Lane 3,5:** AH positive **Lane 4,7-11:** VF positive **Lane 6:** AH negative **Lane 12:** Positive control *C. albicans* ATCC 24433 **MW:** Molecular weight marker Phi X 174 bacteriophage DNA *Hinf* – I digest

Figure 2f: Agarose gel electrophoretogam showing application of ITS PCR on ocular specimens



Lane 1: Negative control II round **Lane 2:** Negative control I round **Lane 3,5,11,13, 16:**AH positive **Lane 4,6,8,9,10** VF positive **Lane 7 :** AH negative **Lane 8:** VF positive **Lane 12:** VF negative **Lane 13:** AH positive **Lane 14:** VF positive **Lane 15:** VF positive **Lane16 :** AH positive **Lane 17:** *C. albicans* ATCC 24433 Positive control **MW:** Molecular weight marker *Hinf* – I digest of Phi X 174 bacteriophage DNA digest

2.4.3 Results of conventional microbiological investigations

The results of conventional microbiological investigations done on clinical specimens is given in Table 2b. The results of conventional microbiological investigations done on clinical specimens is given in Table 2b. The conventional microbiological investigations revealed fungal aetiology in 34 (20.23%) by smear and 42 (25%) by culture. There was only a single failure in smear positive case (corneal scraping 1).Fungi were isolated in 33 specimens in which the direct smear revealed the presence of fungal elements. Culture was additionally positive in 9 cases. The distribution of fungal isolates is shown in figure 2g.

Figure 2g: Distribution of fungi isolated in culture from keratitis and endophthalmitis specimens from August 2003- September 2005

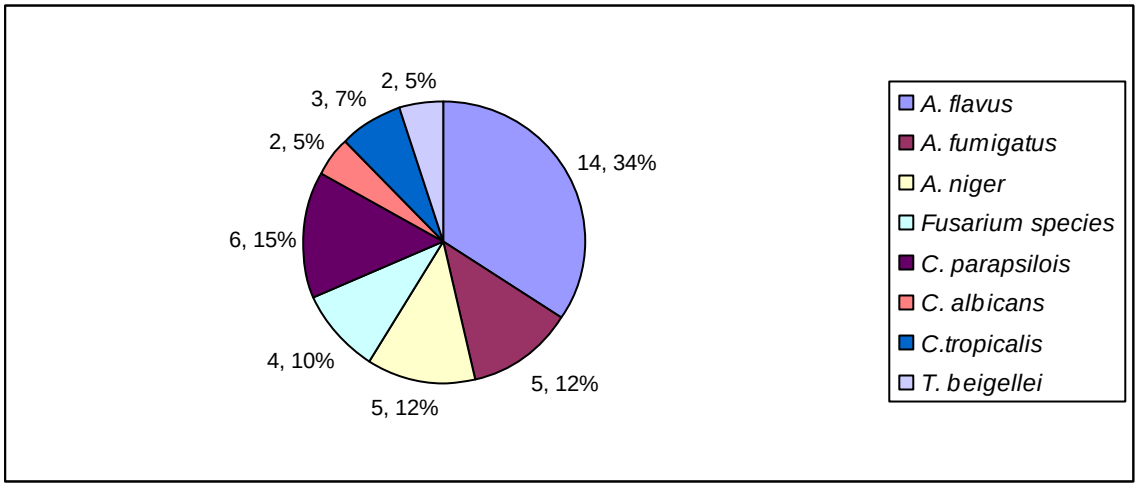


Table 2b: Results of conventional microbiological investigations

Ocular specimens (168)	Conventional microbiological investigations		
	Smear 34 (20.23%)	**Culture 42 (25%)**	**Identification of fungal isolates**
AH n = 83	8	16	*A.flavus* 5, *A.niger* 3, *C.parapsilosis* 4, *C.tropicalis* 2 *C.albicans* 1,*T.beigellei* 1
VF n = 56	11	12	*A.flavus* 2, *A.fumigatus* 2 , *A.niger 2, Fusarium species* 1, *C.parapsilosis* 2, *C.tropicalis* 1, *C.albicans* 1, *T.beigellei* 1
Corneal scraping n = 23	15	14	*A. flavus* 7, *Fusarium* species 3 *A. fumigatus* 3, *A. terreus* 1
Others * n = 6	-	-	-

Note : AH: Aqueous humor, VF: Vitreous fluid, * Fibrin material from anterior chamber 4, Blood 1, Orbital pus 1

2.4.4 Application of PCR on clinical specimens

The results of PCR using ITS primers applied on 168 clinical specimens for detection of fungal genome is shown in Table 2c. Fungal genome was not detected in all the 20 control specimens.

Table 2c: Comparison of results of conventional methods against snPCR targeting ITS region for detection of panfungal genome

Ocular specimens	**Conventional methods**		**Molecular investigations**
	Smear positive	Culture positive	snPCR targeting ITS region Positive
Endophthalmitis (145)	19	28	76*
Corneal scraping (23)	15	14	-
Ocular specimens N = 168	34 (20.23%)	42 (25%)	76 (45.2%)

Note:

- All the specimens positive by conventional methods were detected by PCR
- *snPCR alone detected panfungal genome in 48 specimens.The increase in clinical sensitivity using PCR against conventional methods was found to be statistically significant {P = 0.0001 using Z (normal approximation) Test for two proportion}
- Accuracy of the test was found to be 71.84%

2.4.5 Results of DNA sequencing

The results of DNA sequencing on 28SrRNA and ITS PCR amplicons is shown in Table 2d

Table 2d: Results of PCR based DNA sequencing performed on 26 intraocular specimens for species level identification of fungi

Ocular specimens	RESULTS OF DNA SEQUENCING
16 *	
11 AH	*F. falciforme* 3, *C. albicans* 2, *A. flavus* 2, *F. lichenicola* 1, *C.tropicalis* 1, *A. fumigatus* 1, *Trichosporon beigellei* 1
5 VF	*C. albicans* 2, *A. flavus* 1, *A. fumigatus* 1, *F. falciforme* 1
10 #	
3 AH	*C. albicans* 1, *C. parapsilosis* 2
7 VF	*C. albicans* 2, *A .flavus* 2, *Candida tropicalis* 1, *A. fumigatus* 1, *A. niger* 1

Note:

*** DNA sequencing targeting D2 LSU using Microseq ^{R} fungal identification kit performed in Alcon Research Laboratories, Fort worth, Texas, U.S.A**

 # DNA sequencing targeting ITS region performed in Vision Research Foundation , Sankara Nethralaya, Chennai

2.5 Discussion:

The incidence of fungal infections has dramatically increased in recent years (Sturtevent 2002) and infectious fungal keratitis and endophthalmitis cause extensive ocular morbidity worldwide (Gaudio et al, 2002).Early detection and rapid intervention is a critical element for effective treatment of ocular infections. This has led to the development of culture independent diagnostic tests such as PCR .The potential utility of PCR techniques for improving the diagnosis of fungal infections is well recognized (Okhravi et al, 1998) and the use of PCR for this purpose is expanding. Assays targeting *Candida*, *Aspergillus* species and *Fusarium* species have been tested in vitreous specimens and recently an assay using panfungal primers has been used more extensively.(Jaeger et al 2000, Anand et al,2001)

The ideal marker to detect a fungal infection should be present in all fungal genera (but should contain enough internal variation in its sequence to define a species) and should be a multicopy gene to maximize the sensitivity of detection. PCR using universal fungal primers specific for 28SrRNA gene has been evaluated by Anand et al, 2001. The technique of PCR using primers amplifying conserved region of the 28S rRNA,18s rRNA with further separation of genus and species targeting ITS (ITS) region on the basis of exploiting small but phylogenetically important base pair difference of medically important fungi (Walsh et al, 1995) as well as Real Time PCR (Halliday et al, 2005) is being used for detection of fungal infections.

Fungal endophthalmitis often is difficult to diagnose and missed, unless proper microbiological studies are performed. Hence, laboratory diagnosis is of great importance and is the key to appropriate therapy. Visual symptoms of fungal endophthalmitis include,visual loss, eye pain and irritation,headache, Photophobia, Ocular discharge,Intense ocular and periocular inflammation, decreased visual acuity

The laboratory diagnosis of fungal infection is highly dependent on traditional methods of microscopy and culture. Culture is considered as the ‘gold standard’ but its true sensitivity is not known and it is also time consuming.The eye hospital in which the study was carried out is one of the major referral hospital and majority of the patients referred might have undergone several regimes of therapy including antifungal therapy resulting in negative culture results. In addition, the indolent nature

and localised infection and fastidious nature of the fungi could have attributed to the lower yield by culture. Since PCR is sensitive and requires the mere presence of DNA, more number of samples were positive as against culture in which the presence of viable organism is essential for isolation. These are the valid findings supporting the high positivity by PCR as against negative cultures.

The major draw back of fungal culture is the prolonged period of time, minimum of 2 to 4 days and upto three weeks required for isolation of slow growing fungus on culture medium. In this study it varied from 1 to 15 days with an average of 3 days whereas the results of PCR was available within 5 hours from the time of collection of specimen. The negative results may be due to less sample volume, prior therapy with antifungals and fastidious nature of the organism. Molecular microbiological methods have greater sensitivity and specificity than conventional methods. Multicopy genes, 28SrRNA and ITS region were used as targets to increase the clinical sensitivity and universal panfungal primers were used to broaden the range of detectable fungi. We have proven in our earlier study (Anand et al, 2001) that clinical sensitivity by using PCR has been increased. Our results of PCR on intraocular specimens to detect fungal genome indicates not only high analytical specificity but also increased sensitivity compared to the conventional methods by 30% as against conventional methods and it was found to be statistically significant ($P = 0.0001$ using Z (normal approximation) test for two proportion).However the results could be well validated after confirmation with PCR-RFLP, hybridization with specific probes or sequencing the PCR amplicons. The association of fungal aetiology with endophthalmitis is indeed high (53.57%). In the present study. however the specimens used in the study were collected from patients exhibiting typical signs and symptoms of endophthalmits. Further, to rule out the presence of bacterial and *P. acnes* genomes which can also cause endophthalmitis, PCR targeting 16SrRNA of bacteria and *P. acnes* were applied on 168 specimens. Mixed genomes were detected only in cases of post traumatic endophthalmitis [5.6%]. In all other specimens there were no bacterial and / *P.acnes* genomes detected. Hence the application of ITS PCR on ocular specimens proved to be a rapid detection tool to identify the fungal aetiology and aided in timely institution of sight saving specific therapy.

In this study, PCR was standardized targeting the ITS primers and the results of PCRs were validated using smear, culture in culture positive cases and by DNA sequencing in culture negative cases.By application of snPCR rapid detection was available within six hours of specimen collection. Furthermore, the results of PCR correlated well with specimens which were culture positive for fungus. Other proposed molecular techniques for fungal identification such as the use of RFLP (Williams et al 1995, Morace et al 1997, hybridization with specific probe (Sandhu *et al* 1995, Lindsley *et al* 2001 and specific npCR (Hidalgo *et al*, 2000) could be useful in confirming specific fungal infection. The development of snPCR for detection of panfungal genome proves to be useful rapid diagnostic test for detection of fungal infection.

2.6 Conclusion

The conventional laboratory methods are time consuming and less sensitive. The application of snPCR targeting ITS regionwas found to be a rapid (within 6 hours of specimen collection) tool in detection of ocular mycoses

2.7 Future prospects

Optimization of Real Time PCR for quantitation of panfungal DNA in ocular specimens,standardization of Reverse Transcriptase PCR (RT-PCR) for assessing the transcript level of fungi,design and application of nucleic acid based amplification techniques for simultaneous detection of fungi and the antifungal drug resistance genes by microarray or Real time PCR would aid in successful management of ocular mycoses.

CHAPTER - 3

Development and Application of a Novel Multiplex PCR Targeting 18SrRNA, ITS region ad 28SrRNA for Detection of Panfungal Genome in Ocular Specimens Collected from cases of Fungal Keratitis and Endophthalmitis

3.1 Review of literature

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. So isolation and identification of the fungi and characterization are needed for making a proper diagnosis and understanding the pathogenesis of the condition. The frequency of apparent diagnostic failure (i.e no organism is isolated in culture though an infection is clinically evident) ranges from 20 to 60%.(Gaudio et al, 2002). And endophthalmitis ranges from 4 to 11%(Jaeger et al, 2000).The use of nested PCR in detection of fungi in ocular specimens would help in administration of antifungal drugs and patient management.

3.1.1 Need for nucleic acid based amplification methods

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 which can be submitted for PCR include intraocular fluid (aqueous or vitreous), tears, 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

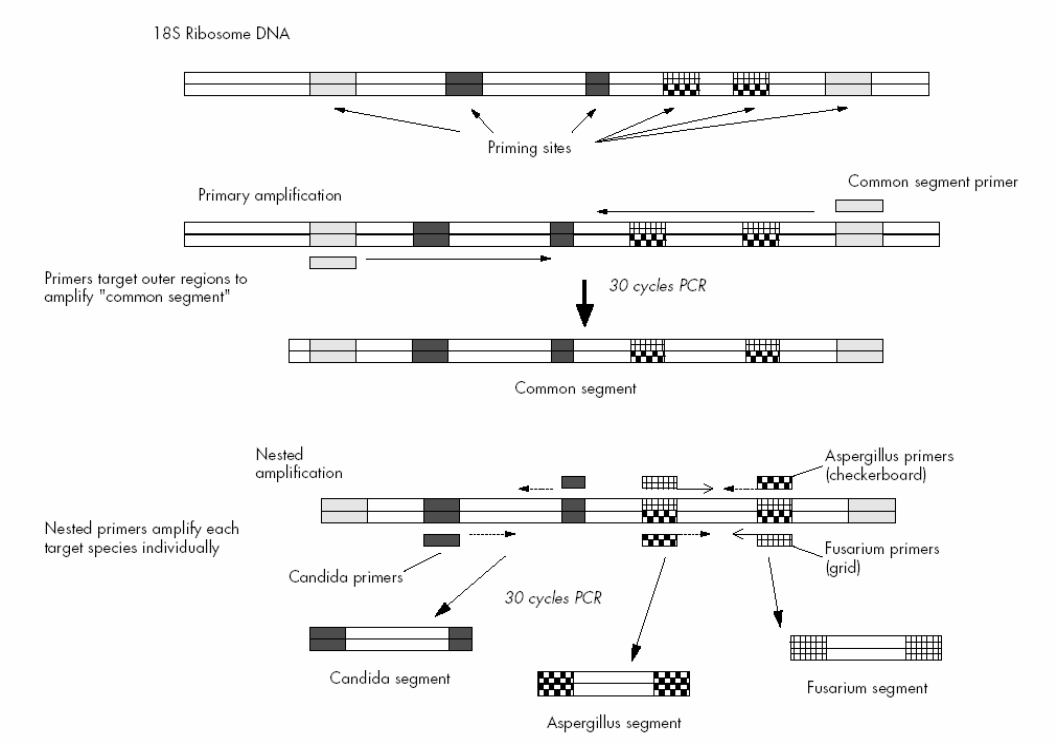
technique and the problems that may arise from the production of false positive results. In most of these studies, insufficient detail has been provided to permit an independent assessment of the adequacy of the techniques used for culture. In the diagnosis of ophthalmic mycoses, PCR would probably be most valuable in providing a positive result in a shorter period than that required for culture (Ferrer et al 2001, Ferrer et al 2002,) and in identification of a fungal isolate which does not sporulate (Badenoch et al, 2001). Although PCR is more advantageous than the estimation of antibodies in serum or ocular fluids because of its extreme sensitivity and specificity, it cannot be used (unlike serological tests, for which serial antibody titers can be studied) to monitor the patient's response to treatment. PCR does not distinguish viable from nonviable organisms; it may therefore be difficult to assess the relevance of a positive PCR result in a healing corneal ulcer, where culture is negative (Alexandrakis et al, 1998), or in locations such as the conjunctival sac, where fungi may be found as transient commensals (Gaudio et al,2002).

A few culture media will suffice to detect and grow the common ocular pathogens, but PCR must be multiplexed for each microorganism that is suspected; the use of panfungal primers may alleviate this problem. Finally, PCR can detect only fungi for which the DNA sequence is known and primers are available; it also does not provide details of cellular morphology of localization (Rajeev et al, 1998).

Universal primers are available for fungi that target the regions of the ITS, once cloned these sequences can be compared to the wealth of other sequences. Although ITS region is the main target, other genes are becoming more widely studied, in particular the beta tubulin gene (Fraaije et al, 1999). Development of taxon specific primers based on these genes is routine and there are many examples in the literature where they have provided discriminatory primers and probes. In some cases where the data is provided inadequate, screening arbitrary regions of the genome is often the next step. Design of primers or probes based on sequence information from this data is often less discriminating as the regions are arbitrarily chosen and there is often little in the database to compare the sequence to therefore, primers and probes designed can often recognize other organisms. (Atkins et al, 2004)

Universal primers common to all the fungi have been used as a promising approach for clinical microbiological diagnosis (White et al , 1990).Thus far two techniques have been reported to separate different fungi detected by universal primers. These include restriction fragment length polymorphism (Hopfer et al, 1993) and hybridization of the amplicon with a specific probe (Maiwald et al, 1994). Hopfer et al, 1993 amplified a segment of ribosomal DNA gene that is highly conserved throughout the fungal kingdom. Using restriction endonuclease digestion of the amplified product , the authors classified medically important fungi into five groups. A similar approach involving the combination of PCR and restriction enzyme analysis was reported by Maiwald et al, 1994.Jaeger et al, 2000 have developed a rapid, sensitive and reliable PCR system for the detection and identification of three fungal genera in ocular specimens. Panfungal primers were designed by using 18S rRNA gene sequences from *C. albicans*, *A. fumigatus*, *F. solani*. (Figure 3a) Ribosomal genes were chosen as targets for amplification as they are highly conserved genes that exist as multiple copies in the fungal genome.

Figure 3a : Schematic representation depicting the target regions of 18SrRNA regions for amplification of panfungal genome



3.1.2 Multiplex PCR (mPCR) targeting ITS region

A mPCR method was developed by Luo et al, 2002 to identify simultaneously multiple fungal pathogens in a single reaction. Five sets of species-specific primers were designed from the internal transcribed spacer (ITS) regions, ITS1 and ITS2, of the rRNA gene to identify *C. albicans*, , *Candida parapsilosis*, *Candida tropicalis*, *Candida glabrata* ,*A. fumigatus*. Another set of ITS primers, CN4 and CN5, were used to identify *C. neoformans*. Three sets of primers were used in one multiplex PCR to identify three different species. Six different species of pathogenic fungi can be identified with two multiplex PCRs. Furthermore, instead of using templates of purified genomic DNA, the PCR was performed directly from yeast colonies or cultures, which simplified the procedure and precluded contamination during the extraction of DNA. A total of 242 fungal isolates were tested, representing 13 species of yeasts, four species of *Aspergillus*, and three zygomycetes. The multiplex PCR was tested on isolated DNA or fungal colonies, and both provided 100% sensitivity and specificity. However, DNA from only about half the molds could be amplified directly from mycelial fragments, while DNA from every yeast colony was amplified. This mPCR method provides a rapid, simple, and reliable alternative to conventional methods to identify common clinical fungal isolates.

Fujita et al 2001, have reported that the internal transcribed spacer 1 (ITS1) and ITS2 regions and the 5.8S ribosomal DNA (rDNA) region of the fungi were amplified by using universal primers ITS1 and ITS4. The ITS2 region was simultaneously amplified by using universal primers ITS3 and ITS4. Since *Trichosporon asahi* and *T. asteroides* showed similar lengths for two amplicons, 29 different gel patterns were demonstrated for 30 yeast species tested on the basis of differences in the lengths of one or two amplicons. Of 75 yeast isolates from clinical materials, 5 isolates (6.8%) which were incompletely identified or not identified by the phenotypic method were identified with our PCR-based method (2 isolates as *Candida guilliermondii*, 2 as *C. krusei*, and 1 as *C. zeylanoides*). No differences in discriminating power or sensitivity were observed between the PCR-AGE method and the PCR-ME method. These methods, prospectively applied to 24 yeast-positive blood culture bottles (16 patients), resulted in the correct detection of 24 yeast strains. In conclusion, multiplex PCR followed by electrophoresis seems to be a promising tool for the rapid

identification of common and uncommon yeast strains from culture colonies and from yeast-positive blood culture bottles (Fujita et al, 2001).

3.1.3 mPCR and liquid hybridization

A procedure based on panfungal PCR and multiplex liquid hybridization was developed by Hendolin et al, 2000 for the detection of fungi in tissue specimens. The PCR amplified the fungal internal transcribed spacer (ITS) region (ITS1-5.8SrRNA-ITS2). After capture with specific probes, eight common fungal pathogens (*A. flavus*, *A. fumigatus*, *C. albicans*, *C.krusei*, *C.glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. neoformans*) were identified according to the size of the amplification product on an automated sequencer. The nonhybridized products were identified by sequencing. The performance of the procedure was examined with 12 deep-tissue specimens and 8 polypous tissue biopsies from the paranasal sinuses. A detection level of 0.1 to 1 pg of purified DNA (2 to 20 CFU) was achieved. Of the 20 specimens, PCR was positive for 19 (95%), of which 10 (53%) were hybridization positive. In comparison, 12 (60%) of the specimens were positive by direct microscopy, but only 7 (35%) of the specimens showed fungal growth. Sequencing of the nonhybridized amplification products identified an infecting agent in six specimens, and three specimens yielded only sequences of unknown fungal origin. The procedure provides a rapid (within 2 days) detection of common fungal pathogens in tissue specimens, and it is highly versatile for the identification of other fungal pathogens.

3.1.4 Need for development of mPCR

Infectious keratitis causes extensive ocular morbidity worldwide. The true extent of visual impairment from this condition is thought to far exceed the recognised prevalence, particularly among agricultural workers in the developing world, where a “silent epidemic” of corneal blindness has been postulated. (Whitcher et al, 1997). Fungal corneal infections in particular occur most frequently in individuals who work in agriculture.(Johnson et al 1997, Deshpande et al 1999).This condition is also associated with diabetes mellitus and the acquired immune deficiency syndrome

(AIDS) Mselle, 1999. Standard microbiological techniques for diagnosing microbial keratitis rely on culturing the organisms in nutrient media. The frequency of apparent diagnostic failure (that is,no organism is isolated though an infection is clinically evident) ranges from 20%(Upadhyay et al, 1991) to 60%.(Wahl et al, 1991). An additional problem is that such techniques require days to weeks for complete results, which can significantly delay appropriate treatment. The potential utility of PCR based techniques for improving the diagnosis of ocular infection is well recognised, (Gordon et al, 1993,Reischl et al, 1997) and the use of PCR for this purpose is expanding.(Alexandrakis et al, 1998, Jaeger et al, 2000, Anand et al, 2001) Assays targeting *Candida, Aspergillus*, and *Fusarium* have been tested preliminarily in vitreous specimens, and an assay using panfungal primers has been used more extensively in India.(Anand et al, 2000) A PCR assay has also been applied to detect *Fusarium* in rabbit corneal infections.(Alexandrakis et al, 2000) .

Fungal endophthalmitis accounts for 4 to 11% of all cases of culture proven endophthalmitis and is usually acquired from an endogenous source **(**Pffugfelder et al, 2000). Fungal endophthalmitis may have a clinical appearance presenting in a similar manner to bacterial endophthalmitis. The time to diagnosis from the onset of symptoms has been reported as varying during which bilateral ocular disease may cause severe morbidity, especially in patients who are already debilitated In cases with a typical history and clinical signs of fungal endophthalmitis, vitreous sampling is implemented. While intraocular specimens are often culture negative, cases that respond to antifungal treatment are considered to be infective in origin (Shin et al, 1999, Sutton et al, 1999). Amplification of target DNA through PCR with sequence specific primers is potentially more sensitive and rapid than microbiological techniques as a number of constraints are removed. A protocol for the rapid detection of fungal DNA in ocular specimens, derived from 3 species; *C. albicans, Aspergillus fumigatus* and *Fusarium solani* has been developed. Two novel pan fungal primers complementary to 18s rRNA sequence present in all 3 species were designed. Pan fungal PCR followed by nested amplification utilizing species-specific primers aids in patient management in cases where fungal endophthalmitis is suspected **(**Pffugfelder et al, 2000). Recently *C. glabrata* has been reported to cause endophthalmitis after penetrating keratoplasty (Grueb et al, 2006) and *A. ustus* causing endophthalmitis after cataract surgery (Yildrian et al, 2006).

The dramatic increase in the opportunistic fungal infections along with the development of new antifungal agents with various spectra of activity and the emergence of antifungal resistance has led to the critical need for diagnostic methods that can rapidly and accurately identify fungal pathogen (Sutton et al, 1999).

The ribosomal DNA genes are found in all micro-organisms and known to accumulate mutations at a slow constant rate over time. Utilization of primers that recognize universally conserved sequences within the rDNA genes has allowed the identification of fungal pathogens to species level. Fungi have a rDNA complex region including a sequence coding for 18 S rRNA, ITS1, 5.8 S, ITS 2, 28 S r RNA gene.

All three genes within the rDNA complex have been used in studies on the molecular evaluation of fungi. The 18 S 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 5.8 S 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 28SrRNA 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 28 S r DNA gene however is conserved among organism

groups limiting the usefulness of this region for species identification (Iwen et al, 2001)

Fungal keratitis and fungal endophthalmitis cause visual impairment worldwide. Standard microbiological techniques for diagnosing microbial keratitis and endophthalmitis rely on culturing the organisms in media which may require days to weeks for complete results which can significantly delay appropriate treatment. Gaudio et al 2002 and Jaeger et al 2000 have designed primers targeting 18 S r RNA for panfungal genome detection followed by species level identification using primers specific for *C.albicans, A.fumigatus ,F. oxysporum.* The use of nested PCR would not only help in rapid detection but also help to identify fungi to species level. ITS 1 and ITS 2 regions flanking the 5.8 S r RNA gene extensive sequence diversity among major groups of eukaryotic organisms and even within species. Various molecular techniques using ITS as molecular targets for identification of fungi have been evaluated – direct sequence analysis, utilization of genus or species specific primers / probes and RFLP. Species specific primers that amplify a specific portion of the ITS region have also been designed and tested (Motoyoma et al, 2000).

The current study aims to use mPCR 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.

3.2 Objectives

- To standardize a multiplex Polymerase chain reaction targeting three regions the small subunit, ITS and the large subunit region for detection of panfungal genome
- To apply multiplex Polymerase chain reaction for detection of panfungal genome in ocular specimens
- To standardize and apply a nested PCR targeting 18SrRNA for detection of *Candida albicans* in ocular specimens
- To standardize and apply a nested PCR targeting 18SrRNA for detection of

Aspergillus fumigatus in ocular specimens

- To standardize and apply a nested PCR targeting 18SrRNA for detection of *Fusarium solani* in ocular specimens
- Application of PCR targeting the small subunit (18S rRNA) ITS region and large subunit (28S rRNA) region on ocular specimens for detection of panfungal genome

3.3 Materials and Methods:

3.3.1 Collection of ocular specimens

Corneal scrapings and intraocular specimens of AH and VF were collected and processed according to the procedure given in Appendix I

3.3.2 Ocular Specimens included in the study

A total of 200 clinical specimens consisting of 100 corneal scrapings and 100 intraocular specimens were included in the study. The specimens were categorized into two groups:

Group I: Corneal scrapings

A total of 100 corneal scrapings, comprising of 50 corneal scrapings from clinically suspected case of keratitis and 50 corneal scrapings from the control group were included in the study.

Group II: Intraocular specimens

A total of 100 intraocular specimens , comprising of 50 (22 Aqueous humor and 28 Vitreous fluid) from clinically suspected cases of endophthalmitis and 50 intraocular specimens comprising of 25 aqueous humor [AH] and 25 vitreous fluid [VF])from the control group were included in the study.

The inclusion and exclusion criteria are given below:

3.3.3 Inclusion and exclusion criteria for ocular specimens for the specimens included in the study:

Inclusion criteria for corneal scrapings **(Patient group)**

- Corneal scrapings positive for fungi either by smear or culture or by both
- Corneal scrapings negative for bacteria, *Acanthamoeba* and fungi by smear and culture

Exclusion criteria for corneal scrapings **(control group)**

- Corneal scrapings positive for bacteria either by smear or culture or by both
- Corneal scrapings positive for acanthamoeba either by smear or culture or by both

Inclusion criteria for intraocular fluids**: (Patient group)**

All intraocular fluids positive by either smear or culture or by both for fungi were included in the study , to compare the results of mPCR. In addition, specimens negative for bacteria by both smear and culture and negative for fungi by smear and culture were used for detection by m PCR.

Exclusion criteria for intraocular fluids **: (Control group)**

Intraocular specimens positive for bacteria either by smear or culture or by both have been excluded.

3.3.4 Conventional mycological investigations:

The control and test specimens were processed as per the procedure given in Appendix I. The fungal isolates were identified according to the standard methods (Forbes, 2000)

3.3.5 Molecular microbiological investigations:

DNA extraction from intraocular fluids and corneal scrapings were carried out by using Biogene and Qiagen kit according to the manufacturer's instructions.

3.3.6 PCR

The primers used for mPCR and nPCR is given in Table 3a. The protocol for uniplex PCR reactions targeting 18SrRNA, ITS and 28SrRNA region is given in Appendix II.

Table 3a: Primer sequences targeting the ribosomal unit of fungi

Primer sequence	Annealing Temperature	Amplified product size
18SrRNA (Panfungal 1st round) Pffor - 5'agggatgtatttattagataaaaaatcaa 3' Pfrev 2 - 5'cgcagtagttagtcttcagtaaatc 3'. **18SrRNA *C. albicans* (Second round)** Cafor2 – 5' gggaggtagtgacaataaataac 3' Carev 3 -5' –cgtccctattaatcattacgat 3' **18SrRNA *A.fumigatus* (Second round)** Asfufor 5' – ccaatgcccttcggggctcct 3' Asfurev 5' –cctggttccccccacag 3' **18SrRNA *F. solani* (Second round)** Fusofor 5' ccaatgccctccggggctaac 3' Fusorev 5' gcataggcctgcctggcg 3'	**58 °C** **66°C** **66°C** **64.5 °C**	**728 bp - *C. albicans*** **743 bp – *A.fumigatus*** **744 bp – *F. solani*** **402 bp *C. albicans*** **520 bp *A. fumigatus*** **565 bp *F. solani***
ITS region (Panfungal 1st round) ITS 1 5' tccgtaggtgaacctgcgg 3' ITS 4 5' tcctccgcttattatgc 3'	**55°C**	**520 -611 bp**
28S rRNA region (panfungal) FU_1 5' tgaaattgttgaaagggaa 3' FU_2 - 5' gactccttggtccgtgtt 3'	**60°C**	**260 bp**

3.3.7 Sensitivity of PCR:

Sensitivity of mPCR,uniplex PCR (uPCR) and nPCR was determined using serial ten fold dilutions of standard strain of *C. albicans* ATCC 24433.

3.3.8 Specificity of PCR :

Specificity of mPCR,uPCR and nPCR primers were verified by using DNA extracts of micro organisms listed in Appendix II.

3.3.9 mPCR targeting the ribosomal unit of panfungal genome:

A single round multiplex PCR was optimized using the primers targeting the small subunit (18S rRNA), the Internal Transcribed Spacer (ITS) region and the large subunit (28SrRNA) using the sets of primers mentioned in Table 3a For a 50µl reaction , 10 µl of 200 µm dNTPs,7.5 µl of 10 X PCR buffer (15 mM $MgCl_2$,500 mM KCl, 100 mM Tris Cl,0.01% gelatin), 7 µl of 10 mM $MgCl_2$,10 picomoles of primers targeting 18SrRNA, ITS and 28SrRNA and 10 µl of template DNA was used. Amplification was carried out in Perkin Elmer thermal cycler (Model 2700). Thermal cycling consisted of initial denaturation at 95˚C for 5 minutes followed by 35 cycles of denaturation at 95˚C for 30 seconds, annealing at 58˚C for 60 seconds and extension at 72˚C for 60 seconds followed by final extension at 72˚C for 10 minutes. The amplified products were detected by agarose gel electrophoresis.

3.4 Results

3.4.1 Conventional methods:

Group I: Corneal scrapings

All the control corneal scrapings did not reveal the presence of infectious aetiology by smear and culture. The distribution of fungi isolated from keratitis is represented in Figure 3b

Group II : Intraocular specimens

All the control intraocular specimens did not reveal the presence of infectious aetiology by smear and culture. The distribution of fungi isolated from endophthalmitis is represented in Figure 3c. The colony morphology of various fungal isolates and lactophenol blue mount are given in Figure 3d.

Figure 3b: Distribution of fungal isolates from corneal scrapings collected from keratitis patients during March – October 2006

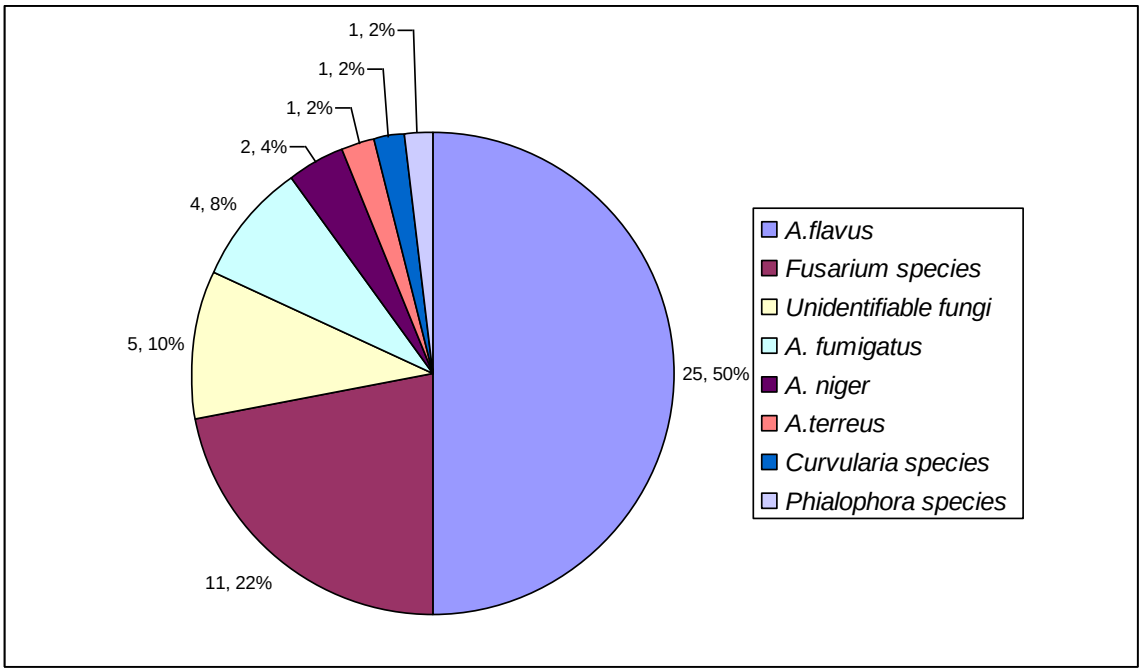


Figure 3c: Distribution of fungal isolates from intraocular specimens collected from endophthalmitis patients during June 2005 – October 2006

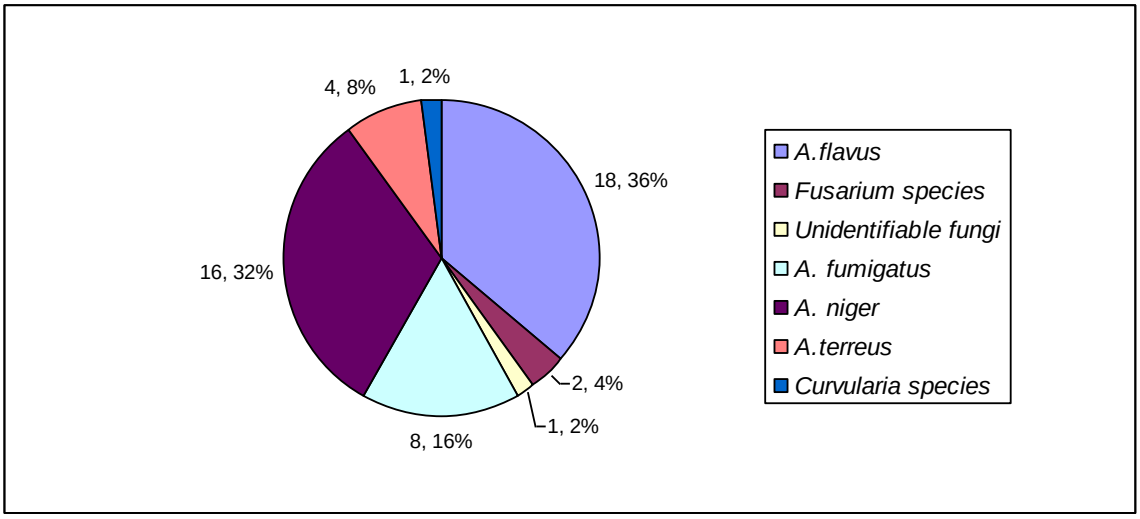
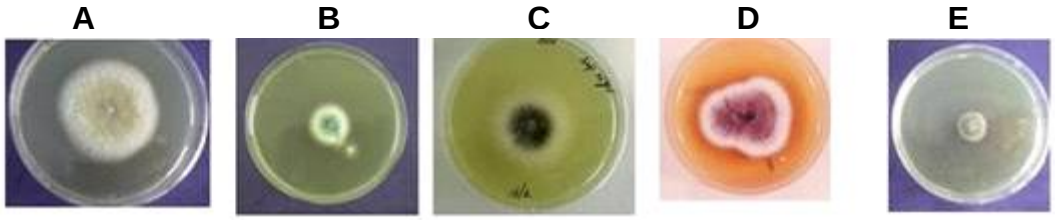


Figure 3d : Colony morphology of fungal isolates on Sabouraud's dextrose agar



A: *A. flavus* : White fluffy growth with yellow spores

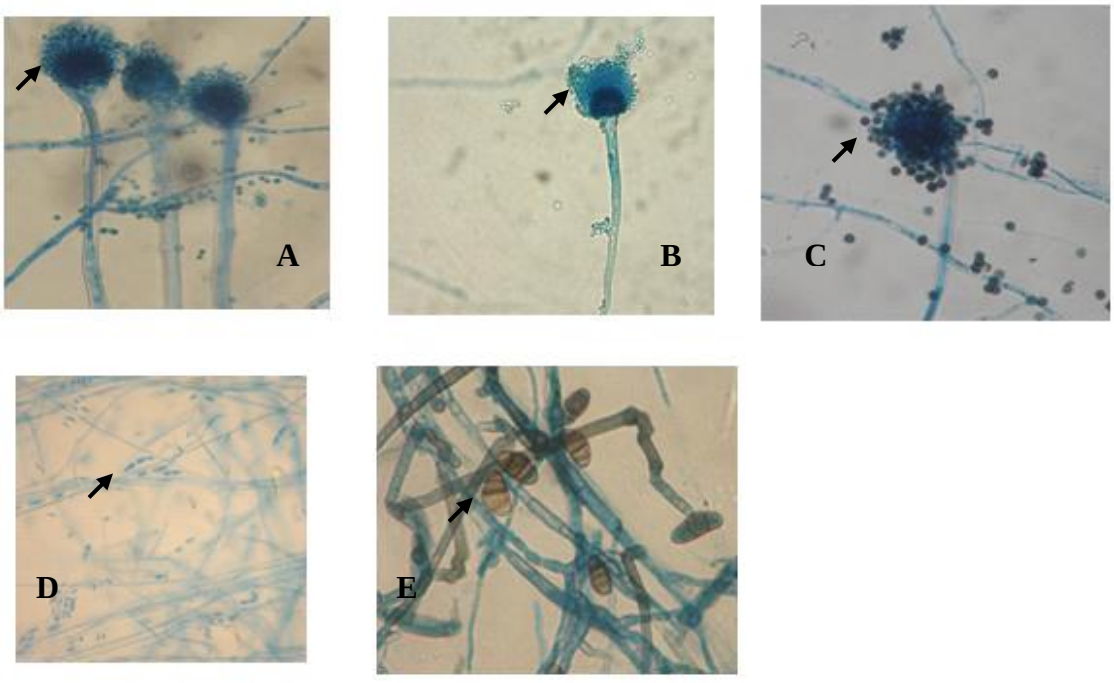
B: *A. fumigatus* : White fluffy growth with green spores

C: *A. niger* : Fluffy growth with black coloured spores

D : *F. falciforme* : Fluffy growth with diffusible reddish pink pigment

E : *Curvularia* species: Fluffy growth with olive green centre later became brown

Lactophenol Cotton blue (LPCB 40 X) mount of fungi



A: *A. flavus* : Conidiophore bearing vesicle, conidia covering the vesicle

B: *A. fumigatus* : Conidiophore bearing vesicle, conidia covering the upper half of the vesicle

C: *A. niger* : Spherical vesicle covered with black conidia

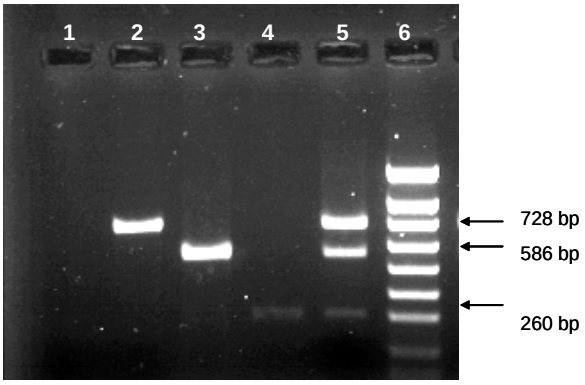
D: *Fusarium species* : Sickle shaped macroconidia and oval macroconidia

E: *Curvularia species :* Clavate conidia with central bulging

3.4.2 Molecular mycological investigations

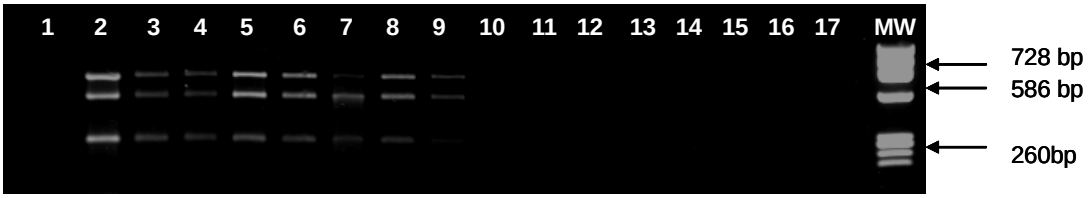
The results of uPCR and mPCR is shown in figure 3e.The results of sensitivity of mPCR performed targeting the three regions, 18SrRNA, ITS and 28SrRNA is shown in figure 3f , 3g, the specificity of the multiplex reaction in figure 3h and the application of the same on ocular specimens is shown in figure 3i and 3j respectively.

Figure 3e: Agarose gel electrophoretogram showing the results of uniplex and multiplex reactions targeting the ribosomal unit using *C. albicans* ATCC 24433



Lane 1: Negative control **Lane 2:** Amplification of *C. albicans* using 18SrRNA primers (728 bp) **Lane 3:** Amplification of *C. albicans* using ITS primers (586 bp) **Lane 4:** Amplification of *C. albicans* using 28SrRNA primers (260 bp) **Lane 5:** Amplification of *C. albicans* using 18SrRNA, ITS and 28SrRNA primers by mPCR **Lane 6**: Molecular weight marker 100 bp DNA ladder

Figure 3f: Agarose gel electrophoretogram revealing the sensitivity of multiplex PCR targeting the ribosomal unit using ATCC *C. albicans* 24433

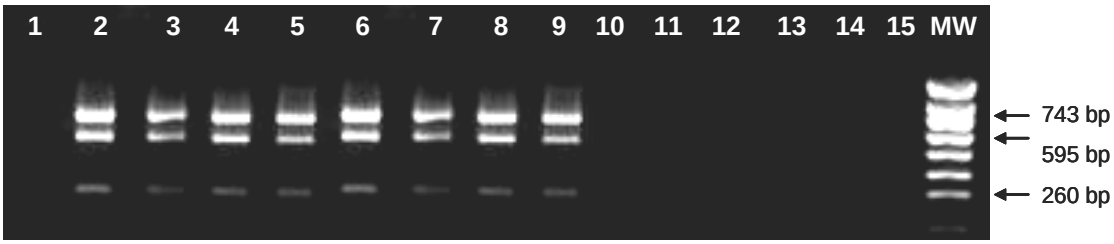


Lane 1: Negative control **Lane 2:** *C. albicans* undiluted DNA

Lane 3 -17: Serial 10 fold dilutions of *C. albicans* DNA (10^{-1} to 10^{-15})

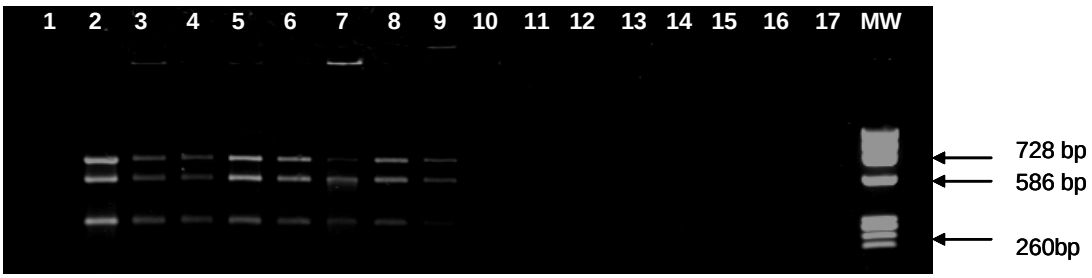
Lane 2-9 : Amplification of *C. albicans* with all three targets **Lane 10-17:** No amplification of *C. albicans* DNA **MW:** Molecuar weight marker *Hae* –III digest of Phi X 174 bacteriophage DNA **. Sensitivity 1 pg**

Figure 3g: Agarose gel electrophoretogram revealing the sensitivity of mPCR targeting the ribosomal unit using ATCC *A. flavus* 204304



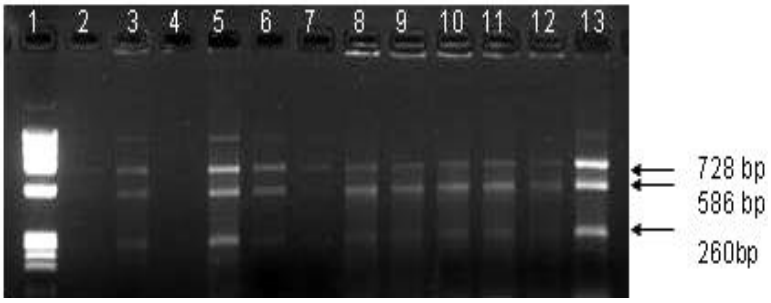
Lane 1: Negative control **Lane 2:** *A. flavus* DNA undiluted **Lane 3 -15:** Serial 10 fold dilutions of *A. flavus* DNA (10^{-1} to 10^{-13}) **Lane 2-9 :** Amplification of *A. flavus* with all three targets **Lane 10-15**: No amplification of *A. flavus* ATCC DNA **MW:** Molecular weight marker *Sau* 3A I digest of puc 18 DNA. **Sensitivity 10 pg**

Figure 3h: Agarose gel electrophoretogram revealing the specificity of mPCR targeting the ribosomal unit of fungi



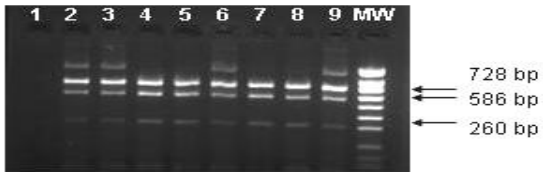
Lane 1: Negative control **Lane 2:** *A. fumigatus* ATCC 10894
Lane 3: *C. parapsilosis ATCC* 22019 **Lane 4**: *C. krusei ATCC 6258*
Lane 5: *C. albicans* ATCC 24433 **Lane 6:** *A. flavus* ATCC 204304
Lane 7: *F. solani* ATCC 36031 **Lane 8:** *Curvularia species* **Lane 9:** *T. rubrum* **Lane 10:** *S. aureus* ATCC 12228 **Lane 11:** *P. aeruginosa* ATCC 27853
Lane 12: *M. tuberculosis* H37RV **Lane 13:** Peripheral blood DNA
Lane 14: *Acanthamoeba polyphaga* ATCC 30461 **Lane 15:** HSV -1 ATCC 733 VR
Lane 16: *N. asteroids* **Lane 17:** *P. acnes* isolate **MW:** Molecular weight marker *Hae* III digest of Phi X bacteriophage DNA

Figure 3i: Agarose gel electrophoretogram showing the results of application of mPCR targeting the ribosomal unit of fungi on corneal scrapings collected from cases of keratitis



Lane 1: Negative control **Lane 2 to 8:** Corneal scrapings positive by mPCR
Lane 9: Positive control *C. albicans* ATCC 24433 **MW:** Molecular weight marker 100 bp DNA ladder

Figure 3j: Agarose gel electrophoretogram showing the results of application of mPCR targeting the ribosomal unit on intraocular specimens collected from cases of endophthalmitis



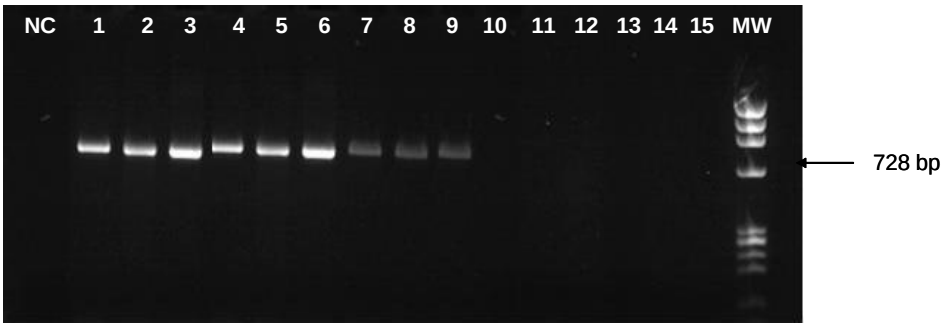
Lane 1: Molecular weight marker *Hae* III digest of Phi X 174 bacteriophage DNA
Lane 2 : Negative control **Lane 3 ,6, 8,9,11,12** : VF positive **Lane 4:** AH negative
Lanes 5,7,10:AH positive **Lane 13:** Positive control *C. albicans* ATCC 24433

3.4.3 PCR targeting 18SrRNA region for detection of panfungal genome

Sensitivity of 18SrRNA PCR

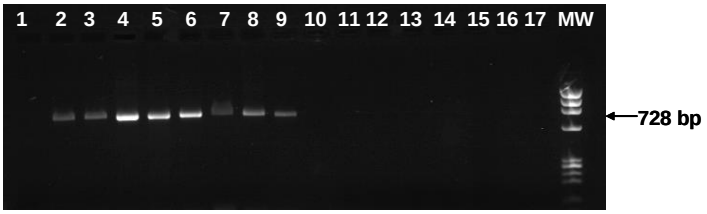
The results of sensitivity of 18SrRNA PCR performed on diluted DNA extracts of standard strains of *C. albicans* was found to be 1 pg and that of *A. fumigatus* was 10 pg. The results of sensitivity of 18SrRNA PCR performed using *C. albicans* is shown in Figure 3k and the results of specificity is shown in Figure 3l.

Figure 3k : Agarose gel electrophoretogram showing the results of sensitivity of 18SrRNA PCR on standard strain of *C. albicans* ATCC 24433



NC: Negative control **Lane 1 :** Undiluted DNA of *C. albicans* **Lane 2 – 15:** Serial 10 fold dilutions of *C. albicans* DNA (10^{-1} to 10^{-14}) **Lane 1- 9** : Positive 728 bp product of *C. albicans* DNA, MW: Molecular weight marker *Hae* –III digest of Phi X 174 bacteriophage DNA. **Sensitivity: 1 pg**

Figure 3l: Agarose gel electrophoretogram showing the results of specificity of 18SrRNA PCR



Lane 1: Negative control **Lane 2:** *C. tropicalis* ATCC 750 **Lane 3:** *C. parapsilosis ATCC* 22019 **Lane 4:** *C. krusei ATCC 6258* **Lane 5:** *C. albicans* ATCC 24433 **Lane 6:** *A. flavus* ATCC 204304 **Lane 7**: *F.solani* ATCC 36031 **Lane 8:** *Curvularia species* **Lane 9:** *T. rubrum* **Lane 10**: *S. aureus* DNA **Lane 11**: *P. aeruginosa* ATCC 27853 **Lane 12**: *M. tuberculosis* H 37 RV **Lane 13:** *Nocardia asteroides* **Lane 14:** *Acanthamoeba polyphaga* ATCC 30461**Lane 15**: Herpes Simplex virus HSV -1 ATCC 733 VR **Lane 16**:Peripheral blood DNA **Lane 17:** *M. fortuitum* **MW:** Molecular weight marker Hae III digest of PhiX 174 bacteriophage DNA

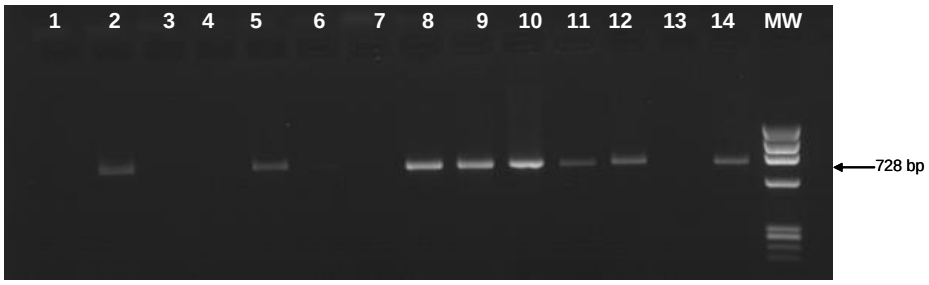
NOTE:

Lane 2 -9 : Amplification seen **Lane 10- 17 :** No amplification was seen

The results of application of 18SrRNA PCR on corneal scrapings is shown in figure 3m and on intraocular specimens is shown in figure 3n. All the specimens positive by

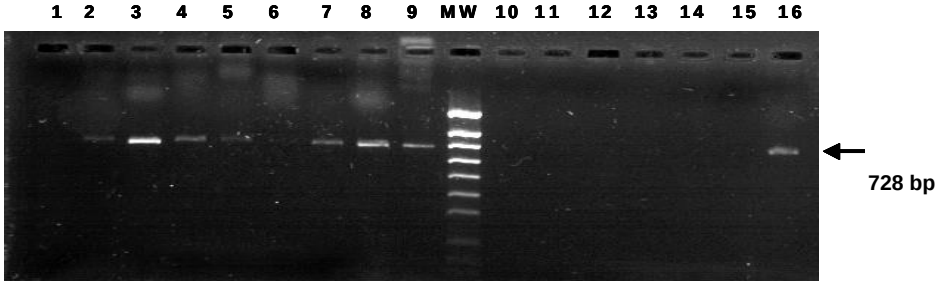
conventional methods were detected by PCR and fungal genome was not detected in control specimens.

Figure 3m : Agarose gel electrophoretogram showing the results of application of 18SrRNA PCR on corneal scrapings



Lane 1: Negative control **Lane 2,5,8,9,10,11,12:** 2986/06 Positive **Lane 3,4,6, 7,13:** Corneal scraping control Negative **Lane 14:** Positive control *C. albicans* ATCC 24433 **MW:** Molecular weight marker *Hae* –III digest of Phi X 174 bacteriphage DNA

Figure 3n : Agarose gel electrophoretogram showing the results of application of 18SrRNA PCR on intraocular specimens

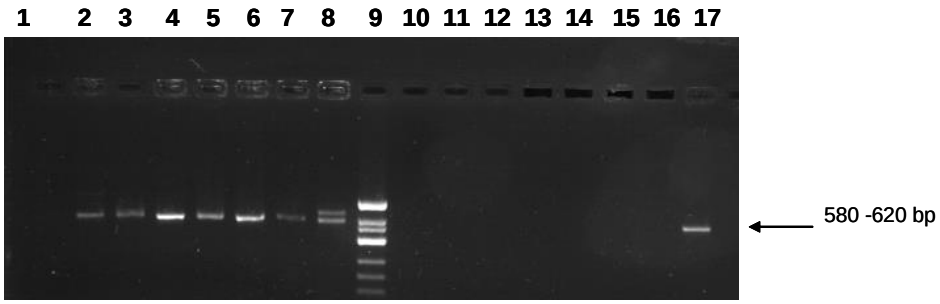


Lane 1: Negative control **Lane 2,4,5,9:** AH Positive **Lane 3,6,7,8:** Vitreous fluid positive **Lane 10,11,12 AH controls** : negative **13,14,15 VF Controls :** Negative **Lane 16:** Positive control *C. albicans* ATCC 24433 **MW:** Molecular weight marker 100 bp DNA ladder

3.4.4 Application of ITS PCR on ocular specimens

The results of PCR targeting ITS region on ocular specimens is given in Figure 3o

Figure 3o: Agarose gel electrophoretogram showing the results of application of PCR targeting ITS region on ocular specimens

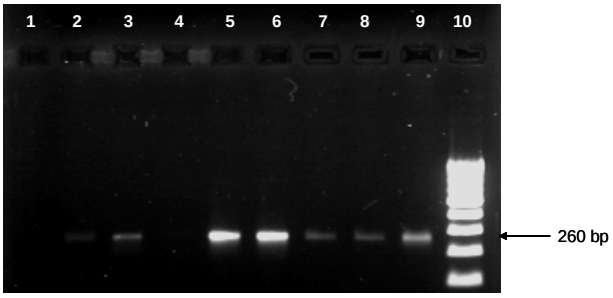


Lane 1: Negative control **Lane 2,6 :** AH positive **Lane 3,7:** VF positive **Lane 4,5, 8** :Corneal scraping Positive **Lane 9:** Molecular weight marker *Hinf* I digest of Phi X 174 bacteriophage DNA **Lane 10-16:**Controls intraocular specimens : Negative **Lane 17:** Positive control *C. albicans* ATCC 24433

3.4.5 Application of 28SrRNA PCR on ocular specimens

The application of 28SrRNA PCR on ocular specimens and on controls is shown in Figure 3p and Figure 3q respectively.

Figure 3p : Agarose gel electrophoretogram showing the results of application of PCR targeting 28SrRNA region on ocular specimens



Lane 1: Negative control **Lane 2,6:** AH Positive **Lane 3,7:** VF positive
Lane 4 :Corneal scraping Negative **Lane 5:** Corneal scraping Positive
Lane 8: Aqueous aspirate positive **Lane 9:** Positive control *C. albicans* ATCC 24433
Lane 10: Molecular weight marker 100 bp ladder

Figure 3q: Agarose gel electrophoretogram showing the results of application of PCR targeting 28SrRNA region on control ocular specimens

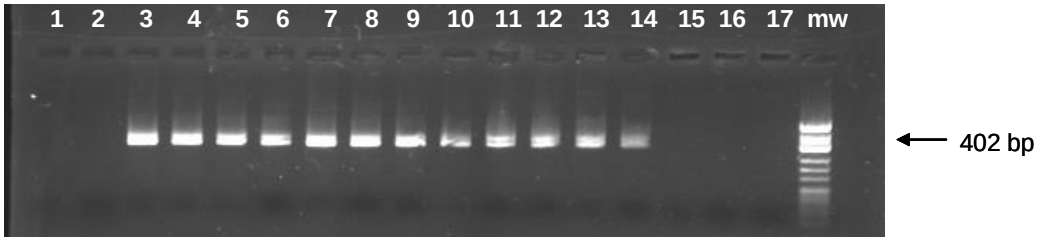
Lane 1: Negative control **Lane 2 – Lane 7:** Control corneal scrapings showing no amplification **Lane 8,9,10 :**Control AH showing no amplification **Lane 11-14 :** Control VF showing no amplification **Lane 15**: Positive control *C. albicans* ATCC 24433 **MW:** Molecular weight marker 100 bp ladder

3.4.6 PCR targeting 18SrRNA specific for detection of *C. albicans*

SENSITIVITY

The results of sensitivity of *C. albicans* PCR after two rounds of amplification is shown in Figure 3r and specificity in figure 3s and the application of PCR is shown in figure 3t

Figure 3r: Agarose gel electrophoretogram showing the results of sensitivity of *C. albicans* PCR targeting 18SrRNA region



Lane 1: Negative control second round **Lane 2 :** Negative control first round

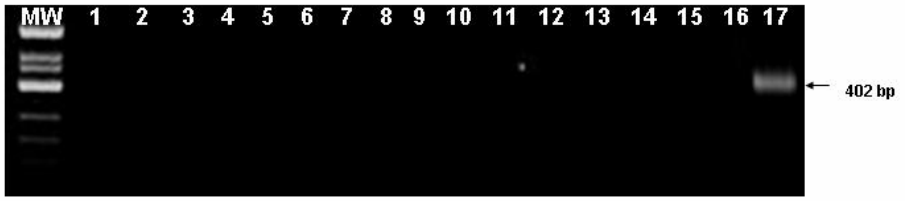
Lane 3: Positive control *C. albicans* ATCC 24433 Undiluted DNA

Lane 4 -14 : Serial 10 fold dilutions of *C. albicans* 10^{-1} to 10^{-14}

MW: Molecular weight marker Phi X bacteriophage DNA *Hinf* I digest

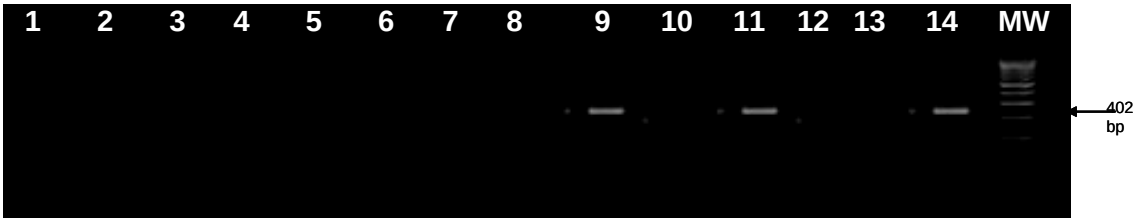
Sensitivity: 50 fg

Figure 3s : Agarose gel electrophoretogram showing the results of specificity of *C. albicans* PCR targeting 18SrRNA region



MW: Hinf I digest of Phi X 174 bacteriophage DNA **Lane 1**: Negative control **Lane 2:** *C.tropicalis* ATCC 750 **Lane 3:** *C. parapsilosis ATCC* 22019 **Lane 4:** *C. krusei ATCC 6258* **Lane 5:***A. fumigatus* ATCC 10894 **Lane 6:** *A. flavus* ATCC 204304 **Lane 7:** *F. solani* ATCC 36031 **Lane 8:** *Curvularia species* **Lane 9:** *T. rubrum* **Lane 10:** *S. aureus* ATCC 12228 **Lane 11:** *P. aeruginosa* ATCC 27853 **Lane 12:** *M. tuberculosis* H 37 RV**Lane 13:** *N. asteroides* **Lane 14:** *Acanthamoeba polyphaga* 30461 **Lane 15**: HSV -1 ATCC 733 VR **Lane 16**:Peripheral blood DNA **Lane 17:** Positive control *C. albicans* ATCC 24433

Figure 3t : Agarose gel electrophoretogram showing the results of application of *C. albicans* PCR targeting 18SrRNA region



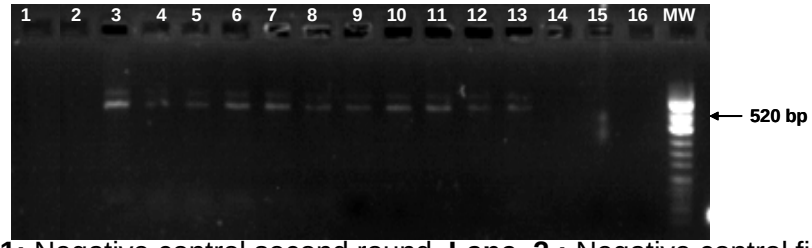
Lane 1: Negative control **Lane 2,6,10,13 :** AH Negative **Lane 3,7**: VF Negative **Lane 4,5,8,12:** Corneal scraping Negative **Lane 9:** Aqueous aspirate Positive **Lane 11:** Vitreous fluid positive **Lane 14 :** Positive control *C. albicans* ATCC 24433 **MW:** Molecular weight marker 100 bp ladder

3.4.7 PCR targeting 18SrRNA specific for detection of *A. fumigatus*

Sensitivity

The results of sensitivity of *A. fumigatus* PCR after two rounds of amplification is shown in Figure 3u and specificity in figure 3v and the application of PCR is shown in figure 3w

Figure 3u : Agarose gel electrophoretogram showing the results of sensitivity of *A. fumigatus* PCR targeting 18SrRNA region



Lane 1: Negative control second round **Lane 2 :** Negative control first round

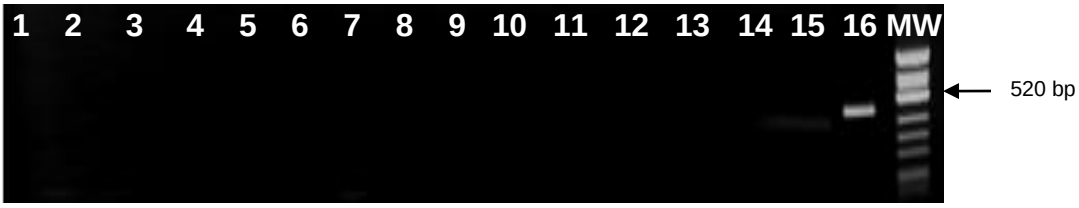
Lane 3: Positive control *A. fumigatus* ATCC 10894 Undiluted DNA

Lane 4 -16 : Serial 10 fold dilutions of *A. fumigatus* 10^{-1} to 10^{-13}

MW: Molecular weight marker Phi X bacteriophage DNA *Hinf* I digest

Sensitivity: 100 fg

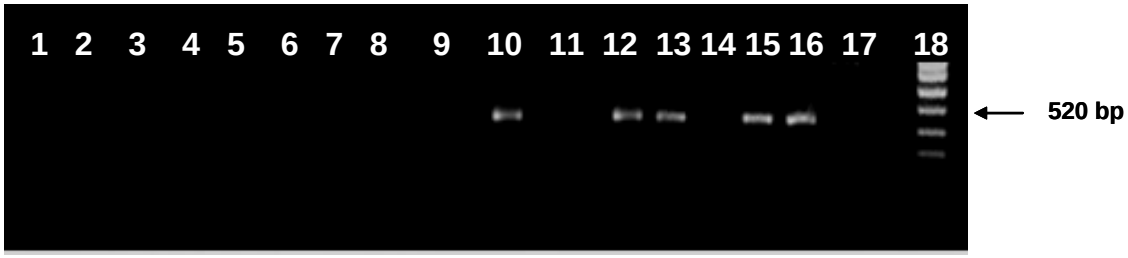
Figure 3v: Agarose gel electrophoretogram showing the results of specificity of *A. fumigatus* PCR targeting 18SrRNA region



Lane 1: Negative control **Lane 2**: *C. tropicalis* ATCC 750 **Lane 3:** *C.parapsilosis ATCC* 22019 **Lane 4:** *C. krusei ATCC 6258* **Lane 5:** *C. albicans* ATCC 24433 **Lane 6:** *A. flavus* ATCC 204304 **Lane 7:** *F. solani ATCC* 36031 **Lane 8:** *Curvularia species* **Lane 9:** *T. rubrum* **Lane 10:** *S. aureus* ATCC 12228 **Lane 11:** *P. aeruginosa* ATCC 27853 **Lane 12**: *M. tuberculosis* H 37RV **Lane 13:** Peripheral blood DNA **Lane 14**: *Acanthamoeba polyphaga ATCC* 30461 **Lane 15:** HSV -1 ATCC 733 VR **Lane 16:** *A. fumigatus* ATCC 10894

MW: Molecular weight marker *Sau* 3A I *puc* 18 DNAI digest

Figure 3w : Agarose gel electrophoretogram showing the results of application of *A. fumigatus* nPCR targeting 18SrRNA region



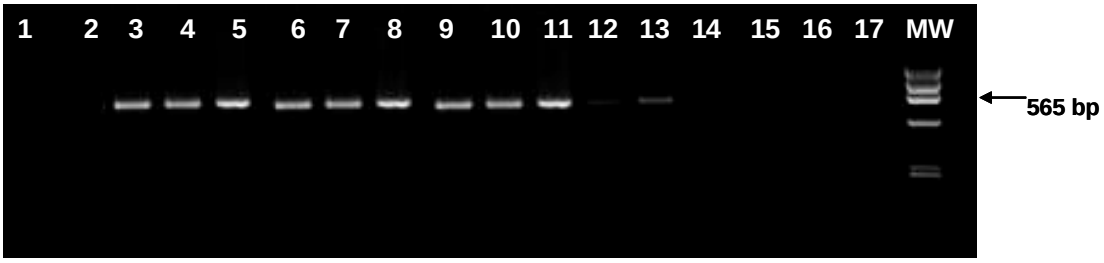
Lane 1: Negative control (second round) **Lane 2:** Negative control (first round)
Lane 3,7,11: VF Negative **Lane 4,5,8:** Corneal scraping Negative
Lane 6,9,14: AH Negative **Lane 10,12,13,15:** Corneal scraping Positive
Lane 16: Positive control *A.fumigatus* **Lane 18**: Molecular weight marker *Sau* 3A I digest puc 18 DNA

3.4.8 PCR targeting 18SrRNA specific for detection of *F. solani*

Sensitivity

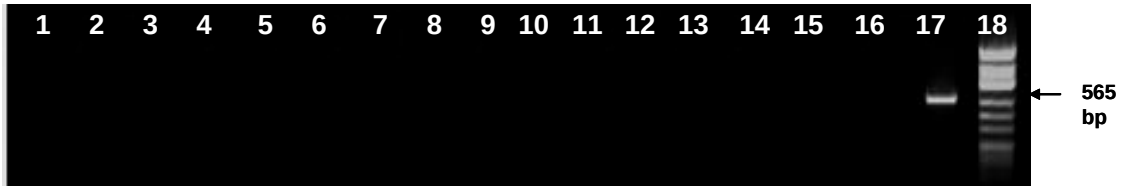
The results of sensitivity of *F. solani* PCR after two rounds of amplification is shown in Figure 3x and specificity in figure 3y

Figure 3x : Agarose gel electrophoretogram showing the results of sensitivity of *F. solani* PCR targeting 18SrRNA region



Lane 1: Negative control second round **Lane 2 :** Negative control first round
Lane 3: Positive control *F. solani* ATCC 36031 Undiluted DNA
Lane 4 -16 :Serial 10 fold dilutions of *Fusarium solani* 10^{-1} to 10^{-15}
MW: Molecular weight marker Phi X bacteriophage DNA *Hae* III digest
Sensitivity 10 fg

Figure 3y: Agarose gel electrophoretogram showing the results of specificity of *F. solani* PCR targeting 18SrRNA region



Lane 1: Negative control second round **Lane 2**: Negative control first round **Lane 3:** *C. parapsilosis ATCC* 22019 **Lane 4:** *C. krusei ATCC 6258* **Lane 5:** *C. albicans* ATCC 24433 **Lane 6:** *A. flavus* ATCC 204304 **Lane 7:** *F. falciforme* laboratory isolate **Lane 8:** *Curvularia species***Lane 9:** *T. rubrum* **Lane 10:** *S. aureus* ATCC 12228 **Lane 11:** *P. aeruginosa* ATCC 27853 **Lane 12:** *M. tuberculosis* H37 RV**Lane 13:** Peripheral blood DNA **Lane 14:** *Acanthamoeba polyphaga* ATCC 30461 **Lane 15:** HSV 1 ATCC 733 VR **Lane 16:** *A. fumigatus* ATCC 10894 **Lane 17:** *F. solani* ATCC 36031 **Lane 18:** Molecular weight marker *Hae* III digest of Phi X bacteriophage DNA

The genome of *F. solani* was not detected in any of the specimens. The results of application of PCR and mPCR targeting the ribosomal unit on ocular specimens is shown in Table 3b

Table 3b: Results of application of PCR and mPCR targeting the ribosomal unit on ocular specimens

Ocular specimens N = 200	Panfungal genome[@]	nPCR targeting 18SrRNA specific for			mPCR targeting the ribosomal unit
		C. alb	*A. fum*	*F. sol*	
Group I Corneal scrapings 100					
Controls 50	Neg	Neg	Neg	Neg	Neg
Test 50	Pos*	Neg	4[#]	Neg	Pos [@]
Group II Intraocular specimens 100					
Controls 50	Neg	Neg	Neg	Neg	Neg
Test 50	Pos*	8[π]	3[#]	Neg	Pos [@]

NOTE: Neg - Negative**, Pos -** positive**, *C. alb* -** *C. albicans*, ***A. fum* -** *A. fumigatus*, ***F. sol* -** *F. solani*

[@] Panfungal genome: Detection of panfungal genome by uPCR was carried out targeting 18SrRNA, ITS and 28SrRNA region

*: The corneal scrapings and intraocular specimens positive by 18SrRNA PCR were detected by ITS PCR and also by 28SrRNA PCR

4 Corneal scrapings and 3 intraocular specimens revealed the presence of *A. fumigatus* genome by nPCR

π 8 intraocular specimens revealed the presence of *C. albicans* DNA by nested PCR.

@ All the specimens positive by mPCR were detected by individual PCRs.

3.5 Discussion

Fungi were identified as the priniciple aetiological agent of corneal ulceration 44% and 37.6% of cases in India and Ghana (Leck et al 2002) respectively. Earlier studies in the same regions reported a similarly high proportion of fungal keratitis. (Gugani et al 1976,Thomas et al 1987, Foster 1992, Hagan et al 1995, Srinivasan et al 1997,Panda et al 1997, Deshpande et al, 1999, Khanal et al, 2001, Garg et al, 2000). A review of the literature shows that there are distinct patterns of geographical variation in the aetiology of suppurative keratitis and considerable variation in the proportion due to fungi has been documented . In Larsen and Toubro Microbiology Research centre, in fungal keratitis cases *Aspergillus species* constitutes 42% and that of *Fusarium* species is 36%.The proportion of corneal ulcers caused by filamentous fungi increases towards tropical latitudes. In more temperate climates, fungal ulcers are uncommon and are more frequently associated with *Candida* species than filamentous fungi.(, Ormerod et al 1987, O'Day 1987, Tanure et al, 2000). In the present study, The fungal culture isolates included *A. flavus* 25 (50%), *Fusarium* species 11 (22%), Unidentifiable fungi 5 (10%), *A. fumigatus* 4 (8%), *Aspergillus terreus* 1 (2%), *Aspergillus niger* 2 (4%), *Curvularia* species 1 (2%) and *Phialophora* species 1(2%).

Aspergillus spp and *Fusarium* spp are the most frequently reported fungal pathogens isolated from cases of fungal keratitis in the tropics.(Hagan et al, 1995) In both Ghana and south India the most commonly isolated fungal pathogens in the current series were *Fusarium* spp. Other studies in South India have reported *Fusarium spp* to be more common than *Aspergillus* species.(Leck et al, 2002).*Fusarium* spp have also been found to be the principal fungal pathogen in Florida, Paraguay, Nigeria, Tanzania, Hong Kong, and Singapore . *Aspergillus* species predominate in northern India, Nepal, and Bangladesh (Leisegang et al, 1980,Deshpande et al, 1999, Williams et al, 1991) . This phenomenon may be explained by differences in climate and the natural environment. A similar pattern was also observed in Ghana. (Leck et al 2002). Although *Fusarium spp* were the most commonly isolated fungi at all of the centres in Ghana, moulds with enteroblastic conidia adhering in dry chains—for example *Aspergillus* spp and *Paecilomyces* spp, were more frequently isolated from patients in the north of the country where the environment is drier and dustier, than

in the more humid south. As observed by Khairallah et al, 1992 the high proportion of corneal infections caused by *Aspergillus spp* in drier climates may be due to the fact that spores of *Aspergillus spp* can tolerate hot, dry weather conditions. *Aspergillus spp* also predominate in more temperate latitudes. The present study also had a predominance of Aspergillus species (50%) since most of the patients referred to the tertiary referral centre were from the northern and north eastern parts of India.

In the present study, the three PCRs targeting 18SrRNA, ITS and 28SrRNA region was applied on ocular specimens (cases and controls). These three PCRs were carried out as separate thermal cycling reactions. There was absolute correlation with the results of conventional methods and all specimens detected by conventional methods were detected by PCR. In order to reduce the time and cost of performance of these PCRs separately, a multiplex PCR was designed to amplify all these three regions simultaneouly. mPCR was optimized by taking into account a number of critical parameters like the GC content of the primers, the length of the oligonucleotides, $MgCl_2$ concentration and more importantly the annealing temperature. When the thermal cycling reactions were carried out as separate reactions the annealing temperature used for 18SrRNA is 58°C, ITS region is 55°C and 28SrRNA region is 60°C. A wide range of annealing temperature starting from 50°C to 60°C with a single degree rise in temperature was initially attempted. There was no amplification of these three targets when a temperature of 50°C - 54 °C was tried initially. At 55°C, the 18SrRNA and the ITS targets amplified but there was no specific amplification of 28SrRNA at this temperature. At 56°C, 57°C and 59 °C the ITS target showed poor amplification while the other two failed miserably. At, 60 °C the ITS and 28SrRNA region were amplified but no amplification was obtained with 18SrRNA region. The average of calculated annealing temperature of all three primers is 57.6°C. Amplification was tried out at 58°C, in which all the three targets yielded a specific band. Hence an annealing temperature of 58 °C was found to be optimal to amplify the ribosomal targets.

mPCR was also optimized by titrating different concentrations of $MgCl_2$. The $MgCl_2$ concentration was titrated by increasing 0.1 mM i.e. from 1.6, 1.7.1.8.1.9, 2, 2.1, 2.2,

2.3, 2.4 and 2.5 mM. Amplification of three targets occurred at concentrations of 1.7, 1.8, 1.9 mM but with non specific bands. At concentrations 2 -2.5 mM , the 28SrRNA failed to amplify while the ITS and 18SrRNA targets amplified with less intensity. Based on these observations the standardization was carried out by varying 0.05 mM of $MgCl_2$ concentration. A concentration of 1.85 mM was found to be amplifying all the three targets satisfactorily.

The number of cycles and the primer dilution also played an important role in amplification process. The cycling conditions were adjusted starting from 25, 30, 35 and 40 cycles with a final extension of 10 minutes. The 18SrRNA and ITS failed to amplify with 25 cycles while 28SrRNA product was detected. At 30 cycles all the three targets were detected but the ITS band was with less intensity. This led to an increase of 5 cycles,in which the three targets were amplified. A total number of 40 cycles resulted in too many non specific bands and primer dimmer formation. A concentration of 10 picomole of each primer was found suitable. Different amounts of dNTPS and 10X buffer were also tried to optimize this mPCR.

This study demonstrates that fungi can be detected in infected corneas using mPCR technique. Advantages of mPCR as shown here include greater speed than culture methods.Limitations to the mPCR assay used in this study include less sensitivity (single round). mPCR assay used in this study requires 4 hours to generate results, significantly faster than the 2 days to 2 weeks required by any fungal culture technique. While fungal smears can be analysed by light microscopy in minutes, the effectiveness of this technique is more variable, and the results are not definite. The ability of mPCR based assays to detect or rule out the presence of fungi in less time would represent an advance in the management of ocular infections, and may also facilitate efforts to recognise and study fungal keratitis and endophthalmitis.

mPCR allows investigators to analyse specimens far from where they are collected, and thus offers a significant advantage for those conducting field or epidemiological studies of fungal keratitis. This novel mPCR was extremely useful in detecting the infectious agent in the minimal amount of template DNA available. Moreover, this novel mPCR has several advantages over uPCR such as, detection of two or more targets in a single tube, minimizing the use of PCR reagents by one third though the

consumption of *Taq* polymerase increased by 0.6 units , reduction in time and the need for a single thermalcycler for amplification.

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 .

3.6 Conclusions

A novel mPCR targeting the ribosomal unit of fungi was optimized and applied on ocular specimens. This technique was found to be rapid and specific and it can be used as a screening tool for detection of fungal corneal infections.

3.7 Future prospects

The sensitivity of mPCR targeting the ribosomal unit of panfungal genome needs to be increased by designing panfungal primers targeting 18SrRNA and 28SrRNA region.Development of microarray facility targeting a panel of genes for detection and identification of fungi would further initiate rapid and specific aetiological diagnosis of fungal infections.

CHAPTER - 4

Development of a Multiplex Polymerase Chain Reaction (mPCR) for Simultaneous Detection of Eubacterial, *P. acnes* and Panfungal Genome for Aetiological Diagnosis of Infectious Endophthalmitis

4.1 Review of literature

Endophthalmitis is an ocular inflammation resulting from the introduction of an infectious agent into the posterior segment of the eye. Bacterial endophthalmitis is an infection of the interior of the eye that, despite appropriate therapeutic intervention, frequently results in visual loss. Recently, research has begun to elucidate the molecular and cellular events that contribute to the damage that occurs in intraocular infection. During infection, irreversible damage to delicate photoreceptor cells of the retina frequently occurs. Despite aggressive therapeutic and surgical intervention, endophthalmitis generally results in partial or complete loss of vision, often within a few days of inoculation. Infectious agents generally gain access to the posterior segment of the eye following one of three routes: (i) as a consequence of intraocular surgery (postoperative), (ii) following a penetrating injury of the globe (posttraumatic), or (iii) from hematogenous spread of bacteria to the eye from a distant anatomical site (endogenous).Although uncommon, endophthalmitis can also result from keratitis, an infection of the cornea which, if left untreated,can result in corneal perforation and intraocular seeding of organisms (Scott et al, 1996).

4.1.1 Postoperative Endophthalmitis

Postoperative endophthalmitis has been reported following nearly every type of ocular surgery. It occurs most frequently following cataract surgery—the most commonly performed type of ocular surgery. The overall incidence of post-cataract surgery endophthalmitis in the United States, using modern techniques of phacoemulsification and intraocular lens implantation,is about 0.1% (Kattan et al 1991, Jett et al 1998, Schmitz et al, 1999). The incidence following other types of

intraocular surgery has been reported to range between 0.05 and about 0.37% (Kattan et al 1991,Jett et al 1998). In general, those procedures with a higher risk for acute postoperative endophthalmitis (secondary intraocular lens implantation and penetrating keratoplasty [corneal transplantation]) are those with a greater potential for wound leaks with subsequent intraocular bacterial contamination. The etiologic agents of acute postoperative endophthalmitis are generally microorganisms of the eyelid margin and tear film. Although preoperative topical antimicrobial agents can decrease colony counts in the tear film, they do not sterilize the area. In one study, culture of aqueous fluid immediately following cataract surgery revealed a 9% culture positive rate (Ferro et al, 1997). Presumably, low inoculum levels and/or low pathogenicity combined with the innate ocular defenses against infection explain the low rate of clinical infection despite the relatively high prevalence of microorganisms in the eye following surgery. In most series from the United States, coagulase-negative staphylococci are responsible for about 70% of post-cataract surgery endophthalmitis, followed by *Staphylococcus aureus*, viridans group streptococci, other gram-positive microorganisms, and gram-negative microorganisms (Speaker et al 1991, Han et al, 1996). Enterococci are notable among the gram-positive microorganisms both for prevalence and severity of disease (Han et al, 1996). Outcomes following appropriate surgical management are related to a number of factors—most importantly to the level of visual function at the time of clinical presentation. In the Endophthalmitis Vitrectomy Study (EVS), a National Institutes of Health-funded multicenter prospective study, eyes presenting with a visual acuity of only light perception achieved a final vision of 20/40 in 33% of cases, even though the most efficacious management protocol was used. If the presenting vision was better than light perception, over 60% of eyes achieved 20/40 or better vision utilizing the same treatment protocol (Han et al, 1996).Postoperative endophthalmitis may also occur weeks to years following surgery. This delayed infection is likely due either to sequesteration of low-virulence organisms introduced at the time of surgery or to delayed inoculation of organisms.

In the former case, *Propionibacterium acnes* is the most common microorganism encountered, and clinically evident low-to moderate-grade inflammation may occur weeks to months after surgery (Aldave et al 1999,Clark et al, 1999, Samson et al, 2000). In cases with delayed inoculation of microorganisms, organisms gain access

to the eye through either wound abnormalities, suture tracks, or filtering blebs. The most common clinical situation involves antecedent glaucoma filtering surgery (Waheed et al 1998, Soltau et al, 2000). In addition to reflecting the colonization of the preocular tear film, delayed infection of this type is associated with a higher prevalence of streptococcal species.

4.1.2 Post traumatic Endophthalmitis

Penetrating ocular injuries are accompanied by infection at a much higher rate than occurs with surgery. In most series of penetrating injury cases, from 3 to 17% of eyes develop microbial endophthalmitis (O'Brien et al 1995 ,Meredith et al 1999, Jonas et al, 2000). The broad prevalence range is due to factors such as frequency within the series of intraocular foreign bodies, distribution of trauma causes and management strategies. Most authorities agree that the three most important risk factors for posttraumatic endophthalmitis are the presence of an intraocular foreign body, delay in closure of the globe, and location and extent of laceration or rupture of the globe. Posttraumatic-endophthalmitis-associated isolates include a greater variety of organisms than those following ocular surgery and include bacteria derived from the environment. *Bacillus cereus* is ranked second behind staphylococci in prevalence and some cases are polymicrobial (Thompson et al, 1993).

4.1.3 Endogenous Endophthalmitis

Endogenous endophthalmitis results from the introduction of organisms into the posterior segment of the eye as a result of hematogenous spread from a remote primary site of infection.Endogenous endophthalmitis is relatively rare, accounting for only 2 to 8% of all endophthalmitis cases (Greenwald et al, 1986).Populations at greatest risk include immunocompromised patients or those on immunosuppressive therapy, patients with prolonged indwelling devices, and intravenous drug abusers (Shamsuddin et al 1996, Cowan et al, 1987). Common causes of endogenous bacterial endophthalmitis include *S. aureus, B. cereus*, and gram-negative organisms, including *Escherichia coli*, *Neisseria meningitidis*, and *Klebsiella* spp.(Tsang et al, 1996). *Bacillus* spp. are a primary bacterial cause of endogenous

endophthalmitis in intravenous drug abusers and are most likely seeded from contaminated injection paraphernalia and drug solutions (Shamsuddin et al 1996,Greenwald et al, 1986). The most common etiological agent of all cases of endogenous endophthalmitis is the opportunistic fungus *C. albicans* (Romero et al, 1999).

4.1.4 Vitrectomy

Although intravitreal antibiotic therapy can provide effective bacterial killing during endophthalmitis, vitrectomy is an appealing adjunct to management. Vitrectomy (surgical cutting and aspiration of vitreous contents and replacement with balanced salt solution) debrides the vitreous cavity of bacteria, inflammatory cells, and other toxic debris; promotes better diffusion of antibiotics; removes inflammatory membranes; permits earlier visualization of the retina; and may speed recovery of vision (Meredith, 1999). Vitrectomy has been shown to improve visual outcome in severe postoperative EVS-eligible cases (EVS 1995).An ongoing debate exists concerning the appropriate timing for vitrectomy in traumatized eyes. However, most reports agree that vitrectomy should be performed without delay in severe cases of endophthalmitis, especially those involving intraocular foreign bodies (Asrar et al, 1999)

4.1.5 Laboratory investigations - Endophthalmitis

Ideally, infective endophthalmitis would be verified by culture in all cases. Currently, confirmation of the diagnosis of bacterial endophthalmitis is dependent on microbiologic isolation of organisms, but many cultures are negative (21%–63% in the published literature, (Stern et al, 1989, Verbraeken et al 1991, Heaven et al 1992, Okhravi et al,1997). Several reasons have been postulated for this, including small sample size, sequestration of bacteria on solid surfaces (e.g., on intraocular lens, lens remnants, and lens capsule), prior use of antibiotics, and the fastidious nature of some of the organisms that cause intraocular infection.(Kalicharan et al, 1992) These observations suggest that with a more sensitive and specific detection

strategy, a microbiologic diagnosis may be obtained in more cases.(Okhravi et al, 2000)

A sensitive and rapid diagnostic test would not only allow confident verification of the diagnosis (non infective inflammation vs. infection) but also allow early commencement of specific and appropriate treatment. Several investigators have reported the use of panbacterial PCR (Therese et al, 1998, Knox et al 1999, Lohman et al, 2000) and panfungal PCR (Anand et al, 2000) in the analysis of ocular samples from clinical cases with suspected intraocular infection. These studies have focused on using nPCR for the detection of bacteria using panbacterial 16S rDNA gene primers. The present study was undertaken to develop a mPCR for rapid and simultaneous detection of eubacterial, *P. acnes* and panfungal genome

4.2 Objective

To develop and apply a novel mPCR targeting eubacterial, *P. acnes* and panfungal genome for rapid and simultaneous aetiological detection of infectious endophthalmitis

4.3 Materials and Methods

4.3.1 Standard strains used:

S. aureus ATCC 12228, *P. acnes* laboratory isolate,*C. albicans* ATCC 24433

4.3.2 Intraocular specimens used in the study:

Thirty intraocular specimens consisting of 19 Aqueous humor (AH) and 11 Vitreous fluid (VF), collected from 25 patients referred to an ophthalmic hospital, during June-August 2004 with clinical diagnosis of infective endophthalmitis, after cataract or lens surgeries were investigated for detection of the causative infectious agent.

4.3.3 Conventional microbiological investigations

The collection and processing of intraocular specimens was carried out as per the method given in Appendix I

4.3.4 PCR assay conditions for detection of eubacterial, *P. acnes* and panfungal genomes

DNA was extracted from the intraocular specimens of AH and VF by Qiagen kit (Qiagen, Germany, catalogue 51304) method according to the manufacturer's instructions. Nested Polymerase Chain Reaction (nPCR) was carried out using eubacterial primers targeting 16SrRNA [Therese et al*,* 1998] and panfungal genome targeting 28SrRNA[Anand *et al,* 2001] as described previously. For a 50 µl reaction, 8 µl of 200 µm dNTPs, 5 µl of 10 x PCR buffer (5 mM MgCl2, 500 mM KCl, 100 mM Tris Cl, (0.01% gelatin), 0.36 micromole of primers for eubacterial genome: U_1 5' ttggagagtttgatcctggctc 3', rU $_4$ 5' ggactaccagggtatctaa 3' (first round) U_2 5' ggcgtgctta acacatgcaagtcg 3', U_3 5' gcg gctggcacgtagttag 3' (second round), 1 micromole of *P. acnes* primers Pa_1 : 5' aaggccctgcttttgtgg 3' rPa $_3$ 5' actcacgcttcgtcacag 3' (first round) and Pa_1 and rPa_2 5' tccatccgcaaccgccgaa 3' for the second round were used. For panfungal genome detection, 10 picomoles of forward primer FU $_1$ 5' tgaaattgttgaaagggaa 3' and reverse primer FU_2 - 5' gactccttggtccgtgtt 3' were used. The primers and PCR reagents were obtained from Bangalore Genei, India .Amplification of the three genomes was carried out in a single tube using 10 µl of template DNA in Perkin Elmer thermal cycler (Model 2700) with the same thermal profile of Therese et al, 1998 for 25 cycles. Nested amplification for detection of eubacterial and *P. acnes* genomes was carried out with the same thermal profile for 10 cycles.

4.3.5 Sensitivity and specificity

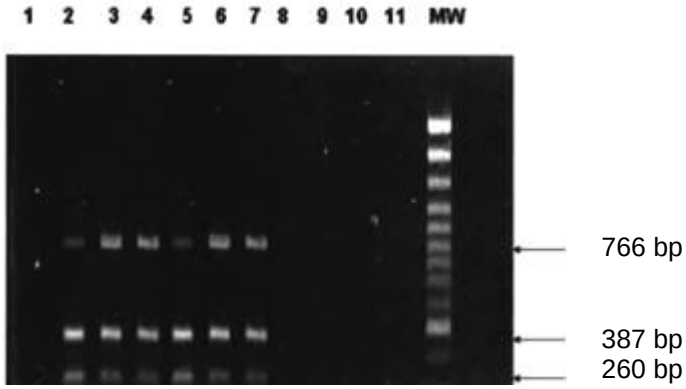
Sensitivity of mPCR was determined using serial ten-fold dilutions of DNA of *S. aureus* (ATCC 12228), laboratory isolate of *P. acnes* and *C. albicans* (ATCC 24433); specificity of mPCR assay was determined using standard ATCC strains of *E. coli* (ATCC 25922), *P. aeruginosa*, (ATCC 7853), *S. aureus* (ATCC 12228), *M. tuberculosis* H37Rv, HSV 1 ATCC 733 VR and human leukocyte DNA.

4.4 Results

4.4.1 Sensitivity and specificity of mPCR

The specificity and sensitivity of both the nPCR tests for detection of eubacterial and *P. acnes* genome were 40 fg and 50 fg as published previously (Therese et al, 1998). The sensitivity of multiplex PCR (mPCR) for detection of eubacterial genome and *P. acnes* genome was 100 fg and for panfungal genome was 0.4 pg. The primers were specific when amplified by multiplex reaction amplifying the respective targets. The results of sensitivity of mPCR after first round of amplification are shown in Figure 4a and after second round of amplification are shown in Figure 4b. The application of eubacterial PCR on ocular specimens is shown in Figure 4c.

Figure 4a: Agarose gel electrophoretogram showing the sensitivity of multiplex PCR using eubacterial, *P. acnes* primers targeting 16SrRNA, and panfungal primers targeting 28SrRNA after first round of amplification



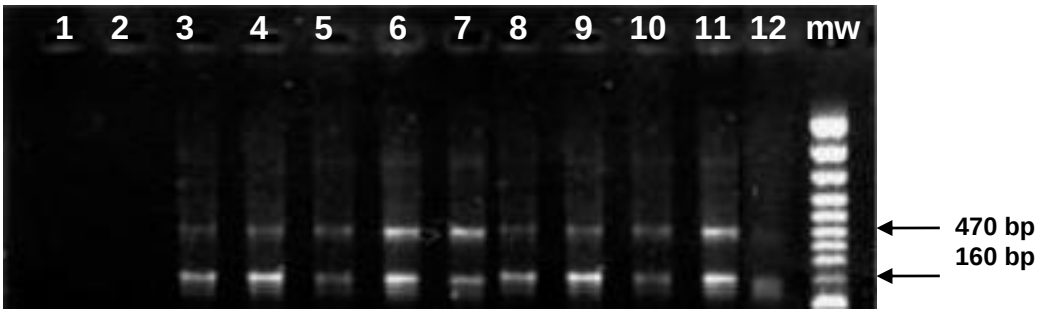
Lane 1: Negative control I round

Lane 2: Positive control : *S. aureus* (ATCC 12228),

laboratory isolate of *P. acnes* and *C. albicans* (ATCC 24433) **Lane 3 – Lane 7** Serial 10 fold dilutions of Positive control DNAs

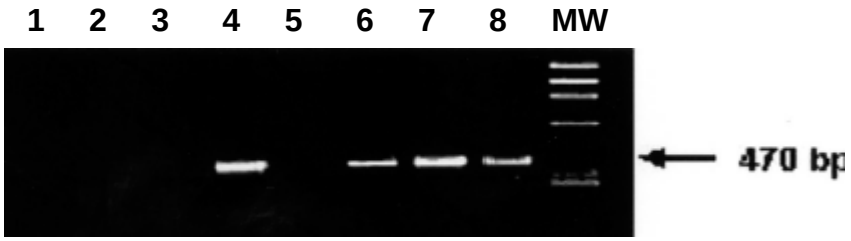
MW : Molecular weight marker 100 bp DNA ladder

Figure 4b: Agarose gel electrophoretogram showing the sensitivity of multiplex PCR using eubacterial, *P. acnes* primers targeting 16SrRNA after second round of amplification.



Lane 1: Negative control II round **Lane 2:** Lane 1: Negative control I round **Lane 3:** Positive control : *S. aureus*, *P. acnes* **Lane 4 – Lane 12:** Serial 10 fold dilutions of Positive control DNA **MW** : Molecular weight marker100 bp DNA ladder , **SENSITIVITY** 100 fg

Figure 4c: Agarose gel electrophoretogram showing the results of nPCR applied on intraocular specimens using eubacterial primers targeting 16SrRNA



Lane 1: Negative control II round **Lane 2:** Negative control I round **Lane 3:** Extraction control **Lane 4:** AH positive **Lane 5:** VF negative **Lane 6 & Lane 7:** VF positive **Lane 8:** Positive Control DNA (*S. aureus* ATCC 12228 strain) **MW :** Molecular weight marker *Hinf* I digest of Phi X 174 DNA

The results of mPCR for detection of eubacterial, *P. acnes* and panfungal genomes and comparison with conventional methods is given in Table 4a. The results of individual eubacterial PCR is shown in Figure 4c and that of *P. acnes* PCR is shown in Figure 4d.

Figure 4d Agarose gel electrophoretogram showing the results of nested PCR applied on intraocular specimens using *P. acnes* primers targeting 16SrRNA

Lane 1: Negative control II round **Lane 2:** Negative control I round

Lane 3: Extraction control **Lane 4,6:** AH positive **Lane 5:** VF negative

Lane 7: Positive Control DNA (*P. acnes*)

MW : Molecular weight marker *Hinf* I digest of Phi X 174 bacteriophage DNA

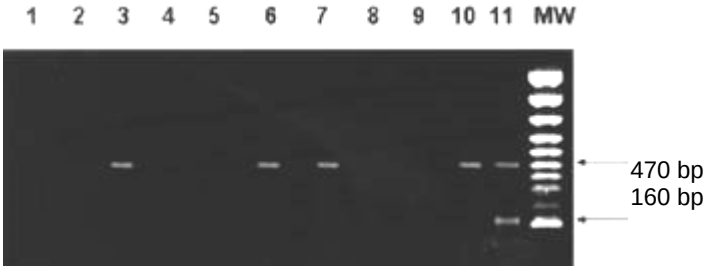
Table 4a: Comparison of results of conventional methods and mPCR applied on intraocular specimens

Intraocular specimens	Clinical diagnosis	Smear	Culture	Results of mPCR		
				Eubacterial	*P. acnes*	Panfungal
AH	P.O.E	GPC	***S. epidermidis***	Pos	Neg	Neg
VF	P.O.E	Neg	**Negative**	Pos	Neg	Neg
AH	P.O.E	GPC	***S. aureus***	Pos	Neg	Neg
VF	P.O.E	GNB	***P. aeruginosa***	Pos	Neg	Neg
AH	Chronic P.O.E	Neg	Neg	Pos	Neg	Neg
VF	P.O.E	Neg	Neg	Pos	Neg	Neg
VF	P.O.E	Neg	Neg	Pos	Neg	Neg
VF	P.O.E	Neg	Neg	Pos	Neg	Neg
AH	P.O.E	Neg	Neg	Pos	Pos	Neg
AH	P.O.E	Neg	Neg	Pos	Pos	Neg
AH	Delayed P.O.E	Neg	Neg	Pos	Pos	Neg
AH	Chronic P.O.E	Neg	Neg	Pos	Pos	Neg
AH	Post traumatic endoph	Neg	Neg	Neg	Neg	Pos
VF	Endog endoph	Neg	Neg	Neg	Neg	Pos

NOTE: AH: Aqueous humor, VF: Vitreous fluid, P.O.E: Post operative endophthalmitis, Post traumatic endoph: Post traumatic endophthalmitis, Endog endoph: endogenous endophthalmitis, GPC: Gram positive cocci, GNB: Gram negative bacilli, Neg: Negative, Pos: Positive

mPCR revealed the presence of eubacterial genome in 12 [(7 AH, 5 VF) 40%],specimens of which eubacterial genome was detected in 9 specimens by PCR alone increasing the clinical sensitivity by 30% as compared to the conventional methods. The results of mPCR applied on intraocular specimens for eubacterial genome detection is shown in Figure 4e.

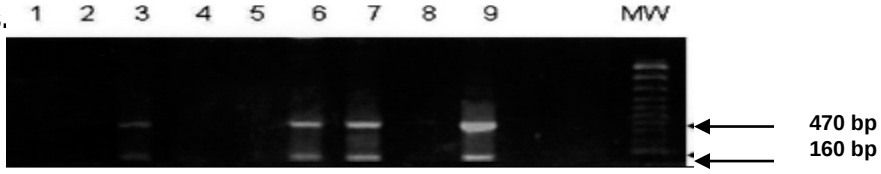
Figure 4e: Agarose gel electrophoretogram showing the results of mPCR applied on intraocular specimens using eubacterial, *P. acnes* and panfungal primers.



Lane 1: Negative control II round **Lane 2:** Negative control I round **Lane 3,7,10:** AH positive **Lane 4,5,8:** VF negative **Lane 6:** VF positive **Lane 7:** AH positive
Lane 9: AH negative **Lane 11:** Positive Control DNAs (*S. aureus, P. acnes*)
MW : Molecular weight marker 100 bp ladder

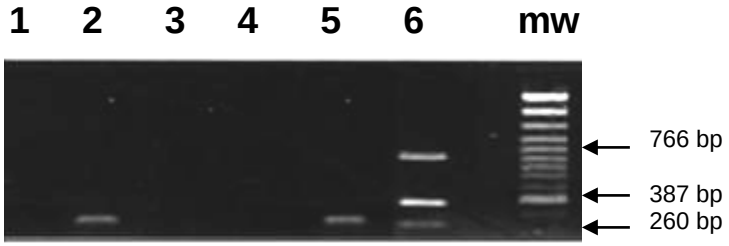
mPCR revealed the presence of *P. acnes* genome in 4 (4 AH,13.3%).The results of mPCR applied on intraocular specimens for *P. acnes* genome detection is shown in Figure 4f and results of mPCR applied on intraocular specimens for panfungal genome detection is shown in figure 4g.

Figure 4f: Agarose gel electrophoretogram showing the results of mPCR applied on intraocular specimens using Eubacterial, *P. acnes* and panfungal primers.



Lane 1: Negative control II round **Lane 2:** Negative control I round **Lane 3:** AH eubacterial positive*, P. acnes* positive **Lane 4:** AH Negative **Lane 5:** VF negative **Lane 6,7**: AH positive for eubacterial and *P. acnes* **Lane 8:** AH Negative **Lane 9:** Positive Control, **MW:** Molecular weight marker 100 bp ladder

Figure 4g: Agarose gel electrophoretogram showing the results of multiplex PCR applied on intraocular specimens using Eubacterial, *P. acnes* and panfungal primers.



Lane 1: Negative control I round **Lane 2**: AH positive **Lane 3:** VF negative **Lane 4:** AH negative **Lane 5:** VF positive **Lane 6:** Positive control (*S. aureus, P. acnes, C. albicans*) **MW:** Molecular weight marker 100 bp ladder

mPCR revealed the presence of panfungal genome in 2 (1 AH, 1 VF 6.3% [Table 4a]). The results of multiplex PCR applied on intraocular specimens for panfungal genome detection is shown in Figure 4g. There was no striking difference in the clinical presentation of cases detected by conventional methods as against those detected only by mPCR

4.5 Discussion

Infective endophthalmitis is a serious ocular infection that can result in blindness. Approximately 70% of cases occur as a direct complication of intraocular surgery.[Endophthalmitis Vitrectomy study group].The diagnosis of infective endophthalmitis is on clinical grounds; but negative cultures are frequently encountered, (21 -63%)[Okhravi *et al*, 2000] resulting in a dilemma. The results of conventional methods of direct smear are available within half an hour and, culture results are available at 48 hours (for bacteria) and at 10 days (for fungus and anaerobic bacteria). In contrast, polymerase chain reaction (PCR) results are available within 8 hours and hence PCR had proved to be a rapid, reliable and sensitive tool in diagnosis of infective endophthalmitis. Multiplex Polymerase Chain Reaction (mPCR) has been widely used for detection and characterization of genes of bacteria [Selvapandian et al*,* 2005, Keya et al, 2001, and viral retinitis.[Priya et al , 2003] It is important to have a rapid and sensitive test that would help resolve the dilemma by detecting the genomes of common infectious agents simultaneously.

In the present study, novel mPCR was developed and applied on to intraocular specimens to detect three infectious genomes. mPCR was evaluated against individual uniplex PCR and results were on par with them. mPCR has several advantages over individual PCR: It is cost-effective as it reduces the total cost by one-third, offers considerable reduction in time required for generating reports and eliminates the need to use separate thermalcyclers for individual uPCR. The consumption of PCR reagents is minimized by carrying out the amplification in a single tube with no additional *Taq* polymerase required to amplify three genomes simultaneously. The annealing temperature for mPCR was determined based on the melting temperature, length (18-24 bases) and GC content of the primers. The annealing temperature of 60^{o}C was optimal for amplifying all three infectious genomes. This novel thermal profile was designed to amplify the three infectious genomes by using 25 cycles for the first round and a reduction of five cycles for the second round without affecting the sensitivity and specificity of the procedure. Moreover, the time period needed for reporting the results was reduced by two hours compared to individual PCRs. By application of mPCR rapid detection was available within five hours of specimen collection as against 8 hours required for each uPCR. Furthermore, the results of mPCR correlated well with intraocular specimens which

were culture positive for bacteria. These findings are comparable to our earlier findings[Therese et al, 1998] and as well as to those published by Hykin et al 1994, and Carroll et al, 1994, Lohmann et al 2000.This novel mPCR was useful in diagnosing the infectious agent in the minimal amount of template DNA available. Moreover, this novel mPCR has several advantages over uPCR such as, detection of two or more targets in a single tube, minimizing the use of PCR reagents, reduction in time and the need for a single PCR machine for amplification. Based on the results of mPCR appropriate therapy was initiated using antibacterial or antifungal drugs as the case may be. To the best of our knowledge, this study is the first of its kind developed for etiological diagnosis of infective endophthalmitis.

4.6 Conclusions

A novel mPCR for simultaneous detection of eubacterial, *Propionibacterium acnes* and panfungal genome was optimized and applied on intraocular specimens. This novel mPCR proved to be a rapid and reliable tool in management of endophthalmitis.

4.7 Future prospects

Further studies are needed to develop this mPCR as nested for detection of panfungal genome. Designing primers targeting inner sets of targeted 28SrRNA region would aid in increasing the sensitivity of panfungal genome detection. Optimization of mPCR targeting 23SrRNA of bacteria, 16SrRNA targeting *P. acnes* and ITS region of fungi can be attempted.sAn other area of research will be to develop a mPCR for simultaneous detection and characterization of genes for drug resistance.

Chapter - 5

Nucleotide Polymorphisms Associated with Internal Transcribed Spacer (ITS) Region of Ocular Isolates of *Aspergillus flavus*

5.1 Review of literature

Strain typing of medically important fungi, i.e., the ability to identify them to the species level and to discriminate among individuals within species, has been galvanized by new methods of tapping the tremendous variation found in fungal DNA. By paying attention to attributes of the life cycle, such as the mode of reproduction and genetic differentiation or isolation, mycologists can tell which intra specific groups are worth identifying and can determine the number and type of markers that are needed to do so. The same evolutionary information can be just as valuable to the selection of fungi for development and testing of pharmaceuticals or vaccines. In the process, we should gain a better basic understanding of the behavior of pathogenic fungi in nature. (Taylor et al, 1999)

5.1.1 Methods of determining strain variations in medically important fungi

The techniques used to study strain variations are multilocus enzyme electrophoresis, electrophoretic karyotype analysis, restriction fragment length polymorphism, randomly amplified polymorphic DNA or arbitrarily primed PCR analysis, Sequence-confirmed amplified region analysis, DNA fingerprinting with repetitive DNA Sequences, Nucleic Acid sequencing of known genes, Amplified Fragment Length Polymorphism Analysis (AFLP).

5.1.2 Multilocus enzyme electrophoresis

Multilocus enzyme electrophoresis (MLEE) hardly needs an introduction. Any protein (locus) that can be selectively stained can be isolated from a collection of individuals and electrophoresed. If the protein bands are variable, they are considered to be alleles on the basis of mobility . The technique is robust and continues to be put to

good use in studies of medically important fungi, e.g., *C. albicans* (Boerlin et al, 1996 and *A. fumigatus* (Rodriguez et al, 1996). An important advantage of this technique is that all alleles are recovered, so that alleles are rarely missing,or null; in other words, the locus is codominant in that both alleles in diploid can be observed. The criticisms of enzyme electrophoresis are that (i) it assays the genotype indirectly, so that much variation at the nucleotide level may go undetected because nucleotide substitutions do not necessarily change the amino acid composition; (ii) changes in amino acid composition do not necessarily change the electrophoretic mobility of the protein and, as a consequence, alleles that are considered to be the same protein alleles from different individuals may represent different gene alleles; and (iii) selection may be acting on the polymorphisms, so that anonymous DNA markers may give a very different picture from allozyme markers, presumably because the former are neutral and the latter are under some sort of selection (Karl et al, 1992, Pogson et al, 1995).

5.1.3 Electrophoretic Karyotype Analysis

Chromosomal size variation is assayed via an electrophoretic technique, electrophoretic karyotype (EK) analysis, which uses electric fields of alternating orientation to move intact chromosomes through the agarose gel matrix. This biologically interesting and complex topic has been reviewed (Zolan et al, 1995). Classic reports for *C. albicans* pointed out the value of this technique (Merz et al 1988), and several other human pathogenic fungi have been studied in this way:
C. immitis (Pan et al, 1992), *H.capsulatum* (Steele et al, 1989), and *C. neoformans* (Wickes et al, 1994). It may be argued that karyotypes should be less variable for sexual species than for asexual ones, due to the need for pairing at meiosis, but fungal EK are known to display some variation due to loss of dispensable chromosomes and to vary in size and gene arrangement, even in sexual species.

5.1.4 Restriction Fragment Length Polymorphism Analysis

Restriction fragment length polymorphism analysis (RFLP) assays the DNA sequence variation of the genome by using restriction endonucleases to sample

short pieces of DNA sequence. Restriction endonucleases recognize specific DNA sequences, usually 4 to 6 nucleotides in length, and cut the DNA in or near the recognition sequence. Alteration of the recognition sequence by nucleotide substitution, insertion or deletion (length mutation), or, for some restriction enzymes, methylation of nucleotides (provided that the DNA is genomic and not amplified or cloned) can prevent the restriction endonuclease from acting and change the fragment pattern .Length mutations in the region between restriction sites also can change the pattern. Any region of DNA (locus) can be used for RFLP analysis if variation (alleles) can be visualized directly due to multiple copies (mitochondrial DNA [mtDNA],rDNA) or if variation can be visualized indirectly either by hybridization to probe DNA or amplification by PCR. If only one variable restriction endonuclease site is present in the DNA fragment, allele scoring is straightforward. When several variable sites are present, mapping the sites will improve the interpretation. RFLPs were the first DNA markers used for fungal evolutionary biology, and they continue to be put to good use in population genetic studies, e.g., for *A. nidulans* (Geisser, 1996). There are many ways in which a restriction endonuclease site can be lost: any of the several nucleotides in the recognition sequence can be substituted or the site can suffer length mutations. Missing sites that have arisen, unknowingly, by different routes confound evolutionary analysis.

5.1.5 Randomly Amplified Polymorphic DNA or Arbitrarily Primed PCR

Randomly amplified polymorphic DNA (RAPD) analysis or arbitrarily primed PCR (AP-PCR) analysis is similar to RFLP analysis in that it assays DNA sequence variation in short regions, but instead of analyzing restriction endonuclease recognition sequences, it focuses on PCR priming regions (Williams et al, 1990). Nucleotide substitutions in the PCR priming regions, particularly the 3’ ends, can prevent primer annealing and PCR amplification. RAPD analysis uses one short PCR primer (10 bp) and a low annealing temperature to generate several fragments in one amplification. If a comparison of amplifications of several isolates shows a band (locus) that varies, alleles are assigned to the presence [1] and absence [0] of

the band. RAPD analysis is technically simple and often detects variation among isolates that are invariant with RFLP analysis. (King et al, 1995, Loudon et al,1993).

Teresa et al 2000, have reported the simultaneous isolation of one *Aspergillus flavus* strain from the aortic prosthesis of a heart surgery patient and another two isolates recovered from a dual-reservoir cooler-heater used in the operating room where this patient was operated on. Genetic typing of these three isolates by RAPD revealed identical genotypes. Eight unrelated control strains of *A. flavus* had eight different genotypes. These results clearly indicated the nosocomial origin of the *A. flavus* strain isolated from the patient. RAPD technique is a rapid and reliable tool to ascertain the epidemiology of infections caused by *A. flavus*.

5.1.6 Sequence-Confirmed Amplified Region Analysis

Starting with variable loci found with RFLP or RAPD analysis, sequence-confirmed amplified region (SCAR) analysis uses DNA sequencing to recover both positive alleles (band present) and null alleles (band absent) and to ensure that alleles from different individuals are homologous. If the RFLP loci are based on DNA hybridization and the variable restriction endonuclease site lies in the probe sequence, it is simple to sequence the probe, design PCR primers to amplify the probe region from every isolate, and use the restriction endonuclease to distinguish the alleles. If, however, the variable restriction endonuclease site lies adjacent to the probe sequence, where it cannot be easily sequenced, or if the variable DNA fragment is a RAPD fragment, where the modified priming site cannot be sequenced, developing the SCAR is more difficult. A robust strategy for finding SCARs involves using RFLPs or RAPDs to generate patterns of bands for a test group of 6 to 10 isolates and then searching for sequence variation among the bands of identical mobility. Sequencing with arbitrary primer pairs (SWAPP) [Burt , et al, 1994]) is a refinement of this method which uses two different, ca. 20-bp RAPD primers and low-stringency PCR annealing temperatures to generate PCR bands from every isolate. These bands, which show no variation in agarose gel electrophoresis, are heat denatured, snap cooled to promote single-strand folding, and then electrophoresed on acrylamide under nondenaturing conditions to see if the folded single strands now show variation. If this single-strand conformational polymorphism

(SSCP) electrophoresis shows variation conformation changes caused by nucleotide substitutions (Orita et al, 1989).The variable bands are retrieved from the gel and sequenced. It is still possible that the positive alleles are independently derived, but this is not likely unless the locus is hypervariable . This approach has been successfully applied to studies of *Coccidioides immitis* (Burt et al, 1994), *C. albicans* (Geiser et al, 1996) and *H. capsulatum* (Carter et al, 1997).

5.1.7 DNA Fingerprinting with Repetitive DNA Sequences

The techniques mentioned so far are used to describe polymorphic loci one at a time, but both the RFLP and PCR approaches can be used to sample many loci at once if the loci involve repeated DNA sequences. With RFLP fingerprinting, the probe is simply a moderately repeated DNA sequence (10 to 20 copies per genome) which hybridizes to restriction fragments containing the repeated DNA. With PCR fingerprinting, the primer recognizes a repeated DNA sequence, also producing a large number of fragments with each reaction. Due to the large number of fragments and the mutability of repeated DNA sequences, both the RFLP and PCR fingerprints are quite variable and provide superior discrimination among individual fungal isolates. In comparisons of different strain-typing methods, the fingerprinting methods are usually the most discriminating (Diaz -Guerra et al,1997). It is tempting to treat many variable fingerprint fragments as an instant multilocus genotype by assuming that each fragment is an independent locus. Unfortunately, the problems of homology of positive alleles and ambiguity about null alleles are magnified with the complex patterns, and the fragments are not necessarily independent. As a result, fingerprints are useful for identifying individual fungi but problematic for estimating genetic or phylogenetic relationships among individuals.

5.1.8 Strain variations encountered in *Aspergillus* species

Aspergillus species are an increasingly important cause of invasive fungal infections in immunocompromised patients (McNeil et al, 2001). Unfortunately, there are few specific clinical signs of invasive aspergillosis and current methods for laboratory diagnosis are less than ideal, particularly in the early stages of the disease (Stevens et al,2002). Given the recent reports of reduced antifungal drug susceptibilities

among some *Aspergillu*s species (Sutton et al,1999), the timely and accurate identification of aspergilli to the species level has become especially important (Ellis et al, 2002). Species identification is also important for epidemiological purposes and as a guide to clinical management (Lutsar et al, 2004). The current laboratory identification of *Aspergillus* species is based on macroscopic colonial and microscopic morphological characteristics (Klich et al 2002, Sigler et al 2003). Over 180 different species in at least 16 subgeneric groups or sections can be distinguished (Peterson et al, 2000, Pitt et al, 2000), including approximately 30 species which are recognized as opportunistic pathogens of humans (De Hoog et al, 2000).

Many clinical laboratories use traditional phenotypic methods of identification and can differentiate only the more common *Aspergillus species*; the delineation of less common species must be referred to specialist laboratories. In addition, species identification by traditional phenotypic methods may require several weeks to accomplish, and in a small number of cases, isolates may not conidiate, obstructing species identification (Klich et al,2002). Therefore, rapid molecular approaches are now being developed to replace the need for culture by detecting and identifying *Aspergillus* species DNA directly from clinical materials.A number of targets for the molecular identification of aspergilli have been investigated including the mitochondrial cytochrome b gene (Spiess 2003), a putative aflatoxin pathway regulatory gene (aflR) (Chang, 1995), the DNA topoisomerase II gene (TOP2) (Kanbe 2002), the 5'tubulin gene (Giese et al, 1999), and various rRNA gene regions. The most promising targets to date have been the 5' end of the large-subunit rRNA gene (D1-D2 region) (Iwen et al, 2001) and the internal transcribed spacer 1 and 2 (ITS1 and ITS2) regions between the small- and large-subunit rRNA genes (White, 1990). The use of DNA sequence diversity in the ribosomal regions as an aid to species identification has been exploited using PCR amplification of targets followed by either fragment length analysis (Turenne et al, 1999), DNA probe hybridization (Willinger et al, 2003), or DNA sequence analysis

In general, DNA-based approaches were found to provide more reliable and faster species identifications than culture based methods. Nonetheless, some diagnostic problems were encountered, resulting from similar amplicon or fragment lengths for

different taxa, unexpected cross-hybridization results and ambiguous sequencing-based identifications because of significant intraspecies heterogeneity or virtually identical sequences for apparently distinct organisms The validation of test specificity was further compromised by the absence of a comprehensive test strain panel; most studies did not include type strains or authenticated culture collection reference strains of the target species or of closely related species in their examinations. Finally, although the D1-D2 and the ITS1 and ITS2 regions have been analyzed separately for some species of *Aspergillus*, a systematic evaluation and comparison of sequences from all three ribosomal regions for their usefulness in the identification and differentiation of the most medically important *Aspergillus* species has not been published to date. Healy et al, 2004 have assessed the utility of the ribosomal D1-D2, ITS1, and ITS2 regions as targets for the molecular identification of 13 potentially invasive *Aspergillus* species (*A. candidus, A.chevalieri, A. flavipes, A.flavus, A.fumigatus, A.granulosus, A.nidulans, A.niger, A. restrictus, A. sydowii, A. terreus, A. ustus, and A. versicolor*). DNA sequence information concerning all three ribosomal regions for each of the above species have used this information to conduct DNA sequence alignments, pair wise nucleotide sequence analyses,comparative GenBank database searches, and an examination for sequence length polymorphisms among *Aspergillus* species. A commercially available repetitive-sequence-based PCR (rep-PCR) DNA fingerprinting assay adapted to an automated format, the DiversiLab system, enables rapid microbial identification and strain typing.This system may be useful for epidemiological studies, molecular typing, and surveillance of *Aspergillus* species.(Healy et al, 2004).

The present study was undertaken to determine the sequence similarities in ITS region of *A. flavus* isolates

5.2 Objective:

To determine sequence similarity in ITS region of *A. flavus* ocular isolates by PCR based DNA sequencing

5.3 Materials and Methods:

Seven ocular isolates of *A. flavus* from aqueous humor {AH (2)}, vitreous fluid {VF (2)}, corneal scraping (1), Eviscerated material (1), corneal button (1) were isolated from 4 patients clinically suspected to have fungal endophthalmitis.

5.3.1 Molecular microbiological investigations:

DNA extraction from isolates

DNA extraction from clinical specimens and fungal isolates was done using Qiamp kit (Qiagen, Germany) according to the manufacturer's instructions.

5.3.2 PCR targeting ITS region

PCR targeting ITS region was carried out on culture isolates of *Aspergillus flavus* according to the method of Ferrer et al, 2001 and the protocol is given in Appendix II

5.3.3 PCR – RFLP

PCR-RFLP was carried out on ITS amplicons (first round products) using restriction enzyme *Hae* - III.For a 25 µl reaction, 2.5 µl of Buffer C, 0.5 µl of Hae – III enzyme and 10 µl of amplified product were added and incubated at 37° C for 3 hours. The digested products were loaded on 4 % agarose gel incorporated with ethidium bromide and the electrophoresis was carried out at 100 volts for 45 minutes. The digested pattern was visualized and documented using gel documentation system (Vilber Lourmat).

5.3.4 DNA Sequencing of ITS amplicons

DNA Sequencing of ITS amplicons was carried out commercially after purification of amplified products in Sunmar Specialities, Bangalore Genei, India.

5.4 Results:

5.4.1 PCR based Restriction Fragment Length Polymorphism (PCR – RFLP)

The results of PCR-RFLP using *Hae* – III applied on ITS amplicons is shown in Figure 5a

Figure 5a: Agarose gel electrophoretgram (4%) showing the results of PCR based RFLP using *Hae* –III on ITS amplicons of *A. flavus* isolates

Lane 1: Undigested product of Aspergillus flavus (595 bp) **Lane 2**: M 278 / 05 (AH) Digested product of Aspergillus flavus (325,182,88 bp) **Lane 3:** M 278 / 05 (VF) **Lane 4:** M 476 / 05 (Corneal button) **Lane 5:** M 549 /05 (Corneal scraping) **Lane 6:** M 573 /05 (Eviscerated material) **Lane 7:** M 1184 / 05 (AH) **Lane 8:** M 426 / 05 (VF) **MW :** Molecular weight marker Phi X bacteriophage DNA *Hinf* –I digest Note: All isolates showed a similar pattern of digestion with *Ha*e –III

The results of DNA sequencing is shown in Figure 5b and the comparison of nucleotide differences is shown in Table 5a The DNA sequence analysis revealed sequence similarity in 3 *A. flavus* isolates obtained from the same patient (Table 5a) .

Figure 5b : DNA sequencing of ITS amplicons of *A. flavus* isolated from vitreous fluid

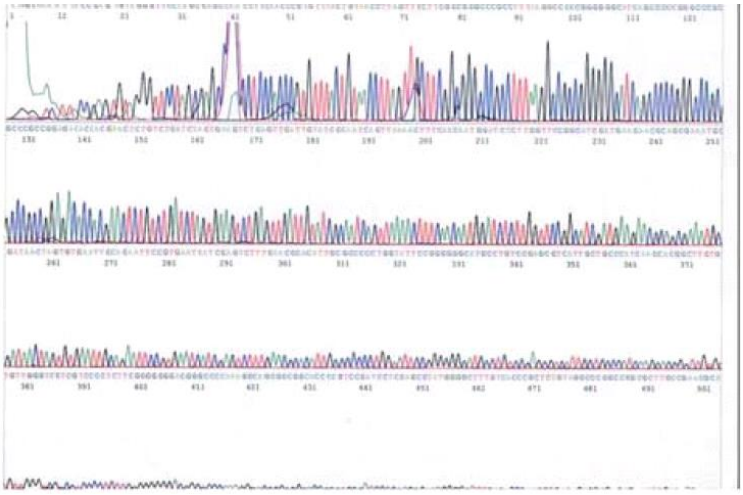


Table 5a : Nucleotide variations in the ITS region of *A. flavus* isolates

Ocular isolates of *A. flavus*	***A. flavus* ATCC 16883 *GenBank Acccession No.* *AB008415*** [#]	**Nucleotide variations in *A. flavus* isolates**
M 278/05 AH*	82 -	A Insertion
	108 A	T Inversion
	111 C	A Substitution
	113 T	G Substitution
	115 G	C Inversion
M 279/05 VF*	117 C	G Inversion
	128,129 TC	- Deletion
M 476/05 CB*	136 -	CC Insertion
	521 T	A Inversion
	525 -	A Insertion
	527 -	C Insertion
	530 -	A Insertion
		C Insertion
M 549/05 Corneal scraping	87 A	G Substitution
	113 T	G Substitution
	118 C	- Deletion
	142 C	G Inversion
M 426 /05 VF	106 C	T Substitution
	122 G	- Deletion
	131 G	C Inversion
	193 T	- Deletion
M 573 /05 Eviscerated material	114 G	C Inversion
	268 A	T Inversion
	361 -372 CGAGCGTCATTGCT	- Deletion
		G Substitution
	383 – 386 A	
M 1184/05 AH	80 T	A Inversion
	106 C	- Deletion
	109 T	A Inversion
	111 A	C Substitution
	242 C	- Deletion

Note: AH Aqueous humor, VF : Vitreous fluid, CB: Corneal button

* The pattern of nucleotide variations observed were the same in 3 *A. flavus* obtained from the same patient

\# The nucleotide positions are denoted according to the position in the Genbank Accession Number AB008415

Accession numbers for the seven isolates: DQ683118, DQ683119, DQ683120, DQ683121, DQ683122, DQ683123, DQ683124

The other isolates exhibited distinct variations in the ITS amplicons. The nucleotide variations in the present study, involved single as well as multiple nucleotide polymorphisms including substitution, insertion, inversion and deletion. *Aspergillus flavus* isolates from aqueous aspirate, vitreous fluid and corneal button obtained from the same patient showed single nucleotide polymorphisms ,inversion in 108,115,117,521 position, insertion in 82,136,525,530,deletion in 128,129 and substitution in 111,113 positions.. The DNA sequencing analysis of other isolates of *Aspergillus flavus* revealed multiple and single nucleotide substitution, multiple deletion and single base insertion and inversion (Table 5a).

5.5 Discussion

Aspergillus species are ubiquitous, commonly occurring in soil, water and decaying vegetation (Teresa et al.,2000). Members of the genus *Aspergillus* are opportunistic pathogens and most infections occur in immuno- compromised patients (Buffington et al,1994). Target for the genus level detection of *Aspergillus* species have included the 18S rRNA gene (Jagger et al.,2000) mitochondrial DNA (Bretagne et al., 1995) , the intergenic spacer region , the Internal Transcribed Spacer (ITS) region (Einsle et al.,1997) and 28 S rRNA region (Anand et al.,2001). The ITS region is located between the 18S and 28S rRNA genes and offer distinct advantages over other molecular targets including increase sensitivity of detection due to the existence of 100 copies per genome. The sequence variation of ITS region has lead to their use in phylogenetic studies of different fungal organisms (Yamakami et al, 1996, Guarro et al, 1999a, White et al,1990).Determining genetic sequence variation at the molecular level is an alternative to culturing *A. flavus*.

Molecular approaches are now being developed to provide a more rapid and reliable identification of fungi compared to traditional phenotypic methods. Ribosomal targets, especially the large-subunit RNA gene (D1-D2region) and internal transcribed spacers 1 and 2 (ITS1 and ITS2 regions), have shown particular promise for the molecular identification of some fungi. An assessment of these regions for the identification of 13 medically important *Aspergillus* species: *A. candidus*, *A. chevalieri*, *A. flavipe*s, *A. flavus*, *A. fumigatus, A.granulosus, A.nidulans, A. niger, A.restrictus*, *A. sydowii*, *A. terreus*, *A. ustus*, and *A. versicolor*. The length of

ribosomal regions could not be reliably used to differentiate among all *Aspergillus* species examined. DNA alignment and pairwise nucleotide comparisons demonstrated 91.9 to 99.6% interspecies sequence identities in the D1-D2 region, 57.4 to 98.1% in the ITS1 region, and 75.6to 98.3% in the ITS2 region. Comparative analysis using GenBank reference data showed that 10 of the 13species examined exhibited a <1-nucleotide divergence in the D1-D2 region from closely related but different species. In contrast, only 5 of the species examined exhibited a <1-nucleotide divergence from sibling species in their ITS1 or ITS2 sequences. Although the GenBank database currently lacks ITS sequence entries for some species, and major improvement in the quality and accuracy of GenBank entries is needed, current identification of medically important Aspergillus species using GenBank reference data seems more reliable using ITS query sequences than D1-D2 sequences, especially for the identification of closely related species.The ribosomal genes demonstrate conserved sequence regions ideal for primer targeting as well as regions of variability useful for species identification. Various molecular techniques like DNA finger printing, RFLP analysis with repetitive probes, polymorphic microsatellite marker analysis and microsatellite length polymorphism have been used to assess the strain variations within *A. flavus (*Healy et al., 2004).

In this report, DNA sequencing technique has been standardized to analyse the genotypic differences existing among *Aspergillus flavus* 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 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 has been investigated including the mitochondrial cytochrome b gene, DNA topoisomerase gene, betaglobulin gene and various ribosomal RNA genes. The most promosing target to date have 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. The ITS region contains hypervariable regions leading to most of the intraspecies sequence diversity (Hinukson et al, 2005).The ITS region have been used as targets for phylogenetic analysis because they generally display 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%.Henry et al., 2000 have reported a variation of 1% in epidemiological analysis of *A.flavus* strains.Alignment of contiguous fungal sequences 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 data base 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 to this in our study, *A. flavus* isolates revealed a variation of 2.3% as compared with the standard strain of *A. flavus* .The sequences have been deposited in GenBank and the accession No. DQ683118, DQ683119, DQ683120, DQ683121, DQ683122, DQ683123, DQ683124 have been 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 polymorphism. 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. Accurate sequence database would not only benefit clinical diagnostic applications but also facilitate the ongoing effort to improve the current taxonomy of *A.flavus* which still lack complete definition by molecular criteria. Upon major improvement of present sequence databases identification to the species level should be feasible through ITS sequence analysis. Such improved databases, should enable a more accurate identity of *A. flavus*, thus promoting further advances

in the molecular diagnosis, epidemiology and clinical management of ocular Aspergillosis (Iwen et al, 2001).The association of this particular pattern of nucleotide polymorphism contributing to the pathogenecity 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* strain in the present study, seems to be novel and the first to be reported in literature to the best of our knowledge.

5.6 Conclusions

The application of PCR based DNA sequencing on ITS region of *A. flavus* isolates revealed novel nucleotide polymorphisms. The association of these polymorphic changes in ocular isolates of *A. flavus* has any role to play in the pathogenic mechanisms needs to be investigated further.

5.7 Future prospects

Strain typing of *A. flavus* could be a prime beneficiary, because every region of the genome (exons, introns, intergenic regions, selfish elements, microsatellites, promoters, enhancers, and categories to be discovered) can be characterized and compared to find regions with the right amount of variation to distinguish evolutionarily meaningful groups. In the process, genome projects would gain information on the diversity in genomic regions, something that is absolutely lacking in the genomic sequence of a single isolate.

CHAPTER - 6

Optimization of PCR Based DNA Sequencing on Internal Transcribed Spacer (ITS) Region for Species Level Identification of Non Sporulating Moulds (NSM)

6.1 Review of literature

Diagnosis of fungal infections usually depends on recovery of fungi from culture of clinical specimens and their identification requires the presence of reproductive structures. Often, fungi cannot be characterized fully because the mold does not sporulate, making identification by microscopic morphology not possible, and potentially increasing time to report an inconclusive result to 21days. While many laboratorians and clinicians assume these fungal isolates are environmental organisms and not clinically significant, to our knowledge no study has systematically attempted to classify these previously unidentifiable fungi in a clinical microbiology laboratory. Phenotypic variants of fungi may not be identifiable or may be misidentified. Typically in the clinical laboratory, isolates that cannot be identified by reproductive structures are described as Mycelia sterilia, a name indicating a filamentous fungus that displays no distinguishing phenotypes recognized by routine clinical laboratory analysis. Phenotypic methods can, in some cases, take weeks, a time frame that is not clinically useful. Rapid identification of molds causing ocular disease could facilitate the timely administration of effective therapy. For example, *A. flavus* and *A. terreus* have been reported to be resistant to amphotericin B (Denning et al, 1997, Seo et al, 1999), and *Aspergillus fumigatus* can become resistant to itraconazole (Sutton et al, 1999).

The identification of filamentous fungi in the clinical laboratory can be challenging; success in this endeavor depends on the organism and the experience of the microbiologist or technologist. The last 20 years or so has seen a growing number of fungal infections coincident with a dramatic increase in the population of severely immunocompromised patients. Another difficulty for microbiologists inexperienced 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. This implies that different concepts have to be applied in fungal taxonomy. Generally, medical mycologists are familiar with only one aspect of pathogenic fungi, i.e., the stage that develops by asexual reproduction. Usually microbiologists ignore or have sparse information about the sexual stages of these organisms. However, the sexual stages are precisely the baseline of fungal taxonomy and nomenclature. It seems evident that in the near future, modern molecular techniques will allow most of the pathogenic and opportunistic fungi to be connected to their corresponding sexual stages and integrated into a more natural taxonomic scheme. (Guaro et al, 1999 b) reproduction within species and the presence of barriers that prevent the cross-breeding of species (Davis et al, 1995). However, application of the biological-species concept to fungi is complicated by the difficulties in mating and in assessing its outcome

Species identification in mycology is based on two main approaches. The character-based concept, or diagnostic approach, defines a species as a group of organisms that have common observable attributes or combinations of attributes. In contrast, the history based concept insists that organisms must be historically related before they can be considered members of any given species (Baum et al,1995). Molecular methods for identification of pathogenic fungi have been validated for use in clinical settings (Einsele et al, 1995,Chen et al, 2000, Fujita et al, 2000, 3, Henry et al, 2000, Chen et al, 2001, Sharmin et al 2002, Ninet et al 2003, Hall et al, 2003, Hall et al, 2004). rRNA genes, including the 28S gene (26S gene in all yeasts), are conserved and possess single nucleotide changes at a relatively low rate, and provide useful phylogenetic information (White et al, 1990). ITS sequences may provide accurate identification of closely related isolates and species which cannot readily be distinguished using 26S or 28S rRNA gene sequences. Henry et al., 2000 demonstrated sufficient sequence diversity at ITS1 and ITS2 to distinguish six medically important *Aspergillus* species from one another. Turenne et al. 1999, and De Baere et al, 2002 used capillary electrophoresis to examine the lengths of ITS2 PCR products for identifying fungi. This method is rapid but does not provide species-specific identification for all organisms tested, demonstrating a need for additional analysis.

The integration of molecular analyses with traditional phenotypic methods of fungal identification can significantly increase the specificity and decrease the turn around time for the identification of clinically important molds. Production of reproductive structures or expression of specific biochemical phenotypes by fungi grown in culture is required for phenotypic analyses. Previously uncharacterized molds are difficult to identify by such methods, yet accurate taxonomic placement of the uncharacterized molds, and hence their identity, can be inferred from their phylogenetic relationships with well-characterized mold isolates.

Molecular analyses of multiple DNA ITS1, ITS2, and 28S DNA sequences of the isolate can identify *C. cladosporioides* to the species and sequevar levels. This analysis can be completed in 24 h once the organism is isolated from a patient specimen. Multilocus analysis allows the separation of possible new species that are not differentiated by standard clinical laboratory phenotypic analyses or single-locus analyses alone.

Rakeman et al, 2005 has developed a rapid method to identify a filamentous fungi cultured on agar medium within 24 h. Isolates were analyzed genotypically if they did not produce reproductive structures within 10 days of isolation or if structures were produced but if phenotypic evaluation did not result in definite identification. Fifty-seven of the 89 isolates produced only sterile hyphae after 10 days and, without DNA sequence analyses, would have been reported as M. sterilia or, if possible, more specifically identified after further incubation. Of these 57, 16 were identified to the species level, including three atypical *A. fumigatus* isolates, and 16 were identified to the genus level. The remaining 25 isolates were assigned a taxonomic orientation based on the relationship of their DNA sequences to those of related organisms in the database. Thus, DNA sequence-based methods can identify isolates that otherwise defy identification by traditional phenotypic analyses.

The integration of multilocus sequence analyses with phenotype- based identification algorithms in a clinical laboratory provides a rapid and definite identification that, in some cases, surpasses the specificity of identification by 28S sequence analyses and phenotypic methods alone. the phenotypically validated ITS DNA sequence database will be useful for identification of routinely isolated molds, previously

unidentified molds, and molds that do not show specific or expected morphological and biochemical phenotypes.

6.1.1 Mycelia Sterilia

Non-sporulating moulds results from unfavorable 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, ascocarps 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 are 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.

The present study was undertaken to identify the non sporulating fungi by PCR based DNA sequencing on ITS region.

6.2 Objective

To identify the non sporulating fungi by PCR based DNA sequencing on ITS region

6.3 Materials and Methods:

6.3.1 Fungal isolates

A total of 50 filamentous fungal isolates from 31 corneal scrapings, 11 corneal buttons, 2 eviscerated material, 2 donor corneal rims, 1 conjunctival scraping, 1 each of vitreous aspirate, necrotic material collected from cornea and infected suture were included in the study.

6.3.2 DNA extraction

After growth of seven days on Sabouraud's dextrose agar, lysates for DNA extraction were prepared from approximately 1 cm^2 mycelia . Briefly, in a biological safety cabinet, mycelia were collected by scraping the slant with a sterile stick in 1 ml of sterile, milli Q water. The material was transferred to a pre-sterilized eppendorf vial and the tubes were centrifuged for 5 minutes at 3000 rpm. The material was resuspended in 200 µL of lysis buffer containing 200mM TRIS, 50mM EDTA,10% SDS, 1M sodium chloride and incubated at 56 ° C for 60 minutes and 150 µL of sodium acetate was added and stored at -20ºC for 20 minutes. This was followed by centrifugation at 14,000 rpm for 10 minutes and the supernatant was transferred into an other tube to which equal volume of isopropanol was added and kept at room temperature for 5 minutes. The samples were centrifuged at 14,000 rpm for 10 minutes and the deposit was washed with 70% ethanol thrice and finally resuspended in 50 µL of TE buffer.

6.3.3 Amplification using ITS primers

PCR targeting the ITS region of fungi was carried out according to the method of Ferrer et al 2001 as given in Appendix II

6.3.4 Sequencing in *ABI Prism 310/3100 AVANT* genetic analyzer

The sequencing of ITS amplicons of NSM were carried out as per the method given in Appendix II

6.4 Results

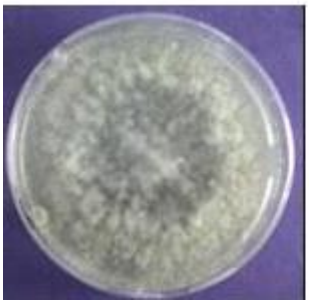
A representative subset of 50 isolates reported as NSM was randomly selected for gene sequencing. PCR based DNA sequencing identified 11 (22%) to genus, 39 (78%) to species. The isolates were identified by sequencing the ITS 1 region identifying 15 different genera and these genera were confirmed by 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

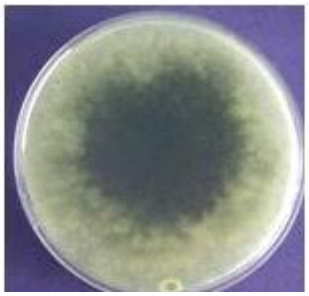
species level and 10 isolates had sequences that shared 95-98% identity to genus level .Of the 50 fungal isolates sequenced 27 were found to be emerging pathogens involving seven genera (*Botryosphaeria species,Lasiodiplodia* species, *Thielavia tortuosa*, *Glomerulla singulata*, *Macrophomina phaseolina*, *Rhizoctina bataticola*, *Podospora* species and 23 as established pathogens involving 8 genera (*Aspergillus*, *Fusarium*, *Bipolaris*, *Pythium*, *Cochilobolus*, *Exserohilum*, *Pseudoallescheria* and *Scedosporium* species) and 12 were found to be, emerging pathogens (plant/soil) involving 5 different genera The identification of NSM based on PCR based DNA sequencing on ITS region is shown in Table 6a. The colony morphology and LPCB mounts of NSM are given in Figure 6a

Figure 6a: Colony morphology and LPCB mounts of non sporulating moulds

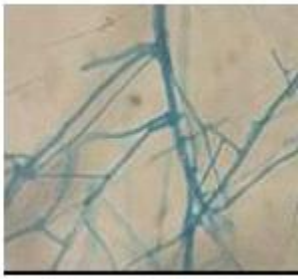
M 5846/06 Corneal scraping : *Botryosphaeria rhodina*



Colony morphology on Sabouraud's dextrose agar Front view



Colony morphology on Sabouraud's dextrose agar Reverse view

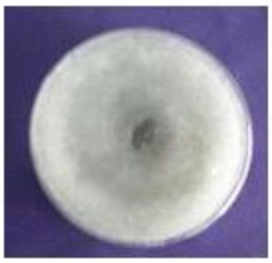


Lactophenol Cotton blue mount

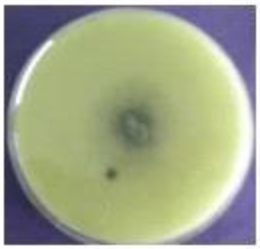
Colony morphology on Sabouraud's dextrose agar Front view
Colony morphology on Sabouraud's dextrose agar Reverse view
Lactophenol Cotton blue mount 40X

Colony morphology on Sabouraud's dextrose agar showing white fluffy growth with greenish black pigmentation on the reverse. LPCB mount (40x) showing the presence of septate hyphae with no spores.

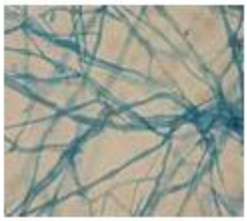
M 3050/01 Corneal scraping : *Pythium insidiosum*



Colony morphology on Sabouraud's dextrose agar Front view



Colony morphology on Sabouraud's dextrose agar Reverse view



Lactophenol Cotton blue mount 40X

Colony morphology on Sabouraud's dextrose agar showing white fluffy growth with greenish black pigmentation on the reverse. LPCB mount (40x) showing the presence of septate hyphae with no spores.

Table 6a: Results of Identification of NSM by PCR based DNA sequencing on Internal Transcribed Spacer (ITS) region

Identification		Ocular specimens
Emerging pathogens 27		
***Botryosphaeria* species**	**10**	
Botryosphaeria rhodina	8	Corneal scraping 4*, Conjunctival scraping 1*, Necrotic tissue 1*, Corneal button 2*
Botryosphaeria dothidea	1	Corneal button 1
Botryosphaeria species	1	Corneal scraping 1
Lasiodiplodia theobromae	**2**	Corneal scraping 1,Eviscerated material 1
Rhizoctonia bataticola	**5**	Corneal scraping 4, Eviscerated material 1
Glomerulla singulata	**3**	Corneal scraping 1, Corneal button 1 Eviscerated material 1
Cochilobolus species	**3**	
Cochilobolus species	2	Corneal scraping 2
Cochilobolus heterophrius	1	Corneal button 1
Macrophomina phaseolina	**2**	Corneal scraping 2
Podospora species	**1**	Corneal scraping 1
Thielavia tortuosa	**1**	Corneal button 1

CONTD…….

Identification		Ocular specimens
Established pathogens		
Pythium insidiosum	**9**	Corneal scraping 6, Corneal button 2, Vitreous aspirate 1
Fusarium species	**7**	
Fusarium solani	2	Corneal scraping 2
F. solani 1, *F. falciforme* 2[#]	3	Corneal scraping 3
Fusarium proliferatum	2	Corneal scraping 1, Corneal button 1
***Exserohilum* species**	**1**	Corneal scraping 1
Aspergillus terreus	**1**	Corneal scraping 1
Aspergillus fumigatus	**1**	Corneal button 1
Scedosporium species	**1**	Infected suture 1
Pseudoallescheria species	**1**	Corneal button 1
Bipolaris species	**2**	Corneal scraping 1 Donor corneal rim 1

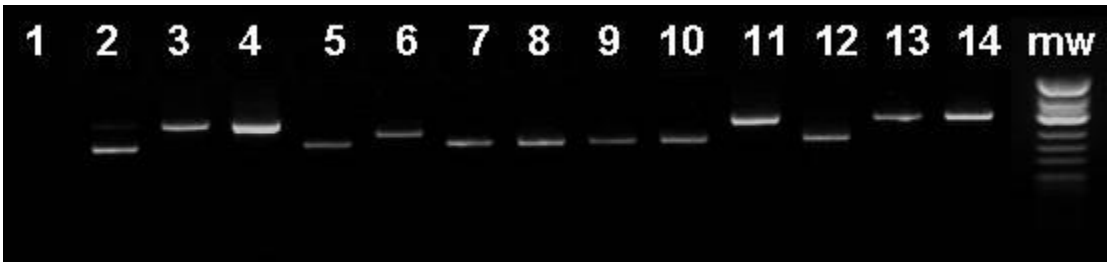
Note:

- * The 5 isolates of *Botryosphaeria rhodina* were obtained from the same patient (Corneal scraping 2, conjunctival scraping, corneal button, necrotic tissue)
- [#] The 3 *Fusarium* species were identified by species specific PCR as ***F. solani*** (1) and ***F. falciforme*** (2) by PCR based DNA sequencing on 28SrRNA gene.
- The sequences of 15 genera of fungi identified were deposited in GenBank and the assignment of Accession Numbers is awaited

.

The ITS amplification performed on non sporulating isolates and agarose gel electrophoresis is shown in Figure 6b,6c and DNA sequencing is shown in 6d

Figure 6b: Agarose gel electrophoresis showing ITS amplified products of non sporulating fungi



- The amplicon size in bp given within paranthesis and the identification of the isolate were determined by PCR based DNA sequencing on ITS region

Lane 1: Negative control **Lane 2***: 5846/06 Corneal scraping *B. rhodina* (525 bp) **Lane 3:** 3050/01 Corneal scraping *P. insidiosum* (785 bp) **Lane 4:** 4562/06 Corneal scraping *P. insidiosum* (733 bp) **Lane 5***: 16/07 : Corneal button *B. rhodina* (525 bp) **Lane 6:**5888/06 Corneal button *P. insidiosum* (740 bp) **Lane 7**: VRF 236/07 Corneal scraping *Bipolaris species* (571 bp) **Lane 8:** 3981/2k Corneal scraping *M. phaseolina* (566 bp) **Lane 9:** 3984/2k Corneal button *Cochilobolus species* (569 bp) **Lane 10:** 2968 /2k Corneal scraping *R. bataticola* (572 bp) **Lane 11:**M 370/07 Corneal button *F. proliferatum* (663 bp) **Lane 12:**M 86/02 Eviscerated material *G. singulata* (557 bp) **Lane 13:**M 383/07 Corneal scraping *Fusarium* species (730 bp) **Lane 14:** M 372/07 Corneal scraping *Exserohilum* species (711 bp) **MW:** *Sau* 3A I digest of Phi X 174 bacteriophage DNA

Note :

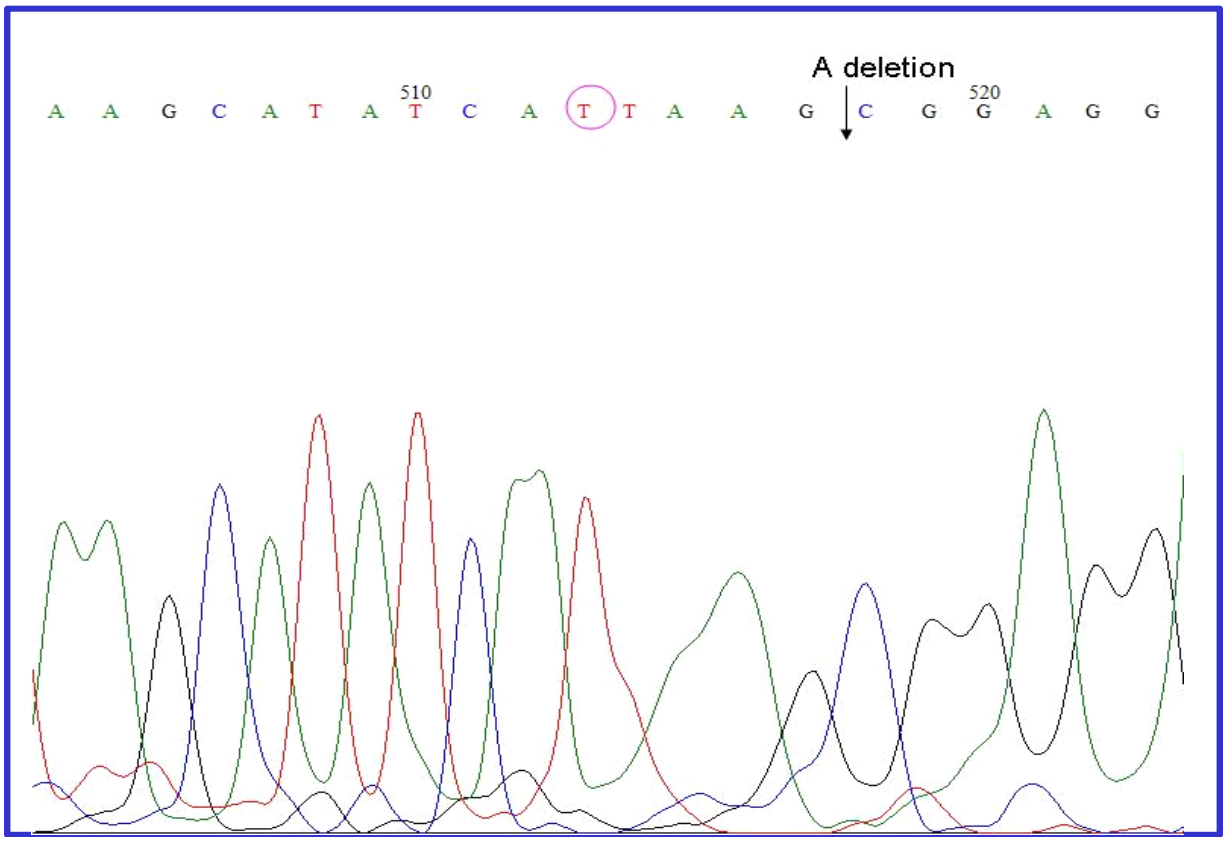
- * specimens collected and processed from the same patient

Figure 6c: Agarose gel electrophoresis showing ITS amplified products of NSM

Lane 1: Negative control **Lane 2*:** 5847/06 Conjunctival scraping *B. rhodina* (525 bp) **Lane 3*:** 5910/6 Corneal scraping *B. rhodina* (525 bp) **Lane 4*:** VRF17/07 Corneal button *B. rhodina* (525 bp) **Lane 5:** 4050/06 Corneal button *B. rhodina* (525 bp) **Lane 6:** 4073 /06 Corneal button *A . fumigatus* (558 bp) **Lane 7:** 4400/2k Corneal button *G. singulata* (566 bp) **Lane 8:** 4225/2k Corneal button *M. phaseolina* (569 bp) **Lane 9:** VRF 40/07 Corneal scraping *G. singulata* (558 bp) **Lane 10:**3890/06 Donor corneal rim *Bipolaris* species (568 bp) **Lane 11:**125/01 Corneal scraping *Botryosphaeria* species (557 bp) **Lane 12:** 380/07 Corneal scraping *Fusarium* solani (549 bp) **Lane 13:** 442 /07 Corneal button *Podospora* species (567 bp) **Lane 14:** 2104/2k Corneal scraping *R. bataticola* (562 bp) **Lane 15:** *F. solani* ATCC 36031 (542 bp) **Lane 16:** *A. fumigatus* ATCC 10894 (538 bp) **Lane 17:** *A. niger* (549 bp) ATCC 16404 **MW :** Molecular weight marker *Hae* III digest of Phi X 174 bacteriophage DNA.

The electrophoretogram of PCR based DNA sequencing on ITS amplicons is shown in 6d and the multalin analysis performed on *Botryosphaeria rhodina* is shown in Figure 6e.

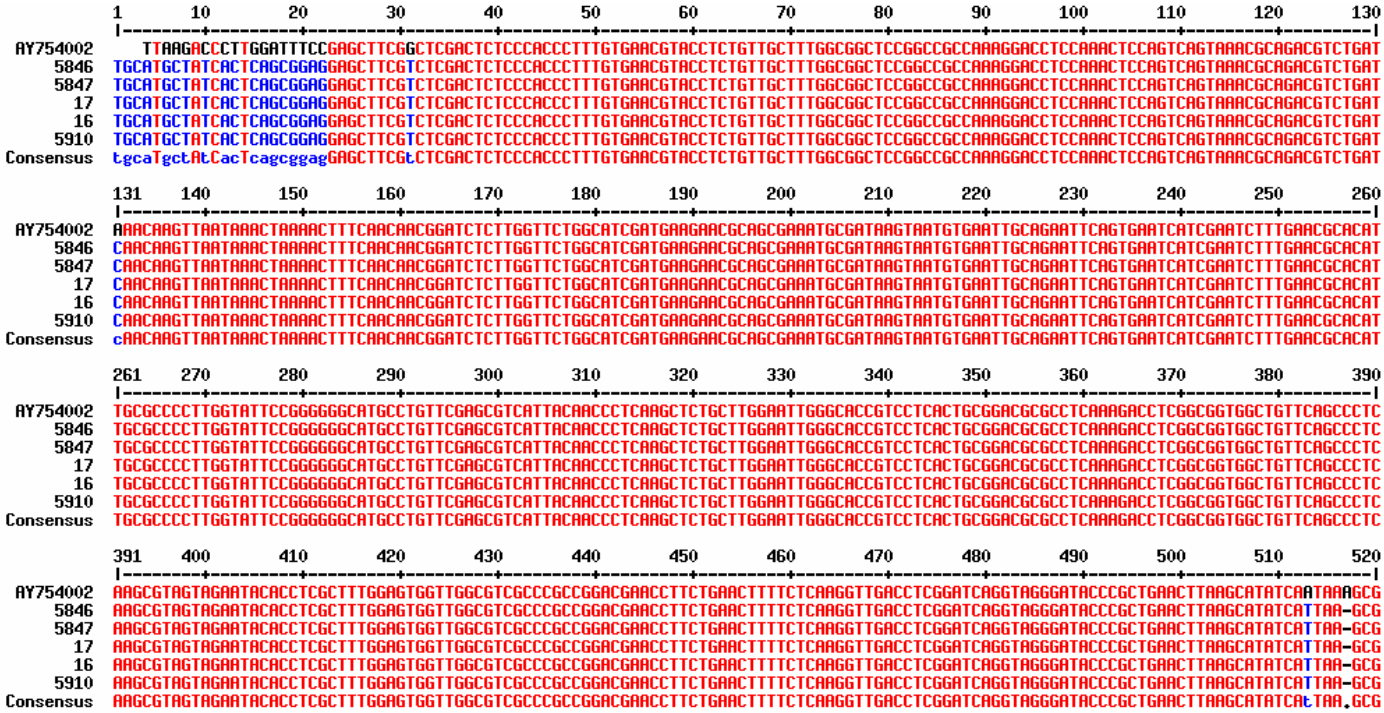
Figure 6d : Electrophoretogram of M 5846 /07 – corneal scraping isolate



Position 513: A to T : single nucleotide polymorphism

Position 518 :A deletion

Figure 6e: Multalin analysis performed between *Botryosphaeria rhodina* Accession Number AY754002 and the *Botryosphaeria rhodina* *isolates* (present study) revealing the single nucleotide polymorphisms in ITS region



NOTE:

- Red colour indicates high consensus
- Blue colour indicates low consensus
- Black colour indicates variation

Multalin analysis performed between *Botryosphaeria rhodina* Accession Number AY754002 and the *Botryosphaeria rhodina* *isolates* revealed the following single nucleotide polymorphisms in ITS region

- 31 (G to T),
- 131 (A to C)
- 513 (A to T)

6.2.4 Discussion

The spectrum of mycotic disease continues to expand well beyond the familiar entities of Candidiasis and aspergillosis. The field of mycology has become a challenging study of infections caused by a wide and diverse array of opportunistic fungi (Pfaller ,2005). 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. To date, sequencing of NSM has been limited to case reports. In ophthalmic settings particularly in tropical countries like India *Fusarium* and *Aspergillus* species are the main aetiological agents of fungal keratitis. (Thomas et al, 1993). 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 pre requisite to identify the fungal genera

DNA-based identification has been utilized successfully to identify pathogenic fungi (Einsele et al 1997 , Zarzoso et al 1999, Chen et al 1999, Boysen et al, 2000, Chen et al 2000, Motoyamo et al, 2000, Ferrer et al 2001, Fujita et al 2001, Zhao et al, 2001, Abliz et al, 2004). In order for molecular identification methods to be successful, it is imperative that phenotypically well-characterized mold isolates from clinical samples are used for developing the method and building the sequence database. In a study conducted by Rakeman et al, 2005, ITS1 and ITS2 DNA sequences were useful for identification of 44 species of pathogenic molds; and validated with 28S DNA sequence and phenotypic information from 143 clinical isolates, 27 type strains, and genetic information from an additional 31 strains.

In the present study, an attempt to identify these NSM was undertaken by targeting the ITS region of these fungi by designing a PCR based DNA sequencing technique. *Botryosphaeria* has been well circumscribed, and can be defined as forming uni- to multilocular ascomata with multi-layered walls, occurring singly or in clusters, often intermixed with conidiomata, which are pycnidial. Asci are bitunicate, with a thick

endotunica, stalked or sessile, clavate, with a well-developed apical chamber, forming in a basal hymenial layer, intermixed among hyaline pseudoparaphyses that are frequently constricted at the septa. Ascospores are hyaline, aseptate, fusoid to ellipsoid or ovoid, bi- to triseriate, mostly without a mucoid sheath or appendages; ascospores turn brown and become septate and even slightly verruculose upon germination (Crous et al, 2006)

The representation and phylogenetic understanding of major groups within *Botryosphaeria* remains poor. Previous analyses based on DNA sequence comparisons have included limited numbers of species, not representing the full anamorph diversity associated with *Botryosphaeria*. The value of the intron-dominated sequences of the ITS, β-tubulin and EF 1-α loci (on which most previous studies were based) to infer phylogenetic relationships across the diversity of the genus, is also unclear. The more conserved mtSSU data have, for example, suggested that *B. dothidea* and *B. corticis* are unrelated to *Fusicoccum* (Zhao et al, 2001) even though they are typically assigned to this genus. *Botryosphaeria* as a single genus is clearly unaligned with evolutionary radiations in the group, as exemplified by the morphologically and phylogenetically distinct anamorph genera linked to it. A preferable approach would be natural unit classification, also referred to as the “genus for genus concept”. Here, morphologically distinct anamorph genera are linked to unique teleomorphs on a one for one basis, correlating with phylogenetic DNA data. (Zipfel et al. 2006) In the present study, multalin analysis on Botryosphaeria revealed polymorphisms at positions 31(G to T),131 (A to C) 513 (A to T), 518 A deletion when compared with the Isolate with Genbank accession number AY754002). The 5 isolates (corneal scraping 2, conjunctival scraping, corneal button, necrotic tissue) obtained from the same patient exhibited similar pattern of single nucleotide polymorphisms. The other isolates exhibited single nucleotide polymorphisma at different positions . The multalin analysis revealed polymorphisms at positions 383 (G to A), 418 (G to C),444 (A to G) when compared with the Genbank sequence in the ITS region of other isolates

Rhizoctonia solani is a group of plant pathogenic fungus of Basidiomycotina with a wide host range, including more than 500 genera of higher plants . Isolates of *R. solani* are usually identified by anamorphic characteristics because of their less

informative teleomorphic characters or lack of them. As a collective species, *R. solani* consists of divergent isolates. The relationships among these groups are not clear. Previous attempts to distinguish AG relationships by esterase zymograms, total soluble protein electrophoresis, serology, DNA restriction fragment length polymorphism, and RNA-RNA hybridizations were unsuccessful. The results of DNA base composition and DNA-DNA hybridization partially agreed with AG classification, with degrees of variation. Studies of DNA reassociation kinetics, isozyme polymorphism ,and ribosomal DNA mapping indicated the presence of subgroup relationships within *Rhizoctonia* (Liu et al,1995). In the present study *Rhizoctonia bataticola* was isolated from Corneal scraping 4, Eviscerated material 1. So far there are no reports of this fungus causing human infections to the best o our knowledge.

Macrophomina phaseolina is the coelomycete synanamorph of "*Rhizoctonia*" *bataticola,* which is shown to be a member of the Botryosphaeriaceae. Conidia also have apical mucous appendages early in their development, which has in the past led to confusion, and the allocation of this species to the genus *Tiarosporella* (von Arx 1981). With age, conidia lose their apical appendages, and become brown and slightly roughened, appearing more Diplodia-like in morphology.(Crous et al, 2006) . In the present study *Rhizoctonia bataticola* was isolated from Corneal scraping 2. So far there are no reports of this fungus causing human infections to the best of our knowledge.

Pfaller et al, 2005 has provided an extensive review of unusual fungal and pseudofungal infections with special mention to pythiosis insidiosi. Pythiosis is a pseudofungal infection of humans and animals caused by *Pythium insidiosum* (Mendoza et al, 1996). Although desribed as an acquatic fungus, this organism is not a true fungus. It belongs to the class oomycetes and family Pythiaceae. It grows in culture as white colonies with submerged vegetatitive and short aerial hyphae. In tissue, *P. insidiosum* exists as a hyaline, pausi-septate, thin walled hyphae or hyphal fragmentsthat branch infrequently. The hyphae are 5 to 7 μm wide with non parallel walls and superficially resemble those of Zygomycetes. Similar to the Zygomycetes , P. insidiosum is angio invasive and stains weakly with GMS and other fungal stains. (McGinnis *et al*, 1997). In contrast to true fungi, the cell walls of *P. insidiosum* are composed of cellulose rather than glucan, mannan and chitin.

Human disease due to *Pythium insidiosum* can be classified into three forms as ocular, cutaneous and arterial. The ocular form of pythiosis is manifested as keratitis that can be severe and result in the formation of corneal perforation. *Pythium insidiosum* is an unusual but serious ocular pathogen. Although the organism grows as a mycelium in tissue, it is not a member of the fungal kingdom and its identification can be a challenge for a routine laboratory. Colonies grew rapidly on the primary fungal medium (plain Sabouraud's dextrose agar, at 25°C). They were white with a yellowish tinge, unusually flat, and difficult to cut and separate from the agar. A few septate hyphae with no spores were seen. These isolates when subjected to DNA sequencing on ITS amplicons revealed a definitive identification of *Pythium insidiosum*. DNA sequencing identified 9 as *Pythium insidiosum* from 6 Corneal scrapings, 2 corneal buttons and 1 vitreous aspirate. The multalin analysis performed on *Pythium inidiosum* isolates with Genbank Accession Number EF016789 revealed nucleotide polymorphisms in positions 100 to 105 (T Insertion), 111(A to G),592 (G insertion), 606 (T to A), 616 (T insertion) With respect to the eye, *P . insidiosum* has been responsible for periorbital infections in Australia (Triscott et al, 1993) and in USA (Shenep et al, 1998) and corneal ulcers in Thailand, (Thianprasit et al, 1996) Haiti, (Virgile et al , 1993) and New Zealand (Murdoch et al, 1997). For the cornea, however, it would be difficult to imagine a successful outcome given the destructive nature of the organism, the slow response if any to most antimicrobial agents, the probable delay in identification, and the need to prevent further tissue invasion. Transplantation may be the best option to treat ocuar pythiosis. (Badonech et al, 2001)

Callister et al, 2004 described a case of a 38 year old pregnant woman with fatal disseminated aspergillosis and multiorgan failure, preceded by a long history of allergic bronchopulmonary aspergillosis. Culture of tissue samples produced a non-sporulating, beige coloured fungus that developed green pigmentation only after three weeks of incubation, identified as *A. fumigatus* by n ucleotide sequencing of the D1–D2 region of the large ribosomal subunit . In the present study, there was an atypical isolate of *A. fumigatus*

Bloom et al, 1992 have reported fungal keratitis caused by *Scedosporium* species (teleomorphic stage *Pseudoallescheria boydii*). The microscopic morphology in ocular samples revealed septate, hyaline, branching hyphae, 2–4 μm wide, similar to other hyaline filamentous fungi. Colonies are flat to dome shaped, floccose or moist, and white to pale or dark gray or black, and attains a diameter of 20 mm (*S. prolificans*) to 40 mm (*S. apiospermum*) in 1 week. Usually the typical microscopic morphology includes long and slender, single or branched, and sometimes conidiophores aggregated into bundles (Graphium state). Conidia (6–12 μm by 3.5–6 μm) are yellow to pale brown, oval with a scar at base, and usually abundant. *S. prolificans* conidiophores are short with inflated base and tapering tip; oval conidia (3–7 μm by 2.5 μm) frequently occur in groups. In the present study , *Scedosporium species* (isolate from infected suture and its teleomorph *Pseudoallescheria species* (isolate from Corneal button) were identified . These two isolates produced only sterile hyphae after prolonged incubation (15 days). The conventional identification could reveal its identity as a hyaline hyphomycete, further definitive identification was possible only by PCR based DNA sequencing on ITS region. *Scedosporium species* and its teleomorph *Pseudoallecheria* species were known to cause fungal keratitis (Bloom et al, 1992, Wu et al, 2002, Thomas et al, 2003), scleritis (Moriaty et al, 1993 Taravella et al, 1997) and endophthalmitis (Pfeifer et al, 1991).

The majority of *Fusarium* species isolated from nature produce their macroconidia on sporodochia. The sporodochial type often mutates in culture, especially on media rich in carbohydrates. Mutations may also occur in nature, but are rare. These mutants may give rise to others so that a mutational sequence occurs. In pathogenic isolates, these mutants frequently exhibit a loss of virulence and the ability to produce toxins may be reduced or lost. Variability and its effect on virulence and taxonomy have been discussed in detail elsewhere (Snyder et al, 1954, Waite et al, 1960). The mutation sequence has never been shown to reverse itself. The two major types of mutants produced from the sporodochial type are the pionnotal type and the mycelial type. The characteristics of the pionnotal type are (i) the production of little or no aerial mycelium, (ii) the production of abundant macroconidia on the surface of the colony causing the surface to appear shiny and wet, (iii) more intense pigmentation of colonies than of the sporodochial colonies, (iv) the production of

longer and thinner macroconidia than those produced by the sporodochial type, and (v) mutants that may be less virulent than the sporodochial type and may also lose the ability to produce toxins. (Snyder et al, 1954, Waite et al, 1960). The *Fusarium* isolates (7) in the present study produced scrace macroconidia and lacked sporodochia, sclerotia and pigmentation in culture. The definitive identification was possible only by PCR based DNA sequencing o ITS amplicons.

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. The estimated cost of identification based on PCR based DNA sequencing is 30 US $ (Rs. 1500) whereas the idenification by conventional methods is 7 US$ (Rs. 350).

When definite fungal identification is clinically indicated, additional targets may be necessary to identify certain genera and species. In summary, ITS sequencing provides a fast, alternative method to conventional identification for nonsporulating molds. The NSM identified in the present study are established plant pathogens. However, the reasons for lack of sporulation on conventional culture media remains an area for emergingresearch in the near future. One of the important features may be the switching off certain genes determining the morphological behaviour of these fungi, when infecting humans. This particular aspect further needs to be validated by monitoring the in vitro and in vivo growth kinetics of these NSM. For specific specimen sources and in the appropriate clinical setting, sequencing the ITS region for NSM that cannot be conclusively characterized by conventional means could serve as a valuable tool to identify clinically relevant pathogens and enable the timely initiation of appropriate antifungal treatment

6.6 Conclusion

Optimization and application of PCR based DNA sequencing of ITS amplicons was found to be a rapid potent tool in identification of NSM. In terms of cost effectiveness, the methodology employed costs 4.2 times more than that of

conventional methods, but a definitive identification becomes possible.This study detected the association of new genera of fungi causing keratitis in Indian population

6.7 Future prospect:

Characterization of NSM, targeting the small and large subunit rRNA would be of immense and potential area of research in the near future. The susceptibility pattern of these NSM can also be investigated which would facilitate to probe into the drug resistance genes expressed by these fungi. Also, monitoring the growth kinetics of NSM *in vivo* would aid in understanding the mode of propogation and the virulence mechanisms exhibited by them to cause a particular disease.

CHAPTER - 7

Characterization of *Fusarium* species by Phenotypic Methods and PCR, PCR-RFLP, DNA sequencing

7.1 Review of literature

Fusarium spp. are opportunistic fungi that can cause life threatening infections, mainly in immunocompromised hosts. While the particular susceptibility of the host is a major determining factor in the establishment of the infection, it is also clear that *Fusarium spp*. possess several cellular and molecular attributes that, together, may confer different degrees of inherent virulence on these organisms. The combination of these virulence factors and the immunocompromised status of the host contribute to the development of invasive fusarial infections. (Nelson et al, 1994)

7.1.1 General Characteristics of *Fusarium* Species

Fusarium species exhibit wide range of colony morphology and may produce three types of spores called macroconidia, microconidia, and chlamydospores.Some species produce all three types of spores, while other species do not. The macroconidia are produced in a specialized structure called a sporodochium in which the spore mass is supported by a superficial cushion like mass of short monophialides bearing the macroconidia .The sporodochium sometimes may be encased in slime. Macroconidia may also be produced on monophialides and polyphialides in the aerial mycelium . A monophialide is a condiophore with only one opening or pore through which endoconidia are extruded, while a polyphialide has two or more such openings or pores. Some conidia are intermediate in size and shape, and these have been referred to as both macroconidia and mesoconidia . These intermediate conidia are found primarily in *F. semitectum* , *F. avenaceum*, and *F. subglutinans* (Nelson et al 1994,). Until more cultures of each species are studied thoroughly, the use of the term mesoconidia is questionable. Microconidia are produced in the aerial mycelium but not in sporodochia. They may be produced in false heads only or in false heads and chains on either monophialides or polyphialides. False heads occur when a drop of moisture forms on the tip of the

conidiophore and contains the endoconidia as they are produced. Microconidia are of various shapes and sizes , and those produced in chains have a truncate base . The third type of spore formed by *Fusarium* species is a chlamydospore, which is a thick-walled spore filled with lipid like material that serves to carry the fungus over winter in soil when a suitable host is not available. The chlamydospores may be borne singly, in pairs, in clumps,or in chains, and the outer wall may be smooth or rough .

7.1.2 Primary characters used to separate species in *Fusarium* Taxonomy

Morphology of the macroconidia

The morphology of the macroconidia is the key characteristic for characterization not only of the species but also of the genus *Fusarium*. Macroconidia of *Fusarium* species are of various shapes and sizes , but the shape of the macroconidia formed in

sporodochia for a given species is a relatively consistent and stable feature when the fungus is grown on natural substrates under standard conditions (Woollenweber, 1989). Dimensions of the macroconidia may show considerable variation within individual species and should be used cautiously as taxonomic criteria.

7.1.3 Secondary characters useful in separating species in *Fusarium* Taxonomy

The following secondary characteristics are useful in describing a species when the cultures are grown under standard environmental conditions of light, temperature, and substrate (Burgess et al, 1981) but should not be regarded as suitable criteria for differentiation of a species: the morphology and pigmentation of the colony, including the presence or absence of sporodochia, sclerotia, or stroma. The pigmentation of colonies grown on carbohydrate-rich media is variable in some species. The pigmentation of the colony may be helpful to someone with experience in *Fusarium* taxonomy but quickly can lead those without prior experience in this area astray. The degree of variation shown by a particular secondary character may differ between species. Although the shape of the macroconidia formed in sporodochia on carnation

leaf agar is a reliable character, the length and width of the macroconidia are less stable features and should be regarded as secondary characters. The macroconidia of a wide range of isolates of *F. culmorum*. are relatively uniform in length, whereas the length of macroconidia of *F. equiseti* varies widely between isolates, even among those from the same geographic location.

7.1.4 Problems in working with *Fusarium* species

Cultural Mutation

The majority of *Fusarium* species isolated from nature produce their macroconidia on sporodochia. The sporodochial type often mutates in culture, especially on media rich in carbohydrates. Mutations may also occur in nature, but are rare. These mutants may give rise to others so that a mutational sequence occurs. In pathogenic isolates, these mutants frequently exhibit a loss of virulence and the ability to produce toxins may be reduced or lost. Variability and its effect on virulence and taxonomy have been discussed in detail elsewhere (Snyder et al, 1954, Waite et al, 1960). The mutation sequence has never been shown to reverse itself. The two major types of mutants produced from the sporodochial type are the pionnotal type and the mycelial type. The characteristics of the pionnotal type are (i) the production of little or no aerial mycelium, (ii) the production of abundant macroconidia on the surface of the colony causing the surface to appear shiny and wet, (iii) more intense pigmentation of colonies than of the sporodochial colonies, (iv) the production of longer and thinner macroconidia than those produced by the sporodochial type, and (v) mutants that may be less virulent than the sporodochial type and may also lose the ability to produce toxins.

The characteristics of the mycelial type are (i) the production of abundant aerial mycelium, (ii) the production of very few to no macroconidia, (iii) the frequent lack of sporodochia, sclerotia, and pigmentation in culture, and (iv) mutants that may be less virulent than the sporodochial type and may also lose the ability to produce toxins .Procedures that reduce mutant populations include (i) initiating cultures from single conidia, (ii) initiating cultures from single hyphal tips, (iii) avoiding media rich in carbohydrates, and (iv) keeping subculturing to a minimum (Nelson et al,1984).

7.1.5 Culture

Potato dextrose agar made according to the specifications of Nelson *et al*.1994, is a valuable medium used principally for noting gross morphological appearances and colony colorations. Because of its high available carbohydrate content,potato dextrose agar generally emphasizes growth to the detriment of sporulation. Cultures grown on this medium sporulate poorly, frequently taking more than a month to do so. The conidia produced are often misshapen and atypical. Consequently, with few exceptions, potato dextrose agar culturesare not used for microscopic observations. Cultures grown on potato dextrose agar are used only in a secondary role.

7.1.6 Ocular infection:

Because of the emergence of *Fusarium* spp. as significant ocular pathogens in humans (Williams et al 1987, Venugopal et al, 1989), several attempts have been made to establish animal models of infection. A short-lived infection that could not be maintained for more than a few days was reported by Jones *et al.* in rabbits (Jones, 1969). Foster and Rebell (Foster et al, 1975) showed that pretreatment with corticosteroids resulted in a more prolonged but still self-limiting disease. Kiryu et al, 1991 showed that in the dexamethasone-treated cornea of rabbits the neutrophils could not ingest and destroy the fungi and that a hypha-in-hypha structure developed. These events were not seen in the absence of dexamethasone treatment. Burda and Fisher (Burda et al, 1991) developed a fusarial corneal infection model in rats. An increased rate of infectivity was observed with steroid pretreatment. Finally, O'Day et al, 1979 developed a rat model of fulminant ocular infection culminating in endophthalmitis that resembled remarkably that encountered in humans. *F. solani* was the infectious agent, and infection could be established and maintained without the need for immunosuppression. *F. solani* is the common aetiological agent causing keratitis in South India (Gopinathan et al, 2002).

7.1.7 *Fusarium* keratitis in contact lens wearers

In a study conducted by Khor *et al*, 2006, 66 patients (68 affected eyes) were diagnosed with *Fusarium* keratitis associated with contact lens wear; the estimated annual national incidence is 2.35 cases per 10,000 contact lens wearers (95% confidence interval, 0.62-7.22). Patients ranged in age from 13 to 44 years (mean [SD], 27.1 [8.4] years), of which 32 (48.5%) were men. The vast majority (65 patients; 98.5%) wore soft, disposable contact lenses; 62 patients (93.9%) reported using 1 brand of contact lens cleaning solution (ReNu, Bausch & Lomb, Rochester, NY), including 42 patients (63.6%) who recalled using ReNu with MoistureLoc. Most patients (81.8%) reported poor contact lens hygiene practices, including overnight use of daily wear contact lenses (19.7%), and use of contact lenses past the replacement date (43.9%). The final best-corrected visual acuity ranged from 20/20 to 20/80. Five patients (5 eyes; 7.4%) required emergency therapeutic or tectonic corneal transplantation. This study showed a new and evolving epidemic of *Fusarium* keratitis associated with contact lens wear in Singapore. In yet another study conducted by Eduardo et al, 2006 involving 34 cases,the microbiologic corneal cultures found *Fusarium oxysporum* in 20 cases, *Fusarium solani* in 3 cases, *Fusarium* species not further identifiable in 10 cases, and no growth in 1 case. There were also similar studies conducted by Maria et al*,* 2006, Chang et al, 2006, Margolis et al*,* 2006 , Dyavaiah et al 2007 on *Fusarium* keratitis

7.1.8 Need for molecular methods for identification of *Fusarium* species

Diagnosis of *Fusarium* at the species level is based on conventional methods, which include the description of colonies on appropriate media (texture, color, and pigment etc.) and microscopic description of conidiogenous cells and conidia. This can be best observed after 2 weeks of incubation, lengthening the time for a definitive diagnosis. Because of important variations of characters, such as pigmentation and growth rate, that are often seen within a given species, only well-trained mycologists are able to ensure the diagnosis.The results are thus frequently inconclusive, as seen in various reports where one-third to one-half of the isolates are not identified at the species level (Henequinn et al, 1997). Identification at the species level is

important for epidemiological purposes and may become absolutely necessary because some new antifungal agents exhibit variable activity against *Fusarium* depending on the species (Sanche et al, 1997, Arikan et al, 1998). The clinical manifestations and histological appearance of *Fusarium* spp. may be indistinguishable from those of organisms causing invasive aspergillosis. Both genera infect profoundly immunocompromised patients, and both are associated with vascular invasion, tissue infarction, and hemorrhage. Recently, Liu et al 1998, reviewed biopsy and cytology specimens from culture-confirmed hyalohyphomycosis caused by *Fusarium, Paecilomyces*, or *Acremonium* species to identify histologic features that distinguish these molds from *Aspergillus* species. Sporadic phialide- and phialoconidium-like structures were present in 16 of 19 cases, including 7 of 10 cases of infection by a *Fusarium* species. Phialoconidium-like structures seen in tissue were either spherical, oval, curved, or elliptical. Several previous studies have used PCR technology to detect fungi. The primers used target three kinds of sequences:(i) sequences coding for an antifungal target, (ii) fungus-specific regions of conserved proteins such as actin, and (iii) re- peated sequences such as those of rDNA and mitochondrial DNA.

With this background, a specific PCR targeting small subunit of *Fusarium* (genus specific) as well as species specific PCR was optimized to identify *Fusarium* species. Additionally a rapid method of PCR-RFLP targeting ITS region was optimized and applied on Fusarium isolates to reveal its species identity. For diagnostic purposes, it is essential that repeated sequences be used as targets to ensure good sensitivity
In this study, we report a rapid method for the identification of significant *Fusarium* species involved in human ocular infections by using rRNA gene (rDNA) sequencing.

7.2 Objective :

- To optimize PCR based RFLP on ITS amplified products for identification of *Fusarium* isolates
- To optimize and apply genus and species specific PCR for identification of *Fusarium* species
- To apply PCR based DNA sequencing on 28SrRNA region for species level identification of *Fusarium* species

7.3 Materials and Methods:

7.3.1 *Fusarium* isolates included in the study

Fifty ocular *Fusarium* isolates from 25 Corneal scrapings, 19 corneal button, 6 eviscerated materials of fungal keratitis patients were included for species level identification.

7.3.2 Identification of *Fusarium* isolates by Conventional mycological methods

All the 50 isolates of *Fusarium* were subcultured on Sabouraud's dextrose agar, (SDA),potato dextrose agar (PDA), yeast extract agar (YEA) and Corn meal agar (CMA) and incubated at 25˚C for 7 days. The macroscopic characteristics like texture, production of diffusible or non - diffusible pigment, colour, reverse pigmentation, presence or absence of sporodochia on all four media were observed. For microscopic identification, standard slide culture technique was performed and identified by the presence of microconidia, macroconidia, chlamydoconidia and presence or absence of monophialides and polyphialides. *Fusarium* isolates were identified using standard mycological methods (Rippon 1988, Sigler 2001) . In brief, *F. falciforme* was identified by production of pink non-diffusible pigment and microconidia, *F. lichenicola* by reddish pink pigment, presence of sporodochia and production of micro and macroconidia, *F. solani* by grey colour colony and characteristic sickle shaped macroconidia, *F. moniliforme* by brown pigment and production of micro, macroconidia and *F. oxysporum* by production of pink centre and white periphery pigment and presence of abundant macroconidia.

7.3.3 Nucleic acid based molecular methods

DNA extraction from *Fusarium* isolates was carried out by phenol chloroform extraction as described by Therese et al, 1998. In brief, extraction was carried out using 200mM Tris, 50mM EDTA, 10%SDS and incubated at 56˚C for 2 hours followed by addition of phenol, chloroform and isoamylalcohol in the ratio of 25:24:1. The aqueous phase was separated to which one tenth volume of 5M Nacl and equal volumes of absolute ethanol was added and incubated at -20˚C for 1 hour. This is followed by washing with 70% ethanol thrice and reconstituted with 30µl of milli –Q water.PCR targeting ITS region was carried out as given in Appendix II.

7.3.4 RFLP on the PCR amplified products

RFLP was carried out on ITS amplicons using *Hae* III enzyme (Sanmar chemicals private Ltd, Bangalore).For a 25 µl reaction, 2.5 µl of Buffer C,0.5 µl of *Hae* – III enzyme and 10 µl of amplified product were added and incubated at 37˚ C for 3 hours.The digested products were loaded on 4 % agarose gel incorporated with ethidium bromide with 0.5 µg/ml and the electrophoresis was carried out at 100 volts for 45 minutes. The digested pattern was visualized and documented using gel documentation system (Vilber Lourmat, U. K.).

7.3.5 DNA sequencing using Microseq D2 Large Sub Unit ribosomal DNA PCR amplification for sequencing

PCR was performed using primers targeting the D2 region of the large subunit of the ribosomal RNA gene using the Micro Seq^{R} D2LSUr DNA PCR Kit (Applied Biosystems, Foster City ,CA). PCR amplicons were sequenced with MicroseqD2 Large Sub Unit ribosomal DNA Fungal Sequencing Kit^{R} (Applied Biosystems, Foster City,CA).

7.3.6 DNA Sequencing and interpretation of results

DNA Sequencing was performed on 8 isolates with a 3100-Avant Genetic Analyser (Applied Biosystems). Phylogenetic analysis was performed using MicroSeq Microbial Identification Software v1.4.1 and Bionumerics software v3.5 (Applied Maths) Gene sequences were compared to the MicroSeq library and to sequences obtained from Gen Bank (www.ncb.nlm.nih.gov.entrez/) for *Fusarium* identification.

7.3.7 Genus specific PCR- *Fusarium* species

For a 50µl reaction , 8 µl of dNTPs,5 µl of 10 X PCR buffer (1.5 mM $MgCl_2$, 500 mM KCl, 100 mM Tris Cl,0.01% gelatin),20 picomoles of P58SL.5'-agtattctggcgggcatgcctgt-3'and P28SL.5'-acaaattacaactcgggcccgaga- and 10 µl of template DNA was used. Amplification was carried out in Perkin Elmer thermal cycler (Model 2700) according to the thermal profile described by Hue *et al,* 1999 .The thermal cycling consisted of 40 cycles with denaturation at 94 ˚ C for 1 minute, annealing at 68˚ C for 1 minute and extension at 72˚ C for 1 minute followed by final extension at 72˚ C for 10 minutes.The amplified product obtained was 329 bp

7.3.8 Species specific PCR targeting *F.* solani, *F. oxysporum* and *F. moniliforme*

For a 50µl reaction , 8 µl of dNTPs,5 µl of 10 X PCR buffer (1.5 mM $MgCl_2$ 500 mM KCl, 100 mM Tris Cl,0.01% gelatin), 30 picomoles of SOL 31 gct acc gag gcc atc aat tca tg SOL 32 tga tgt tgt act tct cct tgc cc for amplification of *Fusarium solani*, OX 31 tga ctt gga tga gac ctt ggc g, OX 32 cag gat tta ccg aca cag ctt ttg for amplification of *Fusarium oxysporum* and MON 1 gag agc tgg atg tac gaa tg, MON 2 cac aga gat ggt tca ctg ag for amplification of *F. moniliforme* were used. The thermal cycling consisted of 40 cycles with denaturation at 94 ˚C for 1 minute, annealing at 62˚ C (*F. solani*), 66˚C (*F. oxysporum*) 60˚C (*F. moniliforme*,) for 1 minute and extension at 72˚ C for 1 minute followed by final extension at 72˚ C for 10 minutes

7.3.9 Sensitivity and specificity of genus and species specific *Fusarium* PCR

The sensitivity of genus and species specific *Fusarium* PCR was determined by serial 10 fold dilutions of *F .solani* ATCC DNA and laboratory isolates of *F. moniliforme*, *F. oxysporum* .The specificity was determined by using DNA extracts of microorganisms given in Appendix II

7.4 Results

7.4.1 Conventional mycological methods:

F. falciforme - **Colony characteristics**: Growth was rapid (72 hours) ,white fluffy cottony growth with reddish brown centre and white periphery. Production of non – diffusible pink pigment. Reverse dark tan in colour and sporodochia were absent. **Microscopic characteristics:** Microconidia abundant , small and oval present in singles, , chlamydoconidia few in number and Macroconidia and monophialides absent

***F. lichenicola* - Colony characteristics**: Growth was rapid (72 hours) ,white fluffy cottony growth with reddish brown centre and white periphery. Production of non – diffusible pink pigment. Reverse reddish brown and sporodochia present

Microscopic characteristics: Microconidia abundant abundant macroconidia 5 -6 μm singly sickle shaped with 2-3 septations chlamydoconidia few in number and monophialides absent

***F.solani* - Colony characteristics**: Growth was rapid (72 hours) ,white fluffy cottony growth with grey centre and white periphery. Production of tan brown non – diffusible pink pigment. Reverse bright red in colour and sporodochia were absent **Microscopic characteristics:** Few microconidia, oval 2 -3μm arranged in clusters. Macroconidia abundant.5-6 μm sickle shaped with 2-3 septations. Small round chlamydoconidia few in number arranged singly. Monophilides absent

***F. oxysporum*- Colony characteristics**: Growth was rapid (72 hours) ,white fluffy cottony growth with pink centre and white periphery. Production of non – diffusible pink pigment. Reverse bright red in colour and sporodochia were absent **Microscopic characteristics:** Abundant microconidia, oval 2 -3μm arranged in clusters. Macroconidia few .5-6 μm sickle shaped with 2-3 septations. Small round chlamydoconidia few in number. Monophilides present

***F. moniliforme*-Colony characteristics**: Growth was rapid (72 hours) ,white fluffy cottony growth with purple centre and white periphery. Dark purple pigment. Sporodochia absent

Microscopic characteristics: Abundant microconidia, small and oval present in singles. Macroconidia few in number. Chlamydoconidia not produced . Branched monophialides.

7.4.2 PCR –RFLP using *Hae* III enzyme

The results of PCR –RFLP is shown in Table 7a.

Table 7a: Results of PCR-RFLP using *Hae* -III for identification of *Fusarium* species.

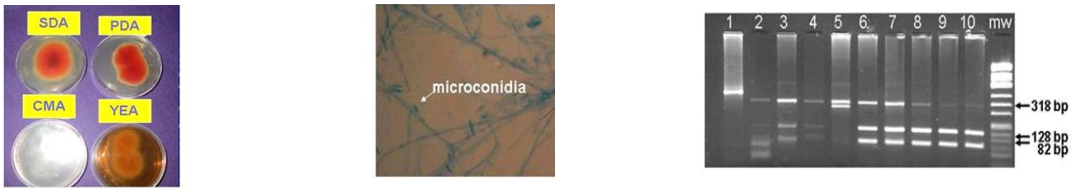
Ocular specimen	PCR-RFLP products	Species level identification using PCR –RFLP on ITS region[#]
Corneal scraping 1 Corneal button 1 Eviscerated material 1	452,152,58 bp	*F. oxysporum*
Corneal scraping 6 Corneal button 4 Eviscerated material 1	232, 188,104 bp	*F. lichenicola*
Corneal scraping 4 Eviscerated material 4	238,132,88 bp	*F.solani*
Corneal scraping 14[*@] Corneal button 10[*@] Eviscerated material 1	318,118,82 bp	*F. falciforme*
Eviscerated material 3	314,140 bp	*F.moniliforme*

- Note [@] Corneal scraping and corneal button were obtained from the same patient
- [#] The results of PCR –RFLP were same as that of identification by conventional methods

The comparative evaluation of phenotypic and genotypic methods using *Hae* –III for identification of *Fusarium* species is shown in Figures 7a, 7b,7c .

Figure 7a: Phenotypic and genotypic identification of *F. falciforme*

1 **2** **3**



1: Colony morphology of *F. falciforme* on SDA and Potato dextrose agar PDA- fluffy growth with pink pigmentation, YEA:intensity of pigment production is reduced, CMA : Scanty growth

2: LPCB mount (40X) of *F.falciforme* : Microconidia abundant small and oval present in singles. Macroconidia absent chlamydoconidia few in number. Monophialides absent.

3: PCR based RFLP using *Hae* -III on ITS amplified products

Lane 1: Undigested product (585 bp) **Lane 3,4,6,7,8,9,10:** Digested product of *F. falciforme* (318,128,82 bp) **Lane 2:** Digetsed product of *C. albicans* Lane mw: Molecular weight marker *Hinf* I digest of Phi X 174 bacteriophage DNA

Figure 7b: Phenotypic and genotypic identification of *F. solani*

1 **2** **3**

1: Colony morphology of *F. solani* on SDA and PDA- fluffy growth with light reddish pink pigmentation, periphery white YEA ,CMA: white fluffy growth

2: LPCB mount (40X) of *F.solani* : Few microconidia, oval 2- 3 <m arranged in clusters. Macroconidia abundant, 5- 6 μm sickle shaped with 2-3 septations. Monophialides absent

3: PCR RFLP using *Hae* III on first round ITS amplified products of *F. solani*

Lane 1: Undigested product (570 bp) **Lane 2:** Digested product of *Fusarium solani* (238,132,88 bp) **Lane MW:** *Hinf* I digest of Phi X 174 bacteriophage DNA

Figure 7c: Phenotypic and genotypic characterization of *F.lichenicola*

1 **2** **3**

1: **Colony morphology of *F.lichenicola*** on SDA and PDA White fluffy cottony growth with reddish brown centre and white periphery. Production of non diffusible pink pigment. Reverse reddish brown Sporodochia present white .YEA no pigment production is less ,CMA scanty growth

2 LPCB mount (40X) of F.lichenicola: Few microconidia,oval 2- 3 microns rranged in clusters. Macroconidia abundant , 5- 6 <m sickle shaped with 2-3 septations.Monophialides absent

3: Agarose gel electrophoretogram of PCR based RFLP using Hae -III on first round ITS amplified products of *F.lichenicola* Lane 1: Undigested product(570 bp) Lane 3,4,5: Digested product of *Fusarium lichenicola* (232,188,104bp) Lane mw:Molecular weight marker Phi X 174 bacteriophage DNA Hinf - I digest

7.4.3 DNA Sequencing

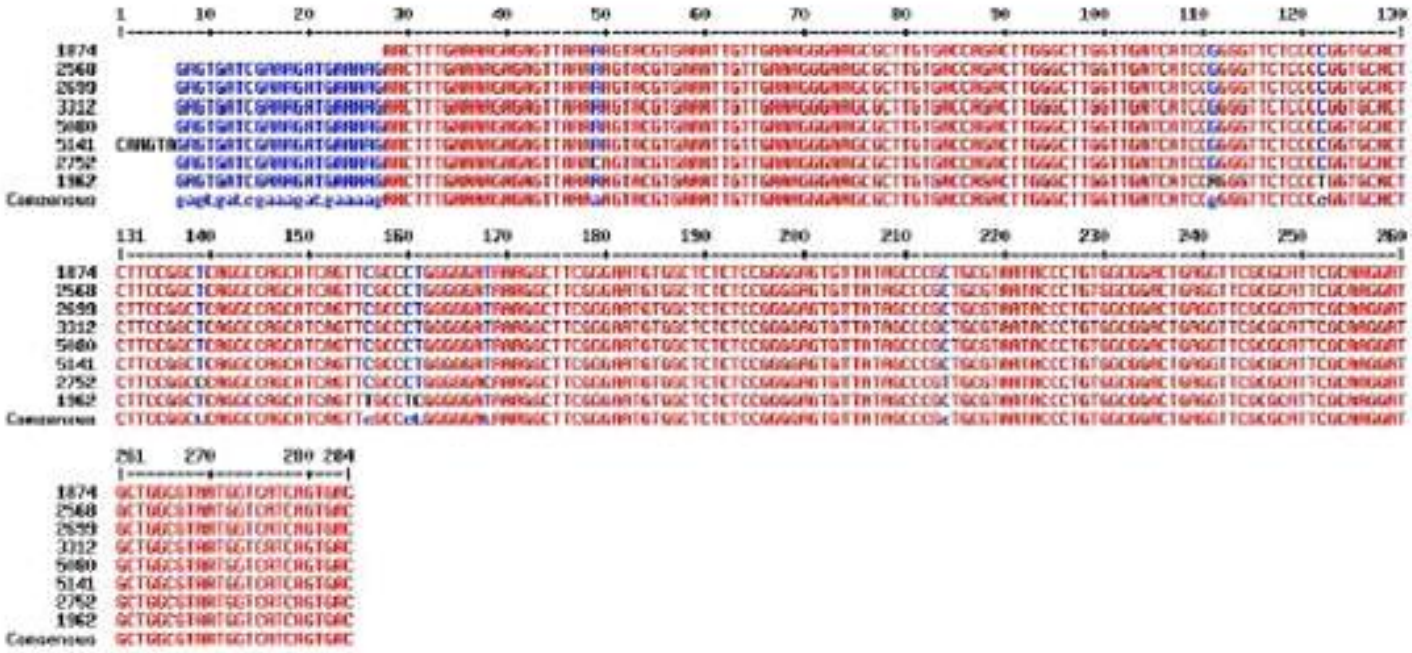
The results of DNA sequencing on 8 *Fusarium* isolates targeting D_2LSU revealed 6 as *F. falciforme* from 4 Corneal scraping and 2 corneal buttons, 1 *F. lichenicola* and *F. solani from* one corneal button. Multalin analysis performed on 6 *F. falciforme* isolates *, F. solani* and *F. lichenicola* with the standard strains revealed 100% sequence homology. The multalin analysis performed with sequences of Fusarium species is shown in Figure 7d

7.4.4 Results of DNA sequencing

Among 50 isolates of Fusarium 20 were subjected to DNA sequencing targeting the 28SrRNA region. A total of 8 isolates were identified using the Microseq kit. The isolates identified by DNA sequencing are *F. falciforme* 6, *F. lichenicola*, 1 and *F.solani* 1

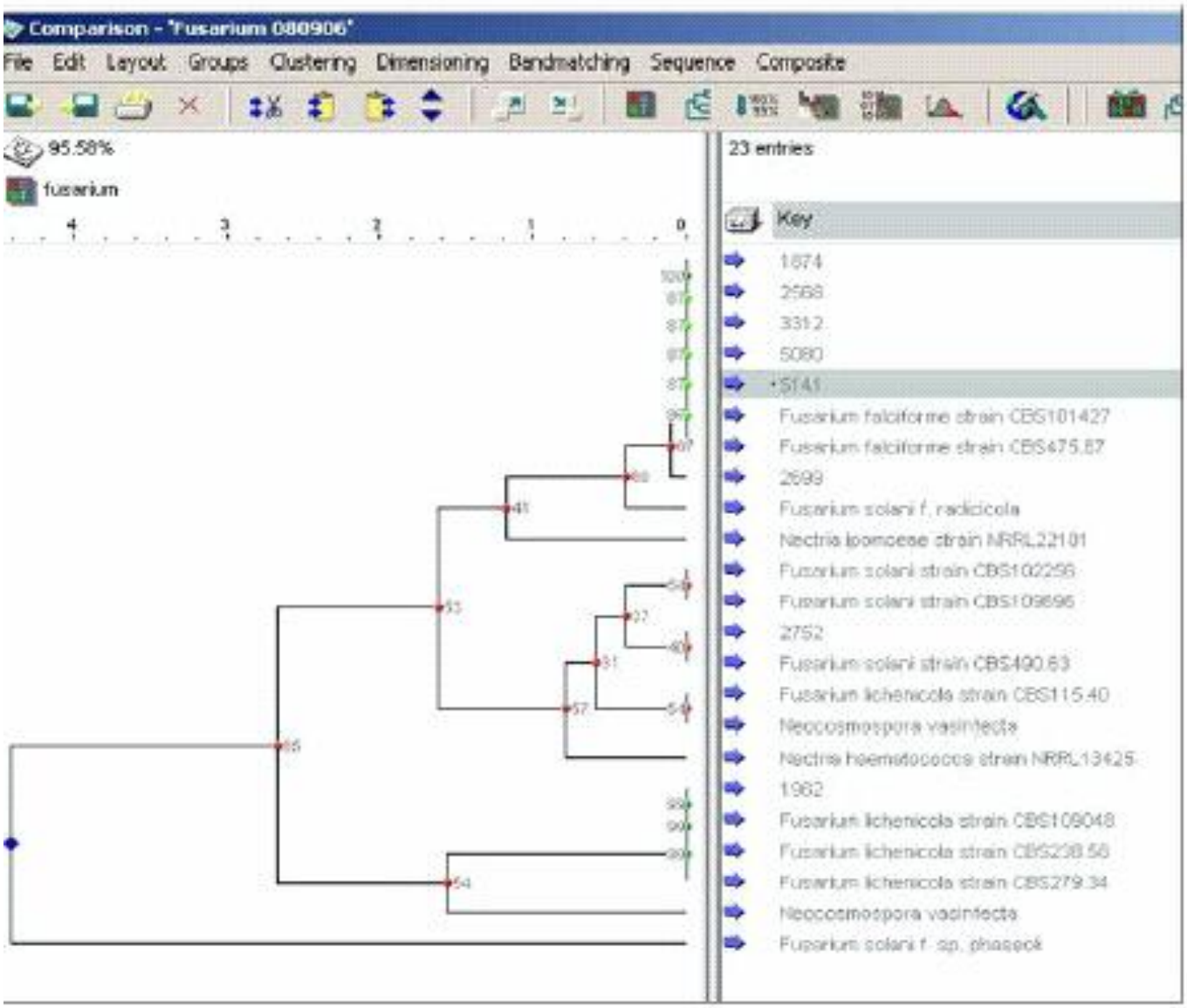
The 12 isolates identified by PCR based DNA sequencing targeting 28SrRNA are *F. falciforme* 5 *F. solani* 3, *F. lichenicola* 2, *F. oxysporum*,1 *F. moniliforme* 1

Figure 7d: Multalin analysis performed on *Fusarium* species



The analysis revealed sequence variations in *F.solani* at positions 50 (C to A),111 (A to G),122 (T to C),139 (C to T),168(T to C),214 (C to T).The sequence variation was also observed in *F. lichenicola* in positions 156 (C to T) and 160,161(CT to TC).The homology between different *Fusarium* isolates is shown in Table 7b. The phylogenetic tree constructed based on *Fusarium* sequences is shown in Figure 7e .

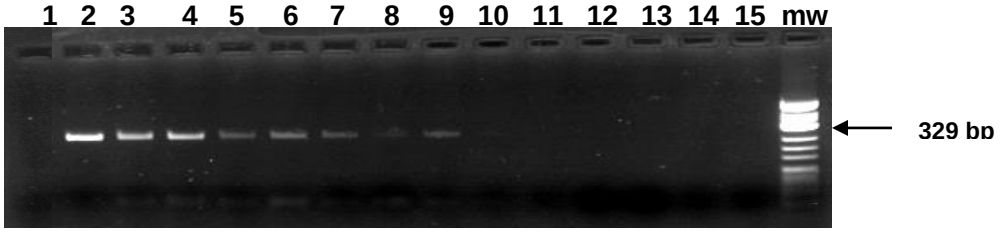
Figure 7e: Phylogenetic tree constructed based on *Fusarium* sequences



7.4.5 Sensitivity and specificity of genus specific *Fusarium* PCR:

The results of sensitivity of genus specific *Fusarium* PCR is shown in Figure 7f. The sensitivity of *Fusarium* specific PCR performed using ATCC *F. solani* DNA had a sensitivity to detect 1 pg of *F. solani* DNA. The results of specificity of *Fusarium* specific PCR performed using *Fusarium* species and laboratory isolates of fungi and other microbial DNA is shown in Figure 7g and 7h. The primers were specific to detect *Fusarium* species amplifying all the isolates of *Fusarium* species.

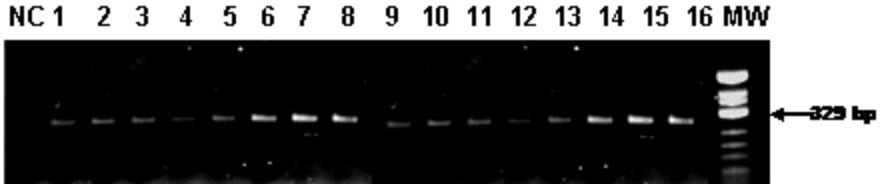
Figure 7f : Agarose gel electrophoretogram showing sensitivity *Fusarium* specific PCR



Lane 1: Negative control **Lane 2:** *F. solani* ATCC 36031 10 µg (undiluted) **Lane 3 - 9:** Serial 10 fold dilutions of *F. solani* DNA ranging from 10 µg to 1 pg showing positivity ; **Lane 11 -15 :** Serial 10 fold dilutions of *F. solani* DNA ranging from 100 femtograms to 10 ottograms of *F. solani* DNA showing no amplification Mw: Molecular weight marker : *Hinf* – I digest of Phi X 174 bacteriophage DNA

Sensitivty: 1pg

Figure 7g: Agarose gel electrophoretogram showing specificity of genus specific *Fusarium* PCR

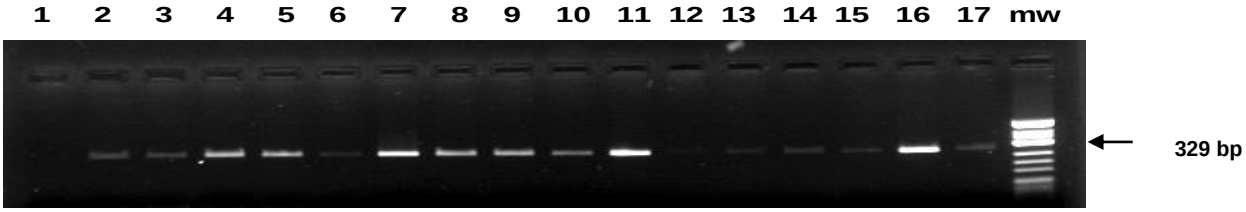


Lane NC: Negative control **Lane 1,2:** *F. lichenicola* (Laboratory isolates) **Lane 3,4,5,9,10,11,13,14,15:** *F. falciforme* (Laboratory isolates) **Lane 12 :** *F. moniliforme* (Laboratory isolate) **Lane 6**: *F. solani* (Laboratory isolate) **Lane 7 :** *F. solani* ATCC 36031 **Lane 8:** *F. oxysporum* (Laboratory isolate) **MW:** Molecular weight marker : *Hinf* – I digest of Phi X 174 bacteriophage DNA

7.4.6 Application of genus specific *Fusarium* species on laboratory isolates

The genus specific *Fusarium* PCR was applied on laboratory isolates of *Fusarium* species obtained from 25 Corneal scrapings, 19 corneal button, 6 eviscerated materials of fungal keratitis The PCR amplified all the 50 strains of Fusarium tested , amplifying 25 *F. falciforme*, 11 *F. lichenicola*, 8 *F. solani*, 3 each of *F. oxysporum* and *F. moniliforme* The application of genus specific Fusarium PCR is shown in Figure 7h

Figure 7h: Agarose gel electrophoretogram showing the application of genus specific *Fusarium* PCR

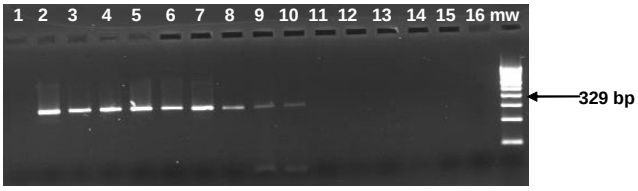


Lane 1: Negative control **Lane 2,3,4,5,6,8:** *F. falciforme* **Lane 7 :** *F. solani* **Lane 9, 10,11 :***F. lichenicola* **Lane12,13,14:** *F. oxysporum* **Lane 15,16 :** *F. moniliforme* **Lane 17:** *F. solani* ATCC 36031 **MW:** Molecular weight marker *Hinf* – I digest of Phi X174 bacteriophage DNA

Sensitivity and specificity of *F. solani* PCR:

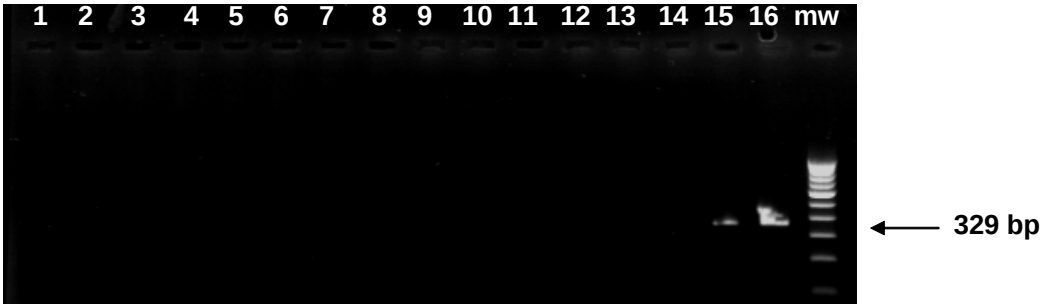
The sensitivity of *F. solani* PCR performed using ATCC *F. solani* DNA had a sensitivity to detect 10 pg of *F. solani* DNA.(Figure 7i). The results of specificity of *F.solani* PCR performed is shown in Figure 7j.

Figure 7i : Agarose gel electrophoretogram showing sensitivity *F. solani* PCR



Lane 1: Negative control **Lane 2 :** *F. solani* ATCC 36031 10 µg **Lane 3 to Lane 16**: Serial 10 fold dilutions of *F. solani* DNA **MW:** 100 bp ladder **Sensitivity : 10 pg**

Figure 7j : Agarose gel electrophoretogram showing specificity of *F. solani* PCR

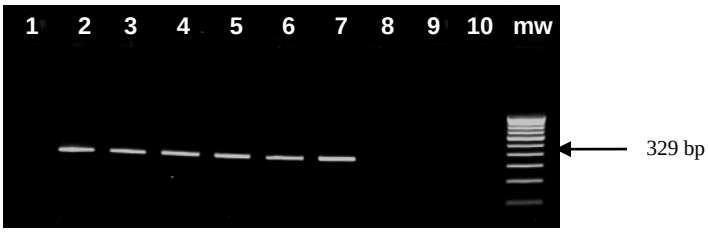


Lane 1: Negative control **Lane 2,3,4,5,6,8:** *F. falciforme* **Lane 7 :** *F. moniliforme* **Lane 9, 10, 11:** *F. lichenicola* **Lane12,13,14:** *F. oxysporum* **Lane 15:** *F. solani (laboratory isolate)* **Lane 16:** *Fusarium solani* ATCC 36031 **MW:** Molecular weight marker 100 bp DNA ladder

7.4.7 Sensitivity and specificity of *F. oxysporum* PCR:

The sensitivity of *F. oxysporum* PCR performed using laboratory isolate of *F. oxysporum* DNA had a sensitivity to detect 10 pg. The results of specificity of *F. oxysporum* PCR performed is shown in Figure 7m. There was no amplification obtained with other fungal, bacterial, viral, parasitic and human DNA.

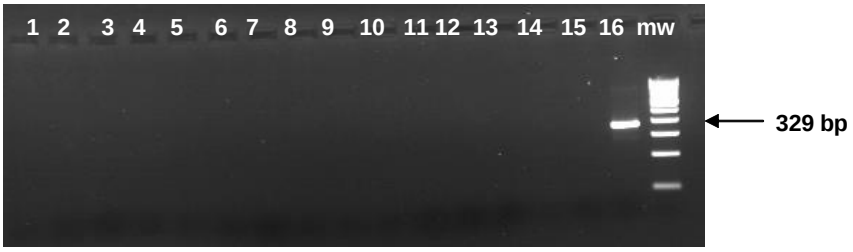
Figure 7k: Agarose gel electrophoretogram showing sensitivity of *F. oxysporum* PCR



Lane 1: Negative control **Lane 2 :** *F.* oxysporum DNA **Lane 3 to Lane 10**: Serial 10 fold dilutions of *F. oxysporum* DNA **Sensitivity : 10 pg**

MW : 100 bp DNA ladder

Figure 7l: Agarose gel electrophoretogram showing specificity of *F. oxysporum* PCR

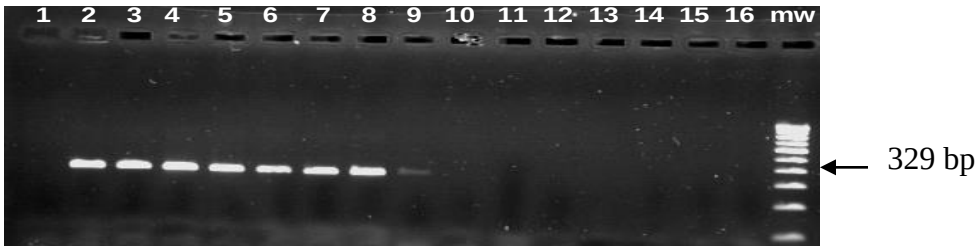


Lane 1: Negative control **Lane 2,3,4,5,6,8:** *F. falciforme* **Lane 7 :** *F. moniliforme* **Lane 9,10,11:** *F. lichenicola,* **Lane 12,13,14,15:** *F. solani* **Lane 16:** *F. oxysporum (laboratory isolate)* Positive control **MW:** Molecular weight marker 100 bp DNA ladder

7.4.8 Sensitivity and specificity of *F. moniliforme* PCR

The results of sensitivity of specific *F. moiliforme* PCR is shown in Figure 7n. The sensitivity of *F. moniliforme* was found to be 0.1 picogram The results of specificity of *Fusarium moniliforme* PCR performed is shown in Figure 7o.

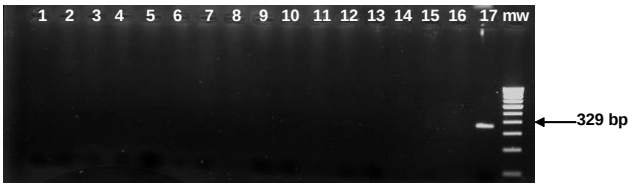
Figure 7m: Agarose gel electrophoretogram showing sensitivity of *F. moniliforme* PCR



Lane 1: Negative control **Lane 2 :** *F.* oxysporum DNA **Lane 3 to Lane 16**: Serial 10 fold dilutions of *F. moniliforme* DNA **MW :** 100 bp DNA ladder

Sensitivity : 0.1 picogram

Figure 7n : Agarose gel electrophoretogram showing specificity of *F.moniliforme* PCR



Lane 1: Negative control **Lane 2, 3, 4, 5, 6,8:** *F. falciforme* **Lane 7** : *F.moniliforme*

Lane 9,10,11: *F. lichenicola* **Lane 12,13,14:** *F. oxysporum* **Lane 15:** *F. solani*

Lane 17: *F. moniliforme (laboratory isolate)* **MW:** 100 bp DNA ladder

7.4.9 Application of species specific *Fusarium* PCR on laboratory isolates

The results of *F. soalni, F.oxysporum and F. moniliforme* PCR s applied on laboratory isolates are shown in Figure 7o , 7p, 7q respectively.

Figure 7o **Figure 7p** **Figure 7q**

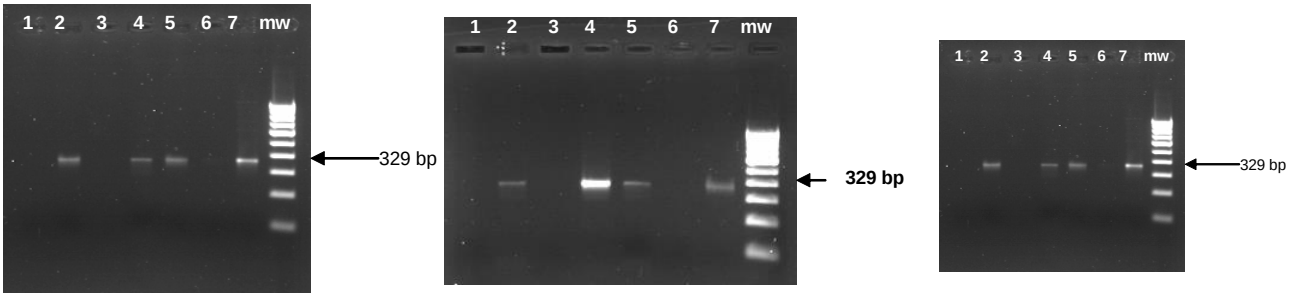


Figure 7o: Agarose gel electrophoretogram showing the results of *F. solani* PCR

Lane 1: Negative control **Lane 2, 4,5:** *F. solani* isolates Positive **Lane 3 ,6 Negative**

Lane 7: *F. solani* ATCC 36031 Positive control **MW:** 100 bpDNA ladder

Figure 7p: Agarose gel electrophoretogram showing the results of *F. oxysporum* PCR

Lane 1: Negative control **Lane 2,4,5:** *F. oxysporum* positive **Lane 3,6:** Negative

Lane 7: Positive control *F. oxysporum* (laboratory isolate) **MW** : 100 bp DNA ladder

Figure 7q: Agarose gel electrophoretogram showing the results of *F. moniliforme* PCR

Lane 1: Negative control **Lane 2,4,5:** *F. moniliforme* positive **Lane 3, 6 :** Negative

Lane 7: Positive control *F. moniliforme* (laboratory isolate) **MW :** 100 bp DNA ladder

The results of application of *Fusarium* species specific PCR is tabulated in Table 7b.

Table 7b Application of *Fusarium* species specific PCR on laboratory isolates

Ocular specimens 50	**Laboratory isolates**	**Result of *F. solani* PCR**	**Result of *F. oxysporum* PCR**	**Result of *F. moniliforme* PCR**
Corneal scraping 4 Corneal button 4	*F. solani*	**Positive**	**Negative**	**Negative**
Corneal scraping 1 Corneal button 1 Eviscerated material 1	*F. oxysporum*	**Negative**	**Positive**	**Negative**
Eviscerated material 3	*F. moniliforme*	**Negative**	**Negative**	**Positive**
Corneal scraping 14 Corneal button 10 Eviscerated material 1	*F. falciforme*	**Negative**	**Negative**	**Negative**
Corneal scraping 6 Corneal button 4 Eviscerated material 1	*F. lichenicola*	**Negative**	**Negative**	**Negative**

7.5 Discussion

Fusarium species are considered to be the third most common fungi isolated from human systemic infections and this genus consists of over 100 species [Ferrer et al, 2005] and the human pathogenic species are *F. solani* and *F. oxysporum* (30% of infections) [Hennequin et al, 1999] and *F. moniliforme* accounting for more than 95% of human infections, while *Fusarium dimerum* [Guarro, 1992 Hennequin et al, 1997) causes 5% of infections. The most common species involved in ocular infections are *F. solani* and *F. oxysporum*. [Alexanderakis et al, 2000, Foroozon et al, 2001 Lowe , 1994, Weissgold et al, 1998] Other species such as *F. dimerum* [Vismer et al, 2001] and *F. verticillioides* [Freidank et al, 1995] are rarely involved in ocular infections.Currently, the identification of members of the genus *Fusarium* is based on the characteristic colony morphology and microscopic characters, which include production of multiseptate sickle shaped conidia called macroconidia, however, recognition may be difficult which if not produced in culture become difficult to identify.[Boutati et al, 1997] Most cases of invasive fusariosis are caused by *F. solani, F. oxysporum*, *F. moniliforme* but in about one third the species is not identified.[Segal et al, 1995] This usually happens with strains isolated from clinical specimens which develop during unfavourable conditions. In this case, the isolates can be confused with genera such as *Acremonium and Verticillium* .

Identification of *Fusarium* to the species level is necessary because new antifungal agents exhibit variable activity depending on the species of *Fusarium* and also is important for epidemiological purposes. Species level identification of *Fusarium* based on conventional methods can be best observed after 2 weeks of incubation, prolonging/delaying the time for a definitive identification. Moreover the precise identification based on the important variations of characteristic features such as pigmentation production of microconidia and chlamydoconidia and growth rate that are often seen within a given species requires expertise of trained mycologists. The results are frequently inconclusive, where one third to one half of the isolates are not identified to the species level.[Paz et al, 1999] The macroscopic and microscopic morphological features of *Fusarium* genus often change in subsequent sub – culture. The species level identification of *Fusarium* is difficult because of their special growth requirements and subtle morphological difference and the need for

correct interpretation of their morphological features [Paz et al,1 999,Liu et al, 1999]. The conventional methods of identification are time consuming requiring a minimum of 7 days as against PCR based RFLP and DNA sequencing in which a definitive result is obtained within 8 hours.

Several molecular methods involving PCR (Jaeger 2000,Paatz et al, 2004,Pujol et al, 1997)and DNA sequencing (Hennequin et al, 1999) have been developed to identify *Fusarium* species .The precise determination of *Fusarium* species remains a prerequisite, for studying the spread of human infections and treatment. The present study was to evaluate the PCR- RFLP and DNA sequencing techniques and compare their results with conventional techniques for identification of *Fusarium* isolates.

The present study was focused on development of a molecular techniques PCR-RFLP and DNA sequencing for rapid species level identification as *Fusarium* species is the second most common isolate in our laboratory, which is a tertiary eye care hospital located in South India. As the ribosomal RNA is highly conserved and universally found in living cells, it was targeted as a powerful taxonomic indicator in this study. The development of molecular techniques for rapid and easy sequencing of long stretches of 18S or 28S have opened the way for systematic exploitation of the remarkable proportion of these molecules as phylogenetic indicators. The present study aims at evaluating the rRNA sequence methodology as a tool for rapid identification of species.[Geralch 1982] The 28SrRNA sequences after DNA sequencing of *Fusarium* species were subjected to BLAST analysis. Based on the highest percentage of similarity, species were identified. Based on the BLAST analysis, a homology table consisting of 8 *Fusarium* isolates was constructed . *F. falciforme* and *F. solani* exhibited 100% homology with the available *Fusarium* sequences in the data base. There is always a subtle difference between *Fusarium* and *Acremonium* species, inorder to make precise identification *F. falciforme* sequences were compared with that of *Acremonium* species (Accession numbers:AY097319 AF130386) .DNA sequencing followed by multalign analysis revealed minor nucleotide changes in positions 390 (C to A) and 555 (A to C).The multalin analysis of *F.lichenicola* (M 1962) revealed 100% homology with standard strains (Accession number AYO97322,AY097323) but differed in positions 43 (A to C),105 (A to G),116 (T to C),150 (T to C),154 ,155(CT to TC),162(T to C), and 208

(C to T), with the standard strain(Accession number AYO97325) Multalin analysis of *F.solani* revealed 100% homology with standard strain (Accession number AY093716) but differed with the other two standard strains (Accession number AY093717,AY093718 in position 178 (T to C) while all *F. falciforme* isolates revealed 100% homology with the standard strains.

Based on the DNA sequences a phylogenetic tree was also constructed as precise knowledge of genetic distances between strains would help in biological manipulations like redefining the taxonomy or identifying a new species.

In this present study results of PCR based RFLP proves to be a reliable method, in which the interpretation of results was easier depending on the pattern of cleavage than the conventional methods where the interpretation of the characteristic features are dependent on the type and quality of media used and it is also highly subjective. *F. falciforme* (50%)emerged as the most common one followed by *F. lichenicola,*(22%) *F. solani*, (16%) *F. oxysporum* and *F.monilifome* (6%). DNA sequencing targeting D2 large subunit of ribosomal RNA gene region revealed a similarity of 95.5%. The identification of *Fusarium* species using two different molecular targets proved to be reliable for precise identification as mismatches or dissimilarity encountered with a particular target could be corrected by the other target. Hence in this study, two molecular targets of 28SrRNA and ITS regions were used to characterize *Fusarium* species.

Molecular analysis of ribosomal ITS region sequences of several *Fusarium* species of medical interest demonstrated that the difference existing in the ITS region is useful in identifying *Fusarium* to species level.[Liu et al, 1998] In contrast to the case with conventional methods, the results could be obtained within 8 hours from a day old culture and are independent of requirement for an experienced mycologist. Hence PCR based RFLP targeting ITS region is a rapid and reliable method for speciating the *Fusarium* isolates on the same day of isolation from any clinical specimen. The genus specific PCR applied on laboratory isolates of 50 *Fusarium* species amplified all the isolates. *F.solani* 8, *F. moniliforme* and *F. oxysporum* 3 each were amplified by the respective species specific PCR. There was no amplification obtained with *F. falciforme, F.lichenicola* when these 3 species specific PCRs were

applied.The results of ITS PCR-RFLP and that of *Fusarium* PCR were found to be concordant. There were no discrepant results and there was no cross reaction of the primers. The *Fusarium* genus specific PCR was specific and the 3 species specific primers of *Fusarium* did not cross react with the other species.The development of genus and species specific PCR would aid in identifying the agent rapidly within 6 hours of isolation. The main advantage is that is it avoids the use of sophisticated instrument like DNA sequencer. This technique proves to be cost effective to identify *Fusarium*, however primers needs to be designed to amplify F. *falciforme and F. lichenicola*

7.6 Conclusion:

To conclude, the application of PCR-RFLP and DNA sequencing technique to identify the ocular isolates of *Fusarium* species was found to be a rapid, reproducible technique to identify *Fusarium* species. The DNA sequencing results targeting the D2 region of large subunit were concordant and confirmed the PCR-RFLP results of identification.Though the DNA sequencing requires additional infrastructure it has an added advantage of determining newly emerging species and redefining the phylogeny

7.7 Future prospect:

The genes coding for production of pigments in *Fusarium* needs to be investigated and primers or probes to detect and identify them by PCRand /or hybridization, microarray would facilitate rapid identification of these organisms.

CHAPTER - 8

In Vitro Antifungal Sensitivity Testing to Determine the Minimum Inhibitory Concentrations of Amphotericin B, Ketoconazole and Fluconazole on Ocular Fungal Isolates

8.1 Review of literature

The frequency of life-threatening fungal infections is rising worldwide. The need for effective antifungal therapies has been more acute since the emergence of AIDS and AIDS related complex, which are often associated with opportunistic fungal infections The failure of drugs to treat fungal infections combined with improvements in performance and standardization of antifungal susceptibility testing have drawn attention to the problem of antifungal drug resistance. It is now clear that antifungal agents can create clinical and epidemiological situations that are analogous to those found with antibiotic-resistant bacteria. Fungi are becoming very important human pathogens, and there are few drugs that can inhibit fungal growth. The epidemiological combination of these two factors predicts that very soon fungi will acquire new genetic determinants that will provide multi drug resistance
(Walsh et al, 1997)

In the 1990s, drug resistance has become an important problem in a variety of infectious diseases including human immuno deficiency virus (HIV) infection, tuberculosis, and other bacterial infections which have profound effects on human health. At the same time, there have been dramatic increases in the incidence of fungal infections, which are probably the result of alterations in immune status associated with the AIDS epidemic, cancer chemotherapy, and organ and bone marrow transplantation. Although extremely rare 10 years ago, antifungal drug resistance is quickly becoming a major problem in certain populations, especially those infected with HIV, in whom drug resistance of the agent causing oropharyngeal candidiasis is a major problem. For instance, 33% of late-stage AIDS patients in one study had drug-resistant strains of *C. albicans* in their oral cavities.

There are no large-scale surveys of the extent of antifungal drug resistance, which has prompted requests for an international epidemiological survey of this problem. beyond testing *Candida* spp. it is clear that the need for meaningful susceptibility test results is as great for the fungi as it is for the bacteria.. Knowledge of mechanisms of antifungal resistance has been valuable in identifying resistant isolates and using them to validate *in vitro* measurement systems. The application of the concepts of pharmacodynamics to antifungal susceptibility testing has provided significant new insights into both the methods for and interpretation of antifungal susceptibility testing. In addition, novel techniques for susceptibility testing provide useful additional tools and insights. (Rex et al, 2001)

8.1.1 Determination of Minimal Inhibitory Concentrations (MICs) by broth dilution method

Determination of MICs by broth dilution method for yeasts is done using M 27 A (National Committee for Clinical Laboratory Standards) method..The test medium used is RPMI 1640 dissolved in 0.165 MOPS buffer pH 7.The standardized inoculum contains 0.5-2.5 x 10^3 CFU / ml. and the incubation temperature is 35°C. The end point is determined as optically clear for Amphotericin B and approximately 80% reduction in growth for other azoles (Horne et al, 1997). Following principles of testing yeasts a proposed Standard method," Reference method for broth dilution of susceptibility testing of conidium forming filamentous fungi NCCLS M - 38 P (NCCLS document, Wayne 1997, 1998) was approved. This method is found to be suitable for *Aspergillus, Fusarium, Rhizopus, P. boydii, S. schenkii, Paecilmyces, Cladosporium, Scedosporium* species. The standardized inoculum contains 0.4-5x10^4CFU / ml and the incubation temperature is 35°C. The end point is determined as optically clear for Amphotericin B and approximately 50% reduction in growth for other azoles (Lees et al, 1995).Khan et al, 2006 have recently reported a semi solid agar dilution method to determine the MIC of antifungal agents.

8.1.2 E test

E test is a proprietary, commercially available method for antimicrobial susceptibility testing. MICs are determined from the point of intersection of a growth inhibition zone

with a calibrated strip impregnated with a gradient of antimicrobial concentration and placed on an agar plate lawned with the microbial isolate under test. This methodology has been adapted to a number of antifungal agents. Both non uniform growth of the fungal lawn and the frequent presence of a feathered or trailing growth edge can make end-point determination difficult. However, with experience and standardized techniques, the correlation between this method and the reference method has been acceptable for most *Candida* spp. and the azole antifungal agents (Warnock et al , 1998)

8.1.3 Definition of Resistance and Role of Susceptibility Testing

Before the development of susceptibility testing of yeasts as outlined by the National Committee for Clinical Laboratory Standards (NCCLS), MIC determinations were inconsistent and varied up to 50,000-fold in different laboratories. NCCLS document M27, first published in 1992, has recently been revised (M27-A) and subsequent studies have demonstrated that the interlaboratory reproducibility of MIC determination approximates that of antibacterial testing. The macrodilution method has set the groundwork for the development of less cumbersome methods adapted for the clinical laboratory. These methods, relying on microdilution with or without colorimetric indicators or agar diffusion, have been shown to be reproducible and consistent with the standardized macrodilution method according to preliminary studies .One complication of the NCCLS protocol is that for certain isolates, the timing of the end point determination can have a major effect (up to 128-fold) on the MIC. A recent study suggests that determining the end point after 24 instead of 48 h ensures that the MICs for these isolates correlate with the in vivo response to azoles. In addition, there have been significant advances in susceptibility testing for filamentous fungi as well as in vitro testing of amphotericin B susceptibility. Historically, clinical resistance has been defined as persistence or progression of an infection despite appropriate antimicrobial therapy. A successful clinical response to antimicrobial therapy typically not only depends on the susceptibility of the pathogenic organism but also relies heavily on the host immune system, drug penetration and distribution, patient compliance, and absence of a protected or

persistent focus of infection (e.g., a catheter or abscess). This is particularly true for fungal infections .The in vitro resistance of an isolate can be described as either primary or secondary. An organism that is resistant to a drug prior to exposure is described as having primary or intrinsic resistance. Secondary resistance develops in response to exposure to an antimicrobial agent. Both primary and secondary resistance to antifungal agents have been observed. A correlation of in vitro susceptibility with in vivo response has been observed for mucosal candidal infections in HIV infected patients. Many groups have noted that the clinical outcome is generally dependent on the in vitro susceptibility of the organism. One group performed fluconazole susceptibility testing on fungal isolates obtained from 87 HIV-infected patients. They observed that persistent oropharyngeal candidiasis correlated with infections caused by yeasts for which the MICs of fluconazole were high. However, host variables become important when in vivo resistanceis compared with in vitro MIC testing, especially in evaluating systemic infections . The more immuno compromised the host, the less reliable is the correlation between in vivo resistance and MIC. The ability of *C. albicans* to form biofilms on surfaces (catheters, teeth, and endothelial cells) has been implicated as a cause of clinical “resistance” despite microbial susceptibility in vitro. One study demonstrated an increased resistance in the cells of the biofilm compared to that in cells growing in suspension (Hawser et al, 1995). Hence it becomes important to practice antifungal sensitivity testing in a routine laboratory similar to that of antibacterial sensitivity testing.

8.1.4 Susceptibilty testing of ocular fungal isolates

The clinical relevance of antifungal susceptibility testing is thought to lie in guiding the clinician in the selection of an appropriate antifungal compound. Such tests have been reported to help in the selection of the appropriate antifungal in different ophthalmic mycoses (Ishibashi et al 1984, Jones et al 1975, Maskin et al 1989, Massry et al 1996). Unfortunately, many of these reports have not provided details of the test procedures used, the criteria by which MICs were deemed significant, details of the severity of the clinical lesions, or the criteria used for authentic diagnosis of mycotic infection. The use of reproducible tests conforming to rigorous standards, such as the approved document (M27A) of the National Committee for Clinical Laboratory Standards (NCCLS) new terminology – Clinical Laboratory Standards

Institute (CLSI) recommended the sensitivity testing of yeasts (Wayne 1997) standard method for susceptibility testing of filamentous fungi, especially *Aspergillus* spp., may clarify in the future whether antifungal susceptibility testing is at all useful in guiding the therapy of ophthalmic mycoses. Interestingly, when the *in vitro* antifungal susceptibilities of nine isolates of filamentous fungi were determined by the NCCLS method in 11 different laboratories and compared to antifungal treatment outcomes in animal infection models, only a limited association between MIC and treatment outcome was seen, due to drawbacks in the models used (Odds et al, 1998). *Curvularia senegalensis* was isolated from a patient with mycotic keratitis, and the MIC of itraconazole for this isolate was found (by a broth microdilution method performed as described by NCCLS guidelines for filamentous fungi) to be 0.25 μg/ml; however, the patient did not respond to antifungal therapy with natamycin or itraconazole (Guarro et al, 1999 c). Recently , voriconazole proved to be a potent drug to treat fungal endophthalmitis.(Sen et al, 2006) Above all, the relationship between in vitro susceptibility data and clinical response to topical antifungal medication needs to be clarified; hitherto, no studies have been performed in this important area.

Though numerous methods are available for antifungal sensitivity testing,a simple and economic technique of agar dilution method was standardized to determine the MIC s of amphotericin B, fluconazole and Ketoconazole on ocular fungal isolates in this study.

8.2 Objective

- To standardize agar dilution method using standard ATCC strains of *Candida* species to determine MIC of amphotericin B, ketoconazole and fluconazole
- To determine MIC of amphotericin B, ketoconazole and fluconazole on ocular fungal isolates by agar dilution method.

8.3 Materials and Methods:

The *in vitro* antifungal susceptibility testing was carried out with the standard strains of *Candida* species and ocular isolates of fungi during the period between

December 1999 – May 2002 in Larsen and Toubro Microbiology Research Centre, Sankara Nethralaya, Chennai, India.

8.3.1 Fungal strains

Standard strains of *Candida* species *C. albicans* ATCC 24433,*C. albicans* ATCC 90028, *Candida parapsilosis* ATCC 90018,*Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6258,*Candida tropicalis* ATCC 750

These strains were included for standardization of *in-vitro* susceptibility testing, based on guidelines of Clinical Laboratory Standards Institute (CLSI) which states that ideal reference strains for quality control have MICs that fall near the mid-range of the concentration for all antifungal drugs.

8.3.2 Ocular isolates of fungi included in the study

Fifty ocular isolates of yeasts and 130 filamentous fungi were included in the study. The distribution of the isolates of yeasts and filamentous fungi from ocular specimens are shown in Tables 8a and 8b The fungi were isolated by the standard procedures (Forbes et al, 2000) from corneal scrapings of keratitis and endophthalmitis patients. The yeasts were identified by the standard germ tube test and fermentation and assimilation of carbohydrate tests. The filamentous fungi were identified based on colony morphology and by Lactophenol cotton blue mount preparation of the slide cultures. The fungal isolates of donor corneal rim (DCR) and conjunctival swab were not associated with any clinical disease. However, these were also included to determine the MICs of amphotericin B,ketoconazole and fluconazole on ocular fungal isolates.

8.3.3 Preparation of stock solutions of antifungal agents

The guidelines of CLSI was followed for preparing the various concentrations of antifungal drugs. The active components in amphotericin B obtained was 78% and ketoconazole and fluconazole were 70%..

- **Amphotericin B**

  The stock solution of amphotericin B (HiMedia, India) was prepared by using 10

mg of amphotericin B dissolved in 1000 mL of dimethylsulfoxide (DMSO). (Makimura et al, 2004) The working solution was prepared by 1 in 1000 dilution. The range of concentrations of amphotericin B was 0.0625-8 µg/mL; DMSO was used to dissolve amphotericin B following NCCLS guidelines.(Pfaller et al, 1997)

- **Fluconazole** (Cipla Pharmaceuticals Limited, India) stock was 2 mg/mL. The aqueous stock solution was used directly without any further dilutions to obtain concentrations ranging from 0.2-102.4 µg/mL
- **Ketoconazole** (Cadila Pharmaceuticals, India) stock solution was prepared by dissolving 2 mg of ketoconazole in 1 mL of methanol according to manufacturer's instructions. Working solution was 1 in 100 dilution to obtain concentrations ranging from 0.025-12.8 µg/mL. When water was used to dissolve ketoconazole, it resulted in precipitation of the drug. Hence, methanol was used to dissolve ketoconazole and diluted 100 times from the stock solution. The intermediate solution was further diluted to the final strength in the test medium. This procedure avoided the dilution artifacts that result from precipitation of compounds with low solubility in aqueous media and the same dilution of methanol was used without the drug for the control plate to ensure that the particular concentration did not inhibit the growth of the fungal strains included .

Table 8a Distribution of ocular isolates of 50 yeasts included in the study for antifungal susceptibility testing by agar dilution method

Ocular fungal isolates	**Cor scrap** **7**	**DCR** **25**	**Conj swab** **12**	**VF** **6**	**Total** **50**
C. albicans	2	5	4	3	14
C. tropicalis	1	4	3	1	9
C. parapsilosis	-	5	2	2	9
C. krusei	1	2	1	-	4
C. guillermondii	-	1	-	-	1
C. lipolytica	1	4	1	-	6
T. beigellei	1	3	1	-	5
R. rubra	1	1	-	-	2

Cor srap: Corneal scraping **Cor button:** Corneal button **DCR:** Donor Corneal rim

Conj swab: Conjunctival swab **VF:** Vitreous fluid

Table 8b Distribution of 130 ocular isolates of filamentous fungi included in the study for antifungal susceptibility testing by agar dilution method

Ocular fungal isolates	Cor Scrap	Cor button	DCR	Evis matl	AH	VF	Others	Total
	47	**23**	**23**	**12**	**3**	**9**	**13**	**130**
A. flavus	15	7	3	4	1	2	4	36
A. fumigatus	8	5	3	1	-	1	1	19
A terreus	3	1	2	2	1	1	2	12
A. niger	2	2	2	1	-	1	1	9
Fusarium sp	14	6	4	4	1	3	1	33
Penicillium sp	2	-	7	-	-	-	2	11
Paecilomyces sp	-	-	1	-	-	-	1	2
Dematiaceous fungi	3	2	1	-	-	-	1	8

Cor srap: Corneal scraping **Cor button:** Corneal button **DCR:** Donor Corneal rim

Evis matl: Eviscerated material **AH :** Aqueous humor **VF:** Vitreous fluid **sp:** species

8.3.4 Preparation of stock solutions of antifungal agents

- **Amphotericin B (**Hi Media, India)
  The Stock I solution of amphotericin B was prepared by using 10 mg of amphotericin B dissolved in 1000 μl of Dimethylsulfoxide (DMSO). The working solution was prepared by1 in 1000 dilution.
- **Fluconazole (**Cipla Pharmaceuticals Limited, India): The concentration of the stock was 2mg/ml. The stock solution was used directly without any further dilutions.
- **Ketoconazole** (Cadila Pharmaceuticals, India): The stock solution was prepared by dissolving 2 mg of ketoconazole in 1ml of methanol. 1 in 100 dilution was used as working solution.

8.3.5 Culture Medium used

The medium yeast nitrogen base (Himedia, India) was dissolved in phosphate buffer pH 7 and it was autoclaved at 110 ° C for 10 minutes and at 10 lbs pressure. 10 ml of medium was dispensed in sterile BHIB bottles and the required concentrations of amphotericin B, fluconazole and ketoconazole (doubling dilutions) were incorporated

in the medium. With each set a growth control without the antifungal agent and solvent control DMSO for amphotericin B and methanol control for ketoconazole were included.

8.3.6 Range of concentrations of the antifungal agents

The MIC was determined by doubling dilutions of antifungal agents for all the three antifungal agents. The range of concentrations of amphotericin B was 0.0625–8 µ g/ml; fluconazole was 0.2 – 819.2 µg/ml and for ketoconazole 0.025 – 12.8 µg/ml

8.3.7 Preparation of standard inoculum

The fungal strains were freshly sub cultured on to Sabouraud's dextrose agar and incubated at 25°C for 3 days. The yeast cells and spores were suspended in sterile distilled water and counted using Neubauer chamber to obtain 10^5 cells/ ml. Ten µl of standardized suspension was inoculated onto the control plates and the media incorporated with the antifungal agents. The inoculated plates were incubated at 25˚C for 48 hours. The readings were taken at the end of 48 hours.

8.3.8 Interpretation of results :

The minimal inhibitory concentration (MIC) of the drug was determined as the minimum concentration of the drug showing no growth as compared to that of the respective fungi in the control plate.

8.3.9 Criteria for susceptibility / resistance

The criteria for susceptibility / resistance for amphotericin B, fluconazole and ketoconazole (Ingroff et al, 2004) for the antifungal drugs is given in Table 8c. Percentile 90 and Percentile 50 of amphotericin B, fluconazole and ketoconazole were calculated for 159 ocular isolates.

Table 8c Criteria for susceptibility / resistance for the antifungal drugs

Antifungal agents	**Susceptible**	**Resistant**
Amphotericin B	≤ 1 μg/ml	> 1μg/ml
Ketoconazole	≤ 0.8 μg/ml	> 0.8μg/ml
Fluconazole	≤ 51.2 μg/ml	> 51.2 μg/ml

8.4 Results:

8.4.1 Standardization of *in vitro* susceptibility testing by agar dilution method

The *in vitro* susceptibility testing of amphotericin B, fluconazole and ketoconazole on fungi was standardized by agar dilution method with standard ATCC *Candida* species. The results of MICs of amphotericin B , fluconazole and ketoconazole on standard strains are given in Table 8d

Table 8d: Results of Minimum Inhibitory Concentrations (MICs) of amphotericin B, Fluconazole and Ketoconazole on ATCC *Candida* species

Standard ATCC Strains of Candida species	**MIC of Amphotericin B μg / ml**	**MIC of Fluconazole μg / ml**	**MIC of Ketoconazole μg / ml**
C. albicans 24433	0.25	12.8	0.4
C. albicans 90028	0.25	25.6	0.4
C.tropicalis 750	0.25	12.8	0.2
C. parapsilosis 90018	0.25	12.8	0.4
C. parapsilosis 22019	0.25	12.8	0.4
C krusei 6258	0.5	51.2	0.4

The standardized *in vitro* antifungal susceptibility testing technique was applied to determine the MICs of amphotericin B, fluconazole and ketoconazole on 180 (50 yeasts and130 filamentous fungi) ocular fungal isolates.

8.4.2 Results of MIC of amphotericin B on ocular fungal isolates

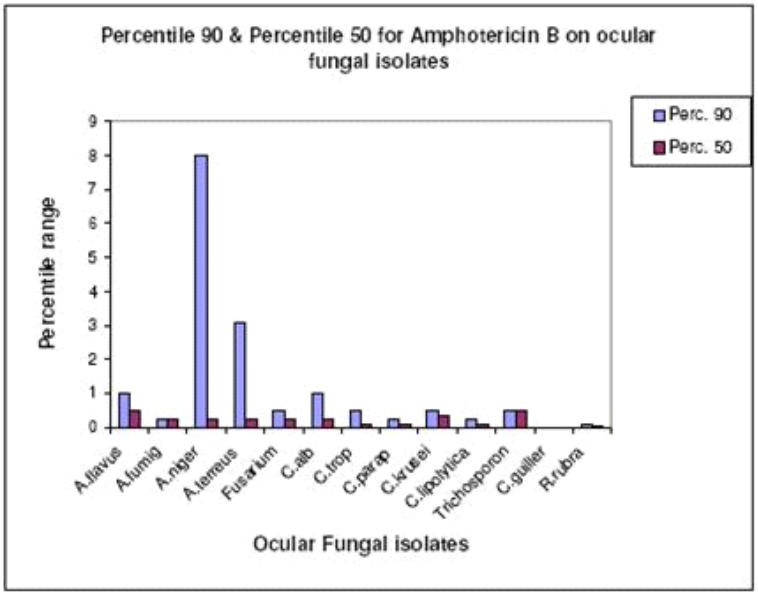
All the 50 Candida species tested were susceptible to amphotericin B . Among the 130 filamentous fungi tested, 124 (95.38%) were susceptible to amphotericin B while 6 (4.61%) were resistant to amphotericin B (Table 8e).

Table 8e : MIC of amphotericin B on ocular fungal isolates

Ocular fungal isolates	**Concentration of amphotericin B in μg / ml**								
	0.0625	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**Total**
Yeasts	12	18	12	6	2	0	0	0	50
Filamentous fungi	8	20	63	24	9	2	2	2	130
Total	**20**	**38**	**75**	**30**	**11**	**2**	**2**	**2**	**180**

The 6 resistant strains were *Aspergillus flavus* (1)*, Fusarium species* (2), were isolated from 3 Corneal scrapings, and *Aspergillus terreus*, *Aspergillus niger*, *Penicillium* species were isolated from DCR (3). Among the yeasts, the MIC of amphotericin B were within the range of 0.0625 – 0.125 μg/ml in 42 (84%) while 91 (70%) filamentous fungi had a MIC range of 0.0625 - 0.25 μg /ml. Percentile 90 and Percentile 50 of amphotericin B have been calculated and represented in Chart 8a..Based on the percentile values of amphotericin B, *A. niger* and *A.terreus* were found to have higher values when compared with other filamentous fungi.

Figure 8a: Percentile 90 and Percentile 50 of amphotericin B on ocular fungal Isolates



A.flavus : Aspergillus flavus
A.fumig : A.fumigatus
A.niger : A.niger
A.terreus : A.terreus
Fusarium : Fusarium species
Trichosporon :Trichosporon beigellei
R.rubra: Rhodotorula rubra

C.alb: Candida albicans
C.trop: Candida tropicalis
C.parap: Candida parapsilosis
C.krusei: Candida krusei
C.lipolytica: Candida lipolytica
C.guillermondii: Candida guillermondii

8.4.3 Results of MICs of fluconazole on ocular fungal isolates

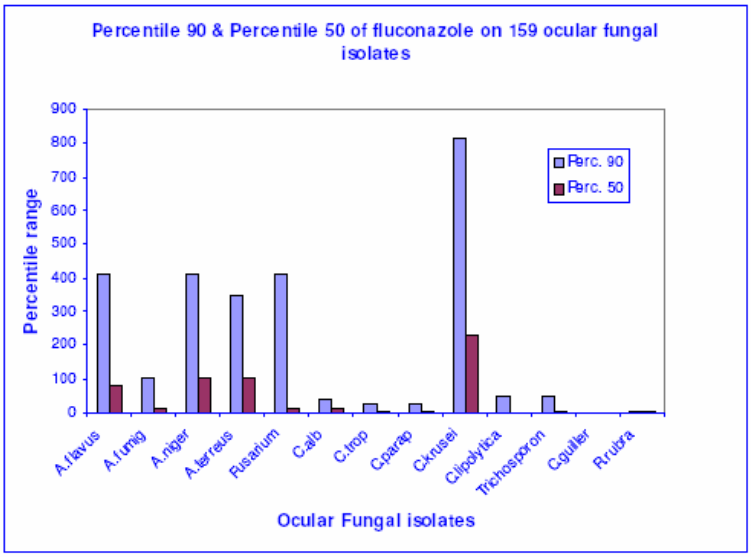
Of the 50 yeasts tested, 48 (96 %) were sensitive to fluconazole with MIC < 51.2 µg/ml , while 2 (4%) were resistant (MIC of fluconazole 102.4- 819.2 µg/ml).The two resistant yeasts *(Candida krusei)* were isolated from DCR and Corneal scraping. Among the 130 filamentous fungi tested, 81 (62.3%) were sensitive to fluconazole while 49 (37.7%) were resistant to fluconazole (Table 8g). The resistant strains were isolated from Corneal scraping (25), Corneal button (12), DCR (12).Among the yeasts 24 (48%) had MIC of fluconazole within the range of 0.2 – 6.4 µg /ml while 39 (30%) filamentous fungi had a MIC range of 25.6 or 51.2 µg /ml. Percentile 90 and Percentile 50 of fluconazole have been calculated and represented in figure 8b *Candida krusei* had the highest percentile value of fluconazole among yeasts and

A. flavus, *A.niger, A.terreus* and *Fusarium* species showed a higher percentile value for fluconazole.

Table 8g: MIC of fluconazole on ocular fungal isolates

Ocular fungal isolates	Concentration of fluconazole in μg / ml									
	0.2-3.2	**6.4**	**12.8**	**25.6**	**51.2**	**102.4**	**204.8**	**409.6**	**819.2**	**Total**
Yeasts	24	4	3	8	9	0	0	1	1	**50**
Filamentous fungi	16	10	16	17	22	21	11	16	1	**130**
Total	**40**	**14**	**19**	**25**	**31**	**21**	**11**	**17**	**2**	**180**

Figure 8b: Prcentile 90 and Percentile 50 of fluconazole on ocular fungal isolates



A.flavus : Aspergillus flavus
A.fumig : A.fumigatus
A.niger : A.niger
A.terreus : A.terreus
Fusarium : Fusarium species
Trichosporon :Trichosporon beigellei
R.rubra: Rhodotorula rubra

C.alb: Candida albicans
C.trop: Candida tropicalis
C.parap: Candida parapsilosis
C.krusei: Candida krusei
C.lipolytica: Candida lipolytica
C.guillermondii: Candida guillermondii

Table 8g : MIC of ketoconazole on ocular fungal isolates

Ocular fungal isolates	Concentration of ketoconazole in μg / ml										
	0.025	**0.05**	**0.1**	**0.2**	**0.4**	**0.8**	**1.6**	**3.2**	**6.4**	**12.8**	**Total**
Yeasts	2	6	8	6	11	12	1	2	2	0	**50**
Filamentous fungi	7	18	23	22	29	20	3	2	3	3	**130**
Total	**9**	**24**	**31**	**28**	**40**	**33**	**4**	**4**	**5**	**3**	**180**

8.4.4 Results of MICs of ketoconazole on ocular fungal isolates

Of the 50 yeasts tested 45 (90%) were sensitive to ketoconazole (MIC of ketoconazole < 0.8μg/ml) while 5 (10%) were resistant to ketoconazole (MIC of ketoconazole > 0.8 μg/ml).The resistant strains were isolated from 2 Corneal scraping (*C.albicans* 1, *C.tropicalis* 1) and 3 DCR (*C.krusei* 2 and *C.albicans* 1). Among the 130 filamentous fungi tested, 120 (92.3%)were sensitive to ketoconazole while 10 (7.6%) were resistant (Table 8g). The resistant strains were isolated from 3 Corneal scraping (*Aspergillus flavus*, *Aspergillus terreus*, *Fusarium* species),4 Corneal button (*Aspergillus flavus, Aspergillus terreus, Fusarium* 2) and 3 DCR (*Aspergillus niger* 2, *Penicillium* species 1). Among the yeasts 29 (58%) had MIC of ketoconazole within the range of 0.2 – 0.8 μg /ml while 71 (54.6%) filamentous fungi had a MIC range of 0.2 – 0.8 μg/ml. Based on the percentile values of ketoconazole, *A. niger* and *A. terreus* were found to have higher values when compared to other filamentous fungi. Among the yeasts tested *C.krusei* had a higher percentile value of ketoconazole.

The distribution of 72 resistant strains of ocular fungal isolates to amphotericin B, ketoconazole and fluconazole are given in Table 8h

Table 8h:Distribution of 72 strains of ocular fungal isolates resistant to amphotericin B, fluconazole and ketoconazole

Ocular isolates resistant to antifungal drugs N = 72		Amphotericin B* N = 6	Fluconazole@ N = 51	Ketoconazole# N = 15
Filamentous fungi	**(65)**			
A. flavus	36	1 (2.7)	18 (50)	2 (5.55)
A. fumigatus	19	0	4 (21)	0
A. terreus	12	1 (11.1)	6 (50)	2 (22.2)
A. niger	9	1 (8.3)	8 (88.8)	2 (16.6)
Fusarium species	33	2 (6.06)	8 (24.4)	3 (9.09)
Penicillium species	11	1 (9.09)	5 (45.5)	1 (9.09)
Yeasts				
(7)		0	0	2 (14.28)
C. albicans	14	0	2 (50)	2 (50)
*C. krus*ei	4	0	0	1 (11.1)
C. tropicalis	9			

* Amphotericin B resistance > 8μg /ml, @ fluconazole resistance > 51.2μg /ml
# ketoconazole resistance > 0.8 μg /ml, figures in parenthesis are in percentage

Figure 8c: Results of MIC of amphotericin B on ocular fungal isolates by agar dilution method

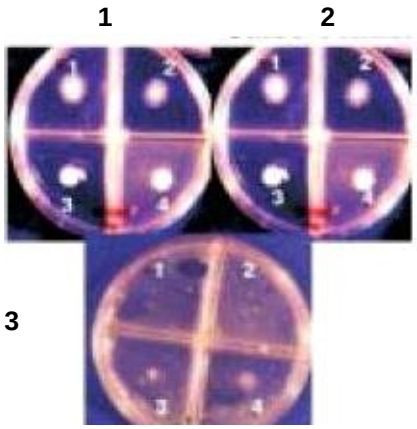


Figure 8c: 1. YNB control: Medium control, **2. DMSO Control:** Solvent control

3. Amphotericin B 1μg /ml : All the four isolates were susceptible to amphotericin B

Isolate 1: *A. niger* **Isolate 2 :** *A. fumigatus* **Isolate 3:***Penicillium* species

Isolate 4: *A. flavus A. niger* and *A. fumigatus* were found to be susceptible to amphotericin B while *A. flavus* and *Penicillium* species were resistant to amphotericin B

Figure 8d: Results of MIcs of fluconazole on ocular fungal isolates by agar dilution method

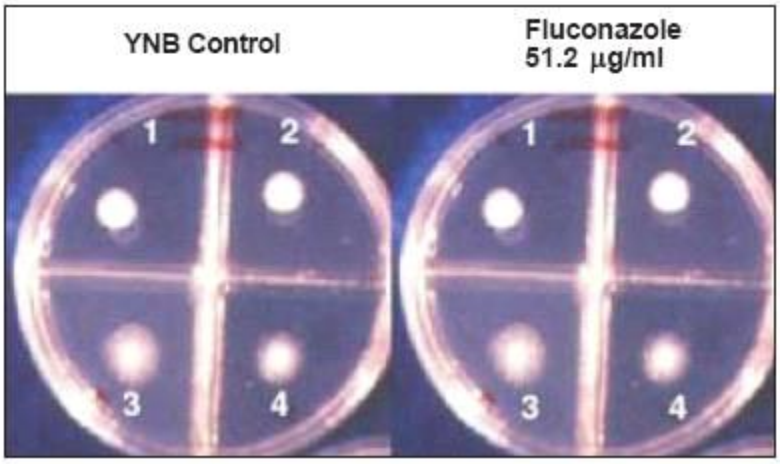


Figure 8d: 1. YNB control: Medium control, **2. Fluconazole 51.2 µg /ml :** All the four isolates were resistant to fluconazole **Isolate 1:** *A. flavus* **Isolate 2 :** *Fusarium species* **Isolate 3:***Penicillium* species **Isolate 4:** *A. niger*

8.5 Discussion

Fungi are opportunistic in the eye, since they rarely infect healthy, intact ocular tissues. An overwhelming number of fungal genera and species have been implicated as cause of ophthalmic mycoses and this number is steadily increasing. Fungal keratitis and endophthalmitis are the major ocular infections in developing countries. Mycotic keratitis apparently occurs much more frequently in India than in developed countries.(Thomas et al, 2003 b) Fungi were identified as the principal aetiological agent of corneal ulceration (44%) in India.(Leck et al, 2002) Fungal keratitis contributes to 6 to 56% (Gopinathan et al 2002) of keratitis and fungal endophthalmitis contributes to 4 to 11% of all cases of endophthalmitis.(Jaeger et al, 2001) The epidemiological pattern of corneal ulceration varies significantly from country to country and even from region to region. *Fusarium* species (47.1%) and *Aspergillus* species (16.1%) are the most common aetiological agents of corneal ulceration in India (Srinivasan et al, 1997) and natamycin (5%) or amphotericin B

(0.15%) remain the drug of choice for superficial keratitis.(Thomas et al, 2003 b) The recent explosion in the rates of opportunistic fungal infections, combined with the increasing number of reports of resistance to the available antifungal agents has propelled interest in clinically relevant methods for antifungal sensitivity testing. The need for antifungal susceptibility testing increases beyond testing *Candida* species because resistance to antifungal drugs have been demonstrated against such diverse fungi as *C.neoformans*, *A. fumigatus, A. terreus, H.capsulatum* and *T. species*. Hence it becomes evident that the need for meaningful susceptibility test result is very important for fungi as it is for bacteria (Ghannoum et al, 1996) Although antifungal susceptibility testing remains less well-established and utilized than antibacterial testing, the scientific support for its validity has benefited greatly by extrapolation from antibacterial testing.(Wanger et al, 1995)

The methods for antifungal sensitivity testing include Clinical Laboratory Standards Institute (CLSI) broth based methodology (M 27-A), CLSI methodology for moulds,(Pfaller et al, 1997) E-test agar based testing methods, flow cytometry and use of viability dyes. The above methods are time consuming and labour intensive, hence a more economical method such as agar dilution have been described (Yoshida et al, 1995) There are only a limited number of antifungal susceptibility testing reports on ocular fungal isolates from India.(Thomas et al 1987, 1988) The present study is focused on standardization of the *in vitro* agar dilution method for determination of minimum inhibitory concentration (MIC) of amphotericin B, ketoconazole and fluconazole for ocular fungal isolates**.**

The present study was designed to develop a cost –effective procedure (1.5 times cheaper than broth dilution) of agar dilution method to determine the MICs of antifungal drugs. Six ATCC strains were included in the study for correlation of the results with agar dilution method standardized in this study with the published reports of other standard methods of susceptibility testing. In a study conducted by Makimura et al 2004, *C. albicans* 90028 and *Candida parapsilosis* ATCC 90018 have been used to evaluate the standard dilution method against the frozen plate method. In yet another study (Arikan et al, 1999) involving proficiency testing, the other *Candida* species, *Candida parapsilosis* ATCC 22019, *Candida tropic*alis ATCC 750 and *Candida krusei* ATCC 6258 were used to evaluate the standard dilution method

against the frozen plate method. Interestingly, the majority of the ocular fungal isolates were susceptible to amphotericin B, followed by ketoconazole and fluconazole. The percentage of resistance to fluconazole and ketoconazole in the present study is comparatively lower than reported by Davey et al 1998 and Yoshida et al 1997. The results of the MIC of the antifungal agents by agar dilution method on standard ATCC strains of Candida species standardized in this study and the published reports of MIC by CLSI broth dilution method were comparable, indicating the suitability of the agar dilution method. The CLSI broth micro dilution method (M-27a) is time consuming, expensive and technically difficult to perform. On the other hand the agar dilution method has two important advantages over the CLS I method. The first is the visual reading based on the intensity of growth showing the clear end point of inhibition.(Rex et al, 2001) The second is that susceptibility testing of large number of fungi is easier and economical as four strains can be tested with one set of agar plates in the agar dilution method whereas each strain needs one set of tubes in the CLSI method. The emergence of antifungal drug resistance has made susceptibility testing important (Iyer et al, 2002) though the applicability of *in-vitro* antifungal sensitivity testing may not directly correlate well with the clinical outcome. The filamentous fungi resistant to amphotericin B in this study were *A. flavus, A. terreus and Fusarium* species. These findings correlated with the study conducted by Arikan et al 1999 in which resistance to fluconazole was observed in 37.7% and to ketoconazole in 8.7% of the filamentous fungi. The percentage of resistance of fluconazole and ketoconazole correlated with the report published earlier. In the present study, *Fusarium* species showed the highest resistance to fluconazole followed by *A. flavus and Penicillium* spp. An important finding in the present study is that all the resistant strains to the three antifungal drugs were from that of external ocular specimens and the percentage of resistance to amphotericin B, fluconazole and ketoconazole were comparable to that of the published reports.(Thomas, 2003) Additionally, among filamentous fungi, *A. niger* followed by *A. terreus* exhibited higher percentage of resistance to amphotericin B, fluconazole and ketoconazole and among the yeasts, *C. krusei* exhibited higher resistance to fluconazole and ketoconazole. Majority of the ocular fungal isolates included in the study were susceptible to amphotericinB when compared to ketoconazole and fluconazole which were effective mainly against yeasts. However, MIC results obtained on ocular fungal isolates by *in-vitro* sensitivity testing are only meaningful

when compared to the ocular tissue concentrations of the drugs obtained after oral, topical and parenteral administration. It should reliably predict the *in vivo* response to therapy in human infections. However, drug pharmacokinetics and drug interactions, factors related to the host immune response and/or the status of the current underlying disease, proper patient management system and factors related to the virulence of the infecting organism and its interactions with both the host and therapeutic agents appear to have more value than the MIC as predictors of clinical outcome.

The ability to generate an MIC is of little value without the corresponding ability to interpret its clinical meaning. However, this process is far from straightforward because (i) MICs are not a physical measurement, (ii) host factors play a critical role in determining clinical outcome, (iii) susceptibility in vitro does not uniformly predict clinical success in vivo, and (iv) resistance in vitro will often, but not always, correlate with treatment failure (Rex ,1997) . The initial publication of the M27 document included interpretive breakpoints for Candida spp. when tested against fluconazole, itraconazole, and flucytosine. The data for each drug had different strengths and weaknesses for determining these interpretive breakpoints, A weakness that applies to all of the MIC-clinical outcome correlation data sets is the relative paucity of clinical outcome data for isolates for which the MICs are elevated. Such potentially resistant isolates are infrequently encountered in most early clinical trials, and this circumstance limits the strength of proposed breakpoints. A second limitation, unique to antifungaldrugs, is that all (itraconazole) or most (fluconazole) of the data were derived from studies of mucosal candidiasis. There are fewer data for invasive candidiasis. Despite these limitations, the general level of clinical correlation achieved to date is similar to that seen with antibacterial agents.

Due to the small patient population of *Aspergillus* infections, the use of animal models is the only method employed in order to detect azole resistance *in vivo*. However, discrepancies have been found with regards to the susceptibility of the same strain when tested in different models. It seems that the response of a strain in animal models depends on the treatment dose and the onset timing of the treatment. In most *in vivo* experiments Itraconazole (ITZ) has shown a dose-dependent response as has posaconazole *in vivo* resistant strain. Another problem in

interpretation when using animal models is the variable ITZ absorption, highlighting the importance of documenting adequate ITZ levels. Furthermore, the *Aspergillus* patient population is heterogeneous. Factors affecting outcome include drug pharmacokinetics ,compliance, drug–drug interactions and severity of disease. Thus, clinical outcome is probably affected not only by the intrinsic susceptibility of a strain against any given antifungal drug, but to an important extent by the state of the patient. For this reason, and the low frequency of resistance, establishing breakpoints for resistance will be difficult based on clinical studies alone. Break points have yet to be formally proposed.

8.6 Conclusion

The agar dilution method can be adopted for *in-vitro* antifungal sensitivity testing, as it is a simple, reproducible, cost effective and easy to perform technique in a routine clinical microbiology laboratory.

8.7 Future Prospects:

Refinements and optimization of the susceptibility testing of azoles against *Aspergillus* will enable a reproducible and clinically predictive test to be made available in the near future. Breakpoint setting will follow. Substantial work needs to be done to identify a methodology for reliably testing *Aspergillus* against polyenes, whether lipid incorporated or not. The availability of new antifungal agents with novel mechanisms of action has stimulated renewed interest in combination antifungal therapies. In particular, and despite the limited clinical data, the high mortality of mold infections and the relatively limited efficacy of current agents have produced significant interest in polyene-, extended-spectrum azole-, and echinocandin-based combinations for these difficult-to-treat infections. Combination antifungal therapy could reduce antifungal killing and clinical efficacy, increase emergingfor drug interactions and drug toxicities, and carry a much highercost for antifungal drug expenditures without proven clinical benefit. Thus, it is important to critically evaluate the role of combination therapy as new data become available.

CHAPTER - 9

Optimization of Nucleic Acid Based Amplification Methods for Detection and Species Level Identification of Dermatophytes from Dermatological Specimens

9.1 Review of literature

The dermatophytes are a group of closely related fungi that have the capacity to invade keratinized tissue (skin, hair, and nails) layers of humans and other animals to produce an infection, because of the inability of the fungi to penetrate the deeper tissues or organs of immunocompetent hosts .Dermatophytes are a group of closely related fungi that invade the keratinized tissue (skin, hair and nails) of humans and other animals to produce an infection, dermatophytosis. Dermatophytes are the main cause of superficial mycoses (Weitzman et al, 1995) and for many years, conventional laboratory methods based on detection of phenotypic characteristics such as microscopy and *in vitro* culture have played an essential role in dermatophyte identification .However, these procedures generally suffer from the drawbacks of being either slow or non specific and such morphologic and physiologic characteristics depend on too many variables like slow growth rate, presence of low threshold of organisms in clinical specimens, prior therapy and production of spores

(Bistis ,1959)

9.1.1 Etiologic agents

The etiologic agents of the dermatophytoses are classified in three anamorphic (asexual or imperfect) genera, *Epidermophyton, Microsporum*, and *Trichophyton*, of anamorphic class Hyphomycetes of the Deuteromycota (Fungi Imperfecti).

9.1.2 Laboratory diagnosis

Both direct microscopy and in vitro laboratory culture of sampled material are necessary to definitively identify the etiologic agent . The specimen should be divided

into two portions for direct microscopy and culture. It is important to understand the limitations of direct microscopy in diagnosing the cause of onychomycosis. The test serves only as a screening test for the presence or absence of fungi but cannot differentiate among the pathogens . Direct microscopy is often time-consuming, because nail debris is thick and coarse and hyphae are usually only sparsely present. The clinician should be aware of the possibility of false-negative results, which occur at a rate of approximately 5 to 15% (, Weitzman et al, 1995 ,Cohen et al, 1992).

When examining a KOH preparation, it is important to observe the hyphae closely to determine if they are typical of dermatophyte fungi or have features of nondermatophyte molds or yeasts. A final caveat concerns the common practice of treating nail infections on the basis of a microscopic preparation alone without culturing the putative pathogen. Although direct microscopy can provide clues about the identity of the microorganism, careful matching of microscopic and culture results is necessary for the clinician to be confident of the diagnosis (Clayton , 1993).

Almost half of all specimens taken from onychomycotic nails fail to yield a pathogen in culture. In onychomycosis, direct microscopy is the most efficient screening technique (Weitzman et al, 1995).The specimen can be mounted in a solution of 20 to 25% KOH or NaOH mixed with 5% glycerol, heated to emulsify lipids (1 h at 51 to 54°C), and examined under 340 magnification. An alternative formulation consists of 20% KOH and 36% dimethyl sulfoxide . The specimen may be counterstained

with chitin-specific Chlorazol black E to accentuate hyphae that are present; this is of particular value if the number of fungal elements is small. This stain is especially useful because it does not stain likely contaminants such as cotton or elastic fibers, which can help prevent false-positive identifications (Elewsky , 1995). Parker blue-black ink also can be added to the KOH preparation to improve visualization, but this stain is not chitin specific. Culture is the only method by which the causative microorganism can be identified. Caution should be used in analyzing culture results, because nails are nonsterile and fungal and bacterial contaminants may obscure the nail pathogen (Weitzman et al, 1995). The dermatophytes and non –dermatophyte

fungi were known to cause dermatomycoses .Of the nondermatophytic filamentous fungi, agents implicated in onychomycosis include members of Scopulariopsis (particularly *S. brevicaulis*) and Scytalidium (the two most common genera), which are both thought to digest keratin in vivo, as well as members of the genera *Alternaria, Aspergillus, Acremonium,* and *Fusarium* (Nolting et al, 1994).

9.1.3 Nucleic acid based techniques

Nucleic acid based techniques rely on the detection of genotypic differences in the pathogenic organism They are intrinsically more specific and precise than those based on phenotypic features as genotypic characteristics are less likely to be affected by external influences such as temperature variation and chemotherapy. Dermatophyte discrimination has met with some success using techniques like arbitrarily primed Polymerase chain reaction (AP-PCR), random amplified polymorphic DNA analysis (RAPD), repetitive sequence PCR (rep-PCR), restriction analysis of mitochondrial DNA and ribosomal DNA (Liu et al,1996, Zhong et al, 1997,Turenne et al, 1999, Jackson et al, 1999, Ferrer et al, 2001, Faggi et al, 2001,Pounder et al, 2005,). These methods are rapid, sensitive, more stable and less influenced by external factors than morphological methods.Molecular methods have been targeted at 18S rDNA (Nascimenta et al 2001, Gupta et al, 2002, Dubach et al, 2001), ITS1 and ITS2 regions (Makimura et al, 1999, Ferrer et al, 2001, Faggi et al, 2001), 5.8S rDNA (Faggi et al, 2001), 28S rDNA (Anand et al, 2001; Ninet et al,2003), non transcribed spacer (NTS) regions (Ferrer et al, 2001,Radford et al, 1998), metalloprotease gene (Jousson et al, 2004), chitin synthase (CHS) gene (Kano et al, 1998), tubulin gene, promoter region within ribosomal intergenic spacer, transcription elongation factor 1, actin gene and calmodulin gene (Gupta et al, 2002). However, approaches towards a single target have not had significant success. Hence a two targeted approach directed against Internal transcribed Spacer region and 18SrRNA was used to detect and identify dermatophytes. This study was designed to optimize a dermatophyte specific PCR targeting 18SrRNA for detection of dermatophytes from dermatological specimens and identify the same by PCR restriction fragment length polymorphism (PCR-RFLP).

9.2 Objectives

- To standardize and apply a dermatophyte specific PCR targeting 18S rRNA for detection of dermatophytes in specimens collected from patients with dermatomycoses

- To apply PCR based RFLP on 18S rRNA for identification of dermatophytes

- To apply PCR on ITS region for detection of fungi in dermatological specimens including dermatophytes

- To apply PCR based RFLP on ITS region for identification of fungi

9.3 Materials and Methods

9.3.1 Dermatophyte strains

Two standard strains comprising of *Trichophyton rubrum*, *Microsporum gypseum* (ATCC) supplied by P.G.I Chandigarh and 10 laboratory isolates consisting of *T. rubrum* 4, *T.mentagrophytes* 3, *M. gypseum* 2 and *E.floccosum* 1 were used in the study.

9.3.2 Dermatological specimens

Dermatological specimens comprising of nail clippings (56), material obtained from blisters (8), hair root (2), material obtained from scaly plaque of foot (1) and skin scraping (1) collected from an external dermatology clinic during the period between April 2006 and November 2006 were included in the study.

9.3.3 Conventional mycological investigations

The dermatological specimens were processed and subsequent identification was carried out according to standard mycological methods (Summerbell ,2003) In brief, the specimens were transferred onto a sterile plate and a part of it was used for

conventional investigations and the other part was used for molecular biological investigations. The specimens were inoculated onto Sabouraud's dextrose agar, Sabouraud's dextrose agar incorporated with 16 µg/ml of chloramphenicol and 500 µg/ml of cycloheximide, potato dextrose agar, and Trichophyton agar No.1 (HiMedia, India) and incubated at 25°C in a cooling incubator (Remi, Mumbai, India). Fungal species were identified on the basis of culture characteristics, pigment production, microscopic examination in lactophenol cotton blue preparation and slide culture wherever necessary. For direct microscopy, the specimens were digested with 40% potassium hydroxide and crushed smears were made. KOH-Calcofluor white wet mount was done and observed under fluorescence microscope for detection of fungi.

9.3.4 Molecular mycological investigations

Fungal DNA extraction: Fungal DNA from the isolates was extracted by following a modification of the Lee and Taylor protocol as described previously (Anand et al, 2001) . The DNA from clinical samples was extracted following the Biogene Kit method (Biogene™ Corporals, USA). In brief, 0.2mL of fungal isolate along with 3µL proteinase-K and 0.2mL TBM™ buffer was cyclomixed and incubated at 56°C for 30 min. It was then spun at 8000 rpm for 1 min in the spin column provided in the kit, after adding 0.2mL of ethanol. After decanting the filtrate and adding 0.5mL of washing solution, it was spun again at 8000 rpm for 1 min and the filtrate was discarded. The third wash was done without the washing solution at 12000 rpm for 3 min. This step was followed by the addition of 0.1mL of elution buffer and incubation at 56°C for 2 min. The DNA was recovered by spinning at 8000 rpm for 1 min and stored at -20°C.

9.3.5 PCR assay targeting 18SrRNA region of dermatophytes

Uniplex Polymerase Chain Reaction (PCR) was carried out using primers DHIL (5' TGC ACT GGT CCG GCT GGG 3'), and DH1R (5' CGG CGG TCC TAG AAA CCA AC 3') (5' ends at positions 631 and 813 respectively, according to the 18S r DNA sequence of *T. rubrum* [X58570]), specific for dermatophytes (Dubach et al, 2001), targeting the D2 subunit of the hypervariable V4 domain in the 18S rDNA region. The expected product length was 160-180 bp. The primers were custom ssynthesized at Bangalore Genei Pvt. Ltd, Bangalore, India. All the PCR steps were carried out in a

50μL reaction volume in 0.2mL thin wall polypropylene tubes (Axygen Inc.,CA) using a Perkin-Elmer Thermal cycler (Model 2700). A 50μL reaction with 200 μm conc of each dNTP, 25pmol of each primer, 1U of *Taq* polymerase (Bangalore Genei, India) and 10μL of template DNA. The PCR profile consisted of denaturation for 3 min at 95°C followed by 35 cycles at 94°C for 1 min, 58°C for 1 min. and 72°C for 40 s and a final extension at 72°C for 5 min.

9.3.6 Analytical Sensitivity

Serial ten-fold aqueous dilution of std. strain of *T.rubrum* ATCC ranging from 10^{-1} to 10^{-10} was used to determine the analytical sensitivity.

9.3.7 Analytical Specificity

Specificity of the primers was tested with *T.rubrum, M.gypseum*, *E.floccosum*, *T.mentagrophytes*, standard strains of *C. albicans* ATCC 24433, *C.tropicalis* ATCC750, *C.parapsilosis* ATCC 22019, *C.krusei* ATCC 6258 and lab isolates of *A.niger, A.terreus, Curvularia* spp.

9.3.8 PCR Assay targeting ITS region

The ITS region was amplified applying PCR to amplify ITS region as described by Ferrer et al, 2001 as per the method given in Appendix II

9.3.9 Application of PCR on dermatological specimens

A dermatophyte specific uniplex PCR, to amplify 18SrRNA and ITS PCR was applied on 68 dermatological specimens..

PCR - RFLP Analysis: The amplified products of dermatophyte specific PCR were subjected to digestion with *Hae* III for 2 hours and that of ITS amplicons were subjected to digestion with the same enzyme for 3 hours.In a reaction volume of 25μL containing 10μL of PCR amplicons, 1μL of *Hae* III(Bangalore Genei, India) and 2.5μL of buffer C. The digested products were resolved using 4% agarose gel electrophoresis gel incorporated with 16μL of ethidium bromide at 100 volts and documented using Gel documentation system (Vilber Lourmat, Cidex, France)

9.4 Results:

9.4.1 Conventional mycological investigations: The conventional mycological methods were standardized by using standard laboratory isolates. The dermatophytes were identified by conventional mycological methods by studying colony characters and microscopic features. The distribution of dermatophyte isolates identified by phenotypic methods is described in Table 9a The colony morphology of dermatophyte fungi and the microscopic characteristics by Lactophenol Cotton Blue mount is provided in Figure 9a

Table 9a. Description of colony and microscopic characteristics of dermatophytes

Species	Colony characters	Microscopic features
M. gypseum	Rapidly spreading.Surface texture of chamois due to multitudes of macroconidia.Pleomorphic.Brown/Pink pigment seen on the reverse side.	Ellipsoidal macroconidia with thin walls and 6-7 cells. Microconidia are not septate.
E.floccosum	Olive-green or khaki color folded colony with a suede-like texture. Pleomorphic.	Microconidia are absent.Blunt and thick-walled macroconidia are seen.
T.rubrum	Fluffy white colony with venous blood color on reverse.Slow-growing colony.Non-diffusing red pigment seen under the colony.	Microconidia are abundant. Long, pencil-shaped macroconidia with transitional forms between hyphae and macroconidia are observed.
T.mentagrophytes	Flat dense downy colony with a tint of cream colored spores seen..Characteristic stellate colony seen on Potato Dextrose Agar (PDA). Pleomorphic	Cigar-shaped macroconidia with thin walls. Abundant round microconidia.

Figure 9a: Colony morphology and microscopic morphology of dermatophytes

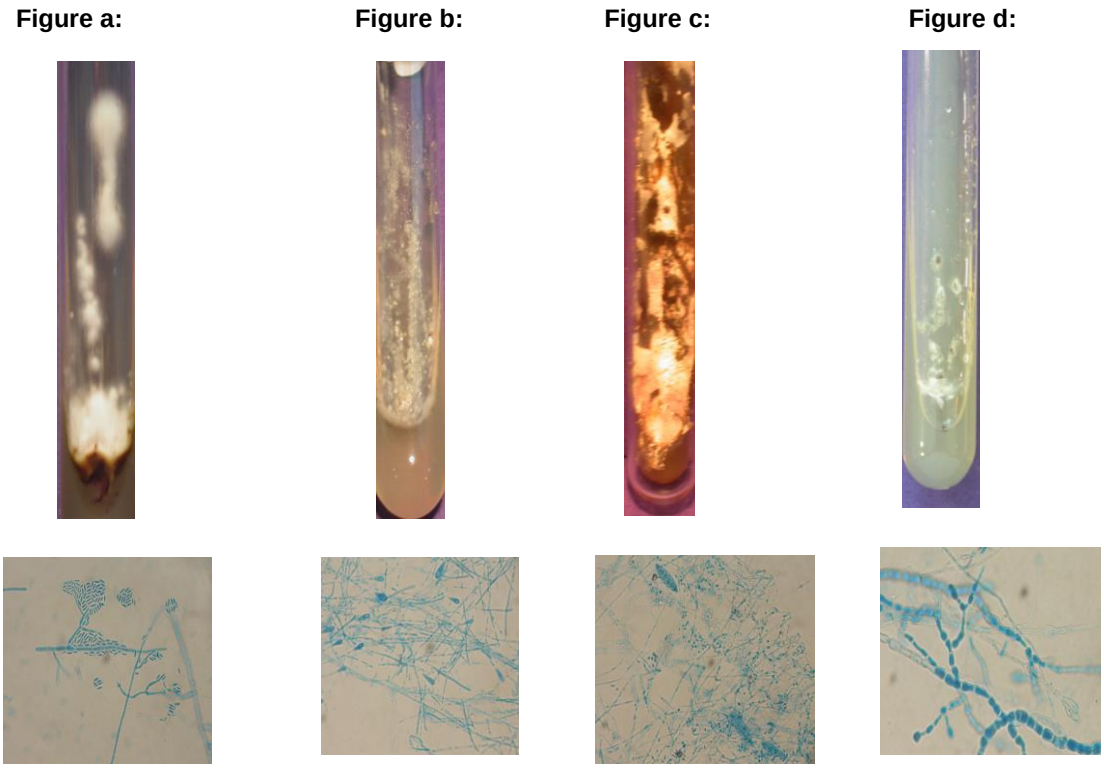


Figure a: Colony morphology: Growth of *Trichophyton rubrum* on Trichophyton agar showing wine red pigmentation. Microscopic morphology: Lactophenol cotton blue mount of *T. rubrum* showing abundant production of microconidia

Figure b: Colony morphology: Growth of *Trichophyton mentagrophytes* on Trichophyton agar showing flat dense downy colony with a tint of cream colored spores Microscopic morphology: Lactophenol cotton blue mount of *T. mentagrophytes* showing abundant production of microconidia

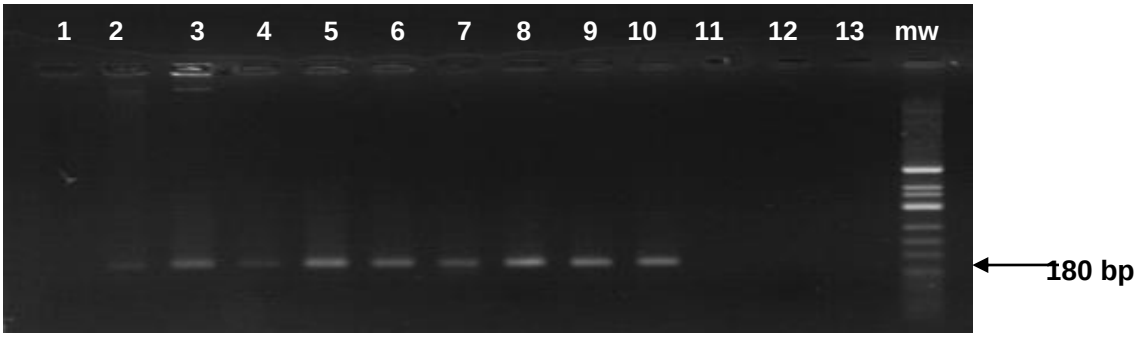
Figure c: Colony morphology: Growth of *Microsporum gypseum* on potato dextrose agar showing brown pigment. Microscopic morphology: Lactophenol cotton blue mount of *M. gypseum* showing production of macroconidia and microconidia

Figure d: Colony morphology: Growth of *Epidermophyton floccosum* on potato dextrose agar showing white colony. Microscopic morphology: Lactophenol cotton blue mount of *M. gypseum* showing blunt and thick walled macroconidia

9.4.2 Dermatophyte specific PCR

The analytical sensitivity of dermatophyte specific PCR performed using serial ten-fold dilution of standard ATCC strain of *T. rubrum* was found to be 10 picograms shown in Figure 9b. The primers were found to be specific amplifying selectively all the dermatophyte isolates tested and not amplifying other non dermatophye fungal DNA (Figure 9c).

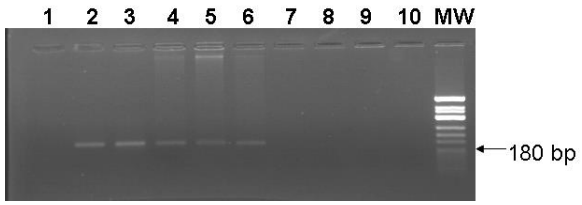
Figure 9b: Agarose gel electrophoresis showing the analytical sensitivity of dermatophyte specific PCR performed using standard strain of *T. rubrum* DNA.



Lane1: Negative control **Lane 2:** Neat DNA of standard strain of *T. rubrum*

Lane 3-13: Serial 10 fold dilutions of *T. rubrum* (10^{-1} to $10^{-11)}$ DNA showing amplification till 10^{-8} dilution. **MW:** *Hinf* I digest of Phi X bacteriophage DNA **Sensitivity**: 10pg

Figure 9c: Agarose gel electrophoresis showing the specificity of dermatophyte specific PCR



Lane 1: Negative control **Lane 2*:*** *T. rubrum* ATCC **Lane 3:** *T. rubrum* iolate

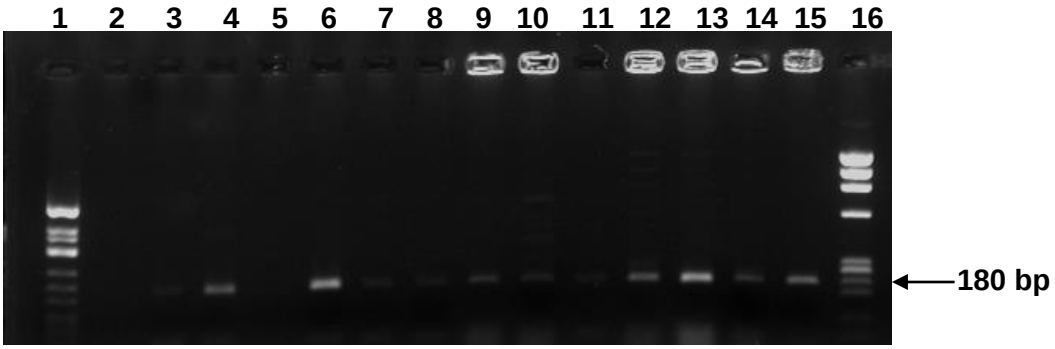
Lane 4: *T. metagrophytes* **Lane 5:** *M. gypseum* **Lane 6:** *E. floccosum* lab isolate

Lane 7: *C. albicans* ATCC 24433 **Lane 8**: *A. terreus* lab isolate**Lane 9:** *Curvularia* species lab isolate **Lane 10:** *A. niger* lab isolate MW: Hinf I digest of Phi X bacteriophage DNA

9.4.3 Application of PCR on dermatological specimens

The application of dermatophyte specific PCR on dermatological specimens is shown in Figure 9d and that of ITS PCR is shown in Figure 9e.

Figure 9d: Agarose gel electrophoresis showing the application of dermatophyte specific PCR on dermatological specimens

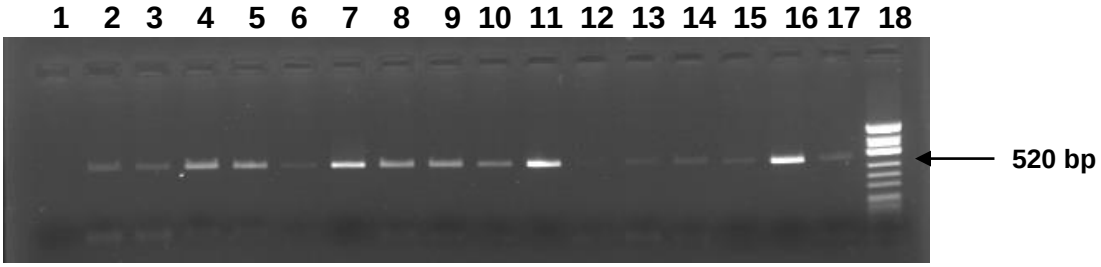


Lane 1: Molecular weight marker *Hinf* I digest of Phi X bacteriophage DNA

Lane 2: Negative control **Lane 3,4,6 -14 :** Positive **Lane 15: Positive control**

T.rubrum :Positive **Lane 16:** Molecular weight marker *Hae* – III digest of Phi X bacteriophage DNA

Figure 9e: Agarose gel electrophoresis showing the application of ITS PCR on dermatological specimens



Lane 1: Negative control **Lane 2,3,4,7-14,16** Nail clipping- Positive

Lane 5,6, 15: Blister top - Positive

Lane 17: Positive control : *T. rubrum* standard strain DNA

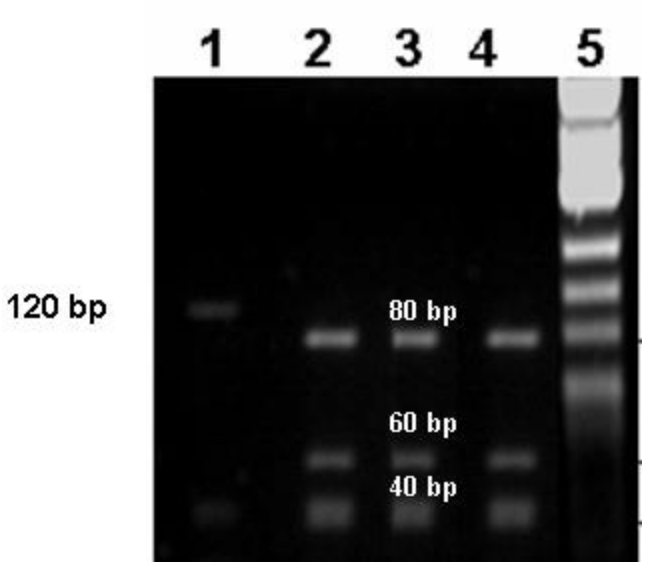
Lane 18: Molecular weight marker *Hae* – III digest of Phi X bacteriophage DNA

Of the 68 specimens subjected to dermatophyte specific PCR, 27 (39.07%) revealed the presence of dermatophyte specific genome. Of the 27 PCR positive specimens, 2 (7.40%) were positive by conventional methods while PCR alone detected the presence of dermatophytes in 13 (19.11%) increasing the clinical sensitivity by 11.71%. All the specimens detected by dermatophyte PCR were also positive by ITS PCR. The results of application of dermatophyte specific PCR revealing the presence of dermatophyte DNA on dermatological specimens is given in Table 2b:

9.4.4 Application of PCR RFLP using *Hae* III enzyme

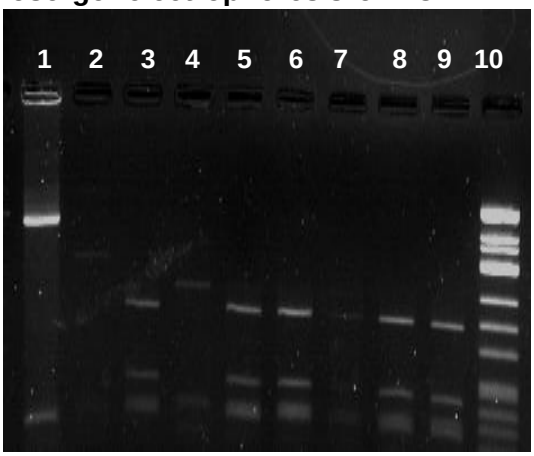
The results of application of PCR RFLP using *Hae* III enzyme dermatophyte specific PCR and ITS PCR amplicons is shown in Figure 8f and 8g respectively

Figure 9f: Agarose gel electrophoresis of PCR RFLP performed on dermatophyte spe



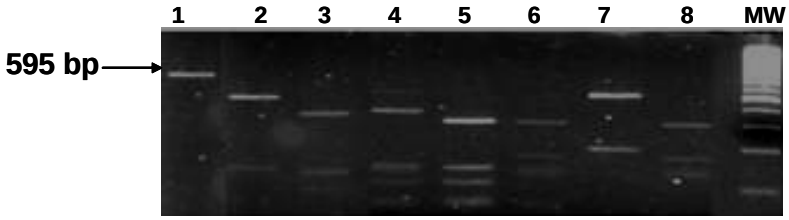
Lane 1 : Digested product of *M. gypseum* (120, 40 bp) **Lane 2,3 :** Digested product of *T. rubrum* (90, 50, 40 bp) **Lane 4 :** Digested product of *T. rubrum* standard strain **MW :** Molecular weight marker *Hinf* –I digest of Phi X 174 bacteriophage DNA

Figure 9g: Agarose gel electrophoresis of PCR RFLP performed on ITS PCR amplicons



Lane 1: Undigested product (595 bp) **Lane 2:** Digested product of *M.gypseum* (420, 95 bp) **Lane 3,5,6, 7,8,9:** Digested product of *T. rubrum* (300,200,95 bp) **Lane 4:** Digested product of *E . floccosum* (350, 95 bp) **MW :** Molecular weight marker *Hinf* –I digest of Phi X 174 bacteriophage DNA

Figure 9h: Agarose gel electrophoresis of PCR RFLP performed on ITS PCR amplicons to identify non – dermatophyte aetiology



Lane 1 : Undigested product *C. albicans* ATCC 24433 595 bp **Lane 2:** Digested product identified as *Aspergillus niger* (440,150 bp) **Lane 3:** Digested product identified as *C. albicans* (331,150,100 bp) **Lane 4:** Digested product identified as *C. tropicalis* (351,150,100,50 bp) **Lane 5:** Digested product identified as *C. parapsilosis* (351,150,100,50 bp) **Lane 6:** Digested product identified as *A. flavus* (325, 182, 88 bp) **Lane 7:** Digested product identified as *Aspergillus niger* (440,150 bp) **Lane 8 :** *C. parapsilosis* (328,156,100 bp) MW: Molecular weight marker *Hinf* I digest of Phi X 174 bacteriophage DNA

By application of PCR RFLP on 27 dermatophyte PCR products, *T. rubrum* was identified in 20 (Nail clipping 15 and blister top 5), *M. gypseum* in 5 (nail clipping 4 and blister top 1) and *T. mentagrophytes* in 1 (Hair root). The results of ITS PCR-RFLP and that of dermatophyte PCR RFLP were found to be concurrent.

Among the 68 specimens tested, 27 (39.7%) revealed the presence of dermatophytes by PCR-RFLP. The presence of non-dermatophyte aetiology was found to be 18 (26.4%) . Fungal aetiology was not to be associated in 16 (23.52%) . The 27 PCR positive specimens were identified to be *T. rubrum* 21, *M. gypseum* 5,

T. mentagrophytes 1.

A comparison of the efficiencies of both conventional and PCR-based mycological methods was done and the results are shown in the Table 8a. Among the 68 specimens tested, 27 (39.7%) revealed the presence of dermatophytes by both methods of PCR-RFLP. Of these 27 PCR positive specimens, 2 (7.40%) were culture positive while PCR alone detected the presence of dermatophytes in 13 (19.11%) increasing the clinical sensitivity by 11.71%. All the specimens in which dermatophyte was detected by PCR targeting 18s RNA region were also positive by PCR for ITS region. RFLP on the amplified products of these 27 dermatophyte positive specimens clearly identified the species as follows: *T. rubrum* 21 specimens (Nail clipping 16 and blister top 5), *M. gypseum* in 5 specimens (nail clipping 4 and blister top 1) and *T. mentagrophytes* in 1 specimen (Hair root). PCR-RFLP on ITS region detected the presence of DNA of non-dermatophytes in 18 (26.4%) clinical specimens and all were culture positive. Fungal DNA was not detected in 15 (22.05%) clinical specimens by both PCR methods and smear and cultures were also negative indicating the specificities of both methods. Overall the clinical sensitivity of PCR targeting 28s RNA increased by 31.67 % over culture method

A comparison of the efficiencies of both conventional and PCR-based mycological methods was done and the results are given in the Table 9b and the clinical diagnoses and the fungi detected given in 9c

Table 9b Comparative analysis of the efficiency of smear, culture and PCR for the detection of dermatophytes in clinical specimens from the lesions of the patients identified clinically as dermatophytosis

PCR and PCR-RFLP identification N= 68	Smear positive culture positive clinical specimens Speciation of fungi N = 15	Smear negative culture positive clinical specimens Speciation of fungi N = 5	Smear positive culture negative clinical specimens Speciation of fungi N = 20	Smear negative culture negative clinical specimens N = 28
PCR on 18 S rRNA region Positive Dermatophyte specific N= 27 (39.07%)[@] PCR –RFLP species identification*	**2 (2.9 %)*** *T. rubrum* 1 *M. gypseum* 1 **15 (22.05%)**	Nil Nil	**12 (17. 64 %)**[@] *T. rubrum* 9 *M. gypseum* 3 **20 (29.41%)**	**13 (19.11 %)**[@] *T .rubrum* 11 *M. gypseum* 1 *T.mentagrophytes* 1
PCR-RFLP on ITS region[#] positive specific for fungi including dermatophytes N = 53 (77.9.%)	2 [£] (2.9 %) dermatophytes	Nil 5 (7. 35 %) non – dermatophyte fungi	12 [˚] (17. 64 %) dermatophytes 8 (11.76 %) non – dermatophyte fungi	13[δ] (19.11 %) dermatophytes
	13 (19.11%)* non – dermatophyte fungi *A. niger* 4 *A. flavus* 2 *A. fumigatus* 3 *Fusarium sp* 1 *C. albicans* 1 *C.tropicalis* 1 *C.parapsilosis* 1	*A.. niger* 3 *A. fumigatus* 1 *Fusarium* sp 1	*A. flavus* 3 *A. fumigatus* 2 *A. niger* 1 *A. terreus* 1 *Fusarium sp* 1	Nil

***** Results indicate 100 % specificity of PCR on 18S rRNA region of the fungus to detect dermatophyte in clinical specimens [@] Clinical sensitivity increased by (17. 64% + 19.11 %) 31.67 % over conventional methods of smear & culture # PCR on ITS region is panfungal genome specific; detects fungi including dermatophyte 2[£] Results of PCR RFLP on ITS region identified the dermatophytes as those of PCR RFLP on 18S rRNA region 12[˚] Results of PCR RFLP on ITS region identified the dermatophytes as those of PCR RFLP on 18S rRNA region13[δ] Results of PCR RFLP on ITS region identified the dermatophytes as those of PCR RFLP on 18S rRNA regionThe statistical analysis was performed to determine the significance of ITS PCR and dermatophyte PCR. The increase in clinical sensitivity - dermatophyte specific PCR targeting 18SrRNA was found to be statistically significant. P value = 0.00001(Fischer exact test).The increase in clinical sensitivity – panfungal specific PCR targeting ITS region was found to be statistically significant. P value $\leq$ 0.0001(Fischer exact test)

Table 9c: Fungi identified from dermatological specimens obtained from dermatomycoses patients

Clinical diagnosis and dermatological specimens		**Fungal aetiology associated with dermatological lesions**		
Nail clipping	**56**	***Dermatophyte fungi identified***	***Non dermatophyte fungi identified***	**PCR and culture negative**
Total onycholysis following trauma	4	*T. rubrum* 1, *M. gypseum* 1*	*C. albicans* 1[#]	1
Onychomycosis	29	*T. rubrum* 8*, *M. gypseum* 3	*A. niger* 6[#,@,$], *A. flavus* 1[#] *A. terreus* 1[$], *C.tropicalis* 1[#] *A. fumigatus* 1[#,] *Fusarium* species 2[#,$]	6
Diabetic distal onycholysis	2	*T. rubrum* 2	-	-
Traumatic onychomycosis	3	*T. rubrum* 2	*C. parapsilosis* 1[#]	-
Chronic paraonychia with onychomycosis	2	*T. rubrum* 1	*A. flavus* 1[#]	-
Distal nail dystrophy	2	-	*A. flavus* 2[$]	-
Subungual hyperkeratosis	1	*T. rubrum* 1	-	-
Ingrown nail	5	*T. rubrum* 1	*A. niger* 1[#], *A. fumigatus* 2[#]	1
Psoriasis of the nail	3	*T. rubrum* 1	*A. fumigatus* 2[$]	-
Lichenplanus of nail	3	*T. rubrum* 1	*A. niger* 1[#]	1
Idiopathic Nail dystrophy	2	-	*A. fumigatus* 1[@]	1
Blister top	**8**			
Pompholyx (vesicular eczema)	2	*T. rubrum* 1	*Fusarium* species 1[@]	-
Oncholysis with renal insufficiency	1	*M. gypseum* 1	-	-
Onychomycosis	4	*T. rubrum* 1	*A.flavus* 1	2
Onychomycosis and brittle nail	1	-	-	1
				1
Others	**4**	-		
Tinea versicolor	2	*T. rubrum* 1 (Skin scraping)	-	1
Depigmented hair	2	*T.mentagrophytes* 1 (Hair root)	-	-

Note :

- * *T. rubrum* (1) and *M. gypseum* (1) were isolated in culture and identified by dermatophyte specific PCR- RFLP and PCR-RFLP on ITS region yielding concordant results

- The other dermatophyte fungi were identified by dermatophyte specific PCR- RFLP and PCR-RFLP on ITS region.

- # The non dermatophyte fungi were detected in smear, isolated in culture and identified by PCR-RFLP on ITS region. The results of PCR-RFLP were concordant with that of conventional identification

- @ The non dermatophyte fungi were isolated in culture (not detected in direct smear) and identified by PCR-RFLP on ITS region. The results of PCR-RFLP were concordant with that of conventional identification

- $ The non dermatophyte fungi were detected in smear and identified by PCR-RFLP on ITS region.

9.5 Discussion:

Dermatophyte fungi are the most common cause of superficial and easily treatable mycoses. Smear examination and culture techniques, the 'gold standard' of dermatophytosis diagnosis (Rebel et al, 1970) have their own disadvantage in not being sensitive and specific. The slow growth rate of dermatophytes, the high rate of contamination and the low sensitivity and specificity make the identification of dermatophytes by conventional methods a laborious 'exercise in contemplative observation. The use of nucleic acid based amplification techniques have proven to be sensitive and specific in detection of dermatophytes.(Binstock et al, 2007)

This study was designed to develop PCR targeting 18SrRNA specific for detection of dermatophytes and subsequent RFLP on the amplicons to reveal the identity of dermatophytes. Apart from that, PCR targeting ITS region was also performed to confirm detection of dermatophyte as fungus. The results clearly demonstrated not only the specificity, but also an increased clinical sensitivity by 31.67 % by this PCR method over the conventional methods of smear and culture and the laboratory procedure. This was also rapid enough to be completed within 24 hours. The slow growth rate of dermatophytes on standard culture media, the high rate of contamination because of long incubation of cultures make the identification of dermatophytes by conventional methods a laborious and often fruitless exercise. PCR targeting 18SrRNA detected dermatophytes in 19.11% among the smear and culture negative clinical specimens and on further speciation by RFLP showed the presence of *T. rubrum*, *M. gypseum* and *T. mentagrophytes*.

Application of PCR on ITS region of the fungal genome indicated the presence of fungus 77.9 % specimens of which 2.94 % were dermatophytes and rest were non-dermatophytes which were identified by RFLP as *A. niger*, *A. flavus*, *A. fumigatus*, *Fusarium* and *C. albicans*, *C. tropicalis*, *C. parapsilosis*. Identification of dermetatophytes by PCR-RFLP using ITS region of the fungal genome was the same as done with PCR-RFLP on 18s RNA region. Of the 68 specimens subjected to dermatophyte specific PCR, 27 (39.07%) revealed the presence of dermatophyte specific genome. Of the 27 PCR positive specimens, 2 (7.40%) were positive by conventional methods while PCR alone detected the presence of dermatophytes in

13 (19.11%) increasing the clinical sensitivity by 11.71%. All the specimens detected by dermatophyte PCR were also positive by ITS PCR The 27 PCR positive specimens subjected to RFLP revealed *T. rubrum* 21, *M. gypseum* 5, *T. mentagrophytes* 1.

The conventional investigations performed on 68 dermatological specimens revealed the presence of fungal elements by smear and culture in 15 (22.05%), smear negative , culture positive in 5 (7.35%), smear positive culture negative in 13 (19.111%) and both smear and culture negative in 27(39.07%).There were a total of 20 (29.41%) specimens positive by culture of which 2 (2.94%) were dermatophytes , 1 each of *T. rubrum* and *M. gypseum* respectively. The other 18 (26.4%) fungal isolates were *A .niger* 7, *A. flavus* 2, *A. fumigatus* 4, *Fusarium* species 2 and 1 each of *C. albicans*, *C. tropicalis*, *C. parapsilosis* 1 respectively.

In the literature, the percentage of the clinical specimens, from patients clinically diagnosed as dermatophytosis, which were identified by conventional methods (direct microscopy and/or culture examination) range from 30% to 70% (Brilhante et al, 2005, Ikit et al, 2005, Marchisio et al,1995,Muggee et al, 2006, Mohanty et al, 1999, Sayed et al, 2006). Even when the sensitivity of culture methods is determined, taking calcofluor white as the standard, culture method could manage a sensitivity of only 59% (Weinberg et al, 2003) while it was a mediocre 32% in another study by Lawry , et al, 2000. This unsatisfactory performance is further compounded by the fact that conventional mycological methods usually have a false negative rate of 30% .

The isolation of dermatophyte by culture is very low i.e 2.94% inspite of using special media like Trichophyton agar,and Dermatophyte medium incorporated with cycloheximide and chloramphnicol and incubation period of 30 days. However 12 (17.64%) specimens revealed the presence of fungi in smear but none of them were isolated by culture. The low yield of culture in the present study could be attributed to low threshold of organisms and /or prior antifungal therapy.Veer et al 2006 have reported *T. rubrum* as the common aetiological agent, similar to the results of the present study.

Another finding in the present study is the association of non – dermatophyte fungi in 18 (26.4%) specimens.The clinical specificity of PCR is also clearly demonstrated by the fact that none of the non-dermatophyte fungi identified by culture were detected by dermatophyte-specific PCR. It could also be observed that one-26.4% showed the presence of a non-dermatophyte fungi but only 2 of the specimens are positive for dermatophytes by culture method. This highlights the comparative difficulty in culturing dermatophytes. It also highlights the high proportion of non-dermatophyte fungi in the clinical specimens. The high proportion of non-dermatophyte fungi could be due to the non-dermatophyte etiology of onychomycosis generally associated with low levels of hygiene and low socioeconomic level. Though generally non-dermatophytes are believed to constitute about 10% of the causative agents of onychomycosis (Poulat et al, 2005) they are quite common in the hot and humid tropical countries such as India (Chi et al, 2005), unlike the West where the non-dermatophyte moulds and yeasts are found as contaminating organisms in dermatophyte onychomycosis, secondary to dermatophytosis (Ellis et al, 1997). This possibility of superinfection or coinfection could be ruled out because none of the non-dermatophyte fungi are positive for dermatophytosis by PCR and none of the PCR positive specimens show the presence of non-dermatophyte fungi. All the PCR positive specimens were either culture negative) or showed growth of the respective dermatophyte (2 specimens). Part of the difficulty in evaluating the role of nondermatophyte fungi cultured from the nail arises because the same fungi are also occasionally found to be pathogens they have the atypical frondlike hyphae associated with nondermatophyte molds or if the same organism is repeatedly isolated. (Elewski,1998) Elewski et al, 1998 have reported *Aspergillus*, *Candida* and *Fusarium* species as the non dermatophytes causing dermatomycoses.

Culture methods also consume quite a lot of time, taking upto 21 days for a definitive diagnosis , which is not arrived at in quite a few cases. Also as William Scherer et al 2004 observed, the conventional mycological methods lack reproducibility. Confronted by such an unenviable condition the physician, to be sure, treats all the suspected patients for dermatophytosis, putting the unfortunate few through an unnecessary course of chemotherapy. Polymerase Chain Reaction (PCR), one of the few techniques which has revolutionized diagnostic medicine in combination with Restriction Fragment Length Polymorphism (RFLP) helps to arrive

at a more specific diagnosis in less time(more than 90% less time) and with high sensitivity . While the results of a PCR-RFLP are available in less than 24 hours from the time of receiving the specimens, results may not be available for upto 21 days or even longer if conventional methods are used. Part of the difficulty in evaluating the role of non dermatophyte fungi cultured from the nail arises because the same fungi are also occasionally found to be pathogens and they have the atypical frond like hyphae associated with nondermatophyte molds or if the same organism is repeatedly isolated. Elewski et al, 1998 have reported *Aspergillus*, *Candida* and *Fusarium* species as the non dermatophytes causing dermatomycoses.

To increase the predictive power of a diagnosis of nondermatophytic invasion of a nail, Summerbell, 1997 suggested that nonfilamentous nondermatophytes identified in nail tissue be categorized as one of the following: contaminant (species growing in culture from dormant propagules on the nails);normal mammalian surface commensal organism; transient saprobic colonizer (colonizer of accessible surface molecules but noninvasive); persistent secondary colonizer (colonizer of material infected by a dermatophyte but incapable of remaining after the dermatophyte is eliminated); successional invader(species that can cause infection after gaining entry into a nail via the disruption caused by a primary pathogen); or primary invader (able to infect and cause onychomycosis in a previously uncolonized nail). Such an analysis has the value of identifying for treatment only nondermatophytic infections that are truly invasive.

As an additional confirmatory technique, definitive identification of nondermatophytic invasion in nails may require theisolation of the agent from successive specimens from the infected region . If the infective pathogen is a dermatophyte,subsequent cultures will most probably grow out the dermatophyte itself, a second contaminant unrelated to the first, or no growth at all. Some investigators believe that claims of an increasing proportion of mixed infections in onychomycosisare exaggerated and have gone so far as to state that nondermatophyte molds and yeasts are usually contaminants secondary to dermatophyte onychomycosis and that their presence need not affect treatment outcome (Ellis et al, 1997).

Polymerase Chain Reaction (PCR), one of the few techniques which has revolutionized diagnostic medicine in combination with Restriction Fragment Length Polymorphism (RFLP) helps to arrive at a more specific diagnosis in less time (more than 90% less time) and with high sensitivity While the results of a PCR-RFLP are available in less than 24 hours from the time of receiving the specimens, results may not be available for up to 21 days or even longer if conventional methods are used. In terms of the number of specimens tested, PCR was more sensitive (19.11%) than conventional mycological method of direct smear examination and culture examination, with respect to the total number of specimens tested. This demonstrates the difficulty of culturing dermatophytes as well as the superiority of PCR-based techniques compared to conventional methods.

Thus, PCR not only brings in better specificity and sensitivity to the detection of dermatophytosis but also aids in the faster and efficient management of patients. In terms of the number of specimens tested, PCR was found to be more sensitive (19.11%) than conventional mycological method of direct smear examination and culture examination, with respect to the total number of specimens tested. This demonstrates the difficulty of culturing dermatophytes as well as the superiority of PCR-based techniques compared to conventional methods. The resolution of PCR-RFLP technique could be further increased by choosing an appropriate molecular target, once the complete genome of all species of dermatophytes has been mapped. The increased resolution will aid the strain-typing of dermatophytes which will help in determining whether a case of recurrent dermatophytosis is due to reinfection or due to drug resistance, which then could be further ascertained once the genes for drug resistance are known leading to better management of the patient.

9.6 Conclusion

The application of two targeted approach – i.e targeting ITS region and 18SrRNA region was found to be useful in detection and species level identification of dermatophyte fungi.This method is preferable since it is rapid within 24 hours of

receipt of specimen as compared to the conventional culture which requires prolonged incubation period of 21 days

9.7 Future prospects

Studies on the pathogenic mechanisms of non –dermatophyte fungi is essential to know its role as a pathogen in dermatomycoses**.** Designing studies to determine the copy numbers of dermatophyte as to non dermatophyte in animal models by real time PCR would define the role of non dermatophyte fungi causing superficial mycoses

10 a Specific Conclusions:

- Rapid identification of NSM by PCR based DNA sequencing, identified fungi belonging to the genera *Botryosphaeria*, *Rhizoctonia, Macrophomina* as the aetiological agents of fungal keratitis in Indian population. The association of these genera with fungal keratitis is the first report to the best of our knowledge

10 b General conclusions:

- Semi nested PCR targeting the ITS region was used as a detection tool in fungal keratitis and endophthalmitis.
- PCR targeting the other ribosomal unit- 18SrRNA and 28SrRNA were also used for detection of panfungal genome.
- Targeting the ribosomal unit was of great importance as these regions are present in multiple copies, conserved and increase the threshold of detection. Particulary, the ITS target exhibits considerable variation within the conserved region enabling the species level identification. This led to the development of a mPCR targeting the ribosomal unit.mPCR targeting the ribosomal unit was optimized and applied on ocular specimens for panfungal genome detection. Another technique of mPCR was optimized to aid simultaneous detection of eubacterial, *P. acnes* and panfungal genome in intraocular specimens.

- PCR based DNA sequencing enabled identification and characterization of the genotypes. The genotypic study on *A. flavus* revealed novel nucleotide polymorphisms in the ITS region.

- PCR based DNA sequencing of ITS region was applied on NSM to facilitate species level identification.Fusarium species constitute the second common aetiological agent of fungal keratitis.
- Phenotypic and gentypic methods of identification was carried out to speciate *Fusarium.*
- A cost effective agar dilution method was standardized to determine the MIC of amphotericin B, fluconazole and ketoconazole.

- Nucleic acid based amplification techniques of PCR, PCR –RFLP were developed to detect and identify the aetiological agents of dermatomycoses. The nucleic acid amplification techniques PCR-RFLP and DNA sequencing proved to be a reliable tool for rapid detection and identification of fungi directly from clinical specimens.

11 Specific Contributions:

11. 1. Identification of Non-sporulating moulds (NSM)

- Rapid identification of NSM by PCR based DNA sequencing, identified fungi belonging to the genera *Botryosphaeria*, *Rhizoctonia, Macrophomina* as the aetiological agents of fungal keratitis in Indian population. The association of these genera with fungal keratitis is the first report to the best of our knowledge

11.2 Optimization of two novel multiplex Polymerase Chain reaction (mPCR) for detection of fungi and bacterial agents from ocular specimens

- A novel mPCR targeting 18SrRNA, ITS and 28SrRNA region was developed for rapid detection of fungi from clinical specimens. Another mPCR was optimized to detect eubacterial, *P. acnes* and panfungal genome for rapid detection of aetiological agents causing infectious endophthalmitis.These two novel mPCRs were optimized and applied on ocular specimens for the first time.

11.3. Nucleotide polymorphisms in *A. flavus*

- The application of PCR based DNA sequencing on ITS region identified novel nucleotide polymorphisms in *A. flavus* isolates. The single and multiple nucleotide polymorphisms associated with ITS region of *A. flavus* were novel and reported in literature for the first time .

11.4. Nucleic acid amplification methods in detection and identification of dermatophytes

- A PCR and PCR-RFLP targeting the 18SrRNA and ITS region were optimized and applied for rapid detection and identification of dermatophytes directly from dermatological specimens for the first time in India.

12 Future scope of work

- Future research in ophthalmic mycoses needs to focus on improvement in diagnostic techniques, development of new antifungal compounds to overcome antifungal drug resistance.Nucleic based amplification techniques involving Real time PCR and Reverse transcriptase PCR needs to be optimized to assess the transcripts produced by the pathogenic fungi. Rapid nucleic acid based amplification methods to detect the drug resistance genes in *A. terreus* and *F. solani* would aid in good therapeutic management.

- Further studies on the growth kinetics of non sporulating moulds,its susceptibility patterns to the in use and emerging antifungal agents is 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

13 References

Abliz P, Fukushima K, Takizawa K, Nishimura K. Identification of pathogenic dematiaceous fungi and related taxa based on large subunit ribosomal DNA D1/D2 domain sequence analysis. *FEMS Immunol Med Microbiol* 2004, 40:41– 49.

Ahmad S, Khan Z, Mustafa AS, Khan ZU. Seminested PCR for diagnosis of candidemia: comparison with culture, antigen detection, and biochemical methods for species identification. *J Clin Microbiol* 2002, 40:2483–2489.

Aldave AJ, Stein JD, Deramo VA, Shah GK, Fischer DH, Maguire JI. Treatment strategies for postoperative *P. acnes* endophthalmitis. *Ophthalmology* 1999, 106:2395–2401.

Alexandrakis G. Jalali S, Gloor P. Diagnosis of *Fusarium* keratitis in an animal model using the PCR. *Br J Ophthalmol* 1998, 82:306–311.

Alexandrakis G, Haaimovicu R, Miller D. Corneal biopsy in the management of progressive microbial keratitis *. Am J Ophthalmol* 2000, 129: 571 -576

Anand AR, Madhavan HN, Therese KL. Use of PCR in the diagnosis of fungal endophthalmitis. *Ophthalmol* 2000, 108:326-30.

Anand AR, Madhavan HN, Sudha NV, Therese KL, PCR in the diagnosis of *Aspergillus* endophthalmitis. *Ind J of Med Research* 2001,114:133-140

Arguello JR, Little AM, Pay AL. Mutation detection and typing of polymorphic loci through double-strand conformation analysis. *Nat Genet* 1998; 18: 192–194.

Arikan S, Chiu ML, Paetznick V, Nagia S, Rex J..Activity of amphotericin, itraconazole and voriconazole against Aspergillus and Fusarium, abstr. J-19. 1998 In Program and abstracts of the 38th Interscience onference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C

Arikan S, Lozano-Chiu M, Paetznick V, Nangia S, Rex JH. Micro dilution susceptibility testing of Amphotericin B, Itraconazole, Voriconazole against clinical isolates of *Aspergillus* and *Fusarium* species. *J Clin Microbiol* 1999, 37:3946-3951.

Arthington B, Yang WL, Meral A, Ciblak,Frade JP, Brandt ME, Rana A,Hajjeh. Comparison of Visual and Spectrophotometric Methods of Broth Microdilution MIC End Point Determination and Evaluation of a Sterol Quantitation Method for In Vitro Susceptibility Testing of Fluconazole and Itraconazole against Trailing and Nontrailing *Candida* Isolates. *Antimicrob agents and Chemother* 2002, 46 : 2477–2481

Arthur SL, Steed L, Apple DJ, Peng Q, Howard G, Gomez EM**.** *Scedosporium prolificans* keratouveitis in association with a contact lens retained intraocularly over a long term*. J Clin Microbiol* 2001, 39: 4579–4582

Asrar A, Al-Amro AMSA, Al-Mosallam AAA, Al-Obeidan S. Post-traumatic endophthalmitis: causative organisms and visual outcome. *Eur J Ophthalmol* 1999, 9:21–31.

Atkins SD, Clark IM. Fungal molecular diagnostics : a mini review *J Appl Genet.* 2004, 45, 3 -15..

Badenoch PR, Coster DJ, Wetherall BL, Brettig HT, Rozenbilds MA, Dreth A, Wagels G. Pythium insidiosum keratitis confirmed by DNA sequence analysis. *Br J Ophthalmol* 2001, 85: 502-503

Baum DA, Donoghue J. Choosing among alternative “phylogenetic”species concepts. *Syst Bot* 1995,20:560–573

Bhartiya P, Daniell M, Constatinou M, Islam FA, Taylor HR. Fungal keratitis in Melbourne . *Clin Experiment Ophthalmol* 2007 35:124-30.

Binstock JM. Molecular biology techniques for identifying dermatophytes and their possible use in diagnosing onychomycosis in human toenail: a review. *J Am Podiatr Med Assoc*. 2007, 97:134-44

Bistis GN. Pleomorphisms in the dermatophytes. *Mycologia*. 1959, 51:440-4.

Bloom PA,Laidlaw DA, Easty DL, Warnock DW..Treatment failure in a case of fungal keratitis caused by *Pseudallescheria boydii*. *Br J Ophthalmol* 1994*,* 76:367–368

Boerlin P, Boerlin-Petzold F, Goudet J, Durussel C, Pagani JL, Chave JP, Bille J. Typing *C. albicans* oral isolates from human immunodeficiency virus-infected patients by multilocus enzyme electrophoresis and DNA fingerprinting. *J Clin Microbiol* 1996,34:1235–1248.

Bougnoux ME, Dupont C, Mateo J, Saulnier P, Faivre V, Payen D, Chanoine MHN.. Serum is more suitable than whole blood for diagnosis of systemic candidiasis by npCR. *J Clin Microbiol*. 1999, 37:925–930.

Boutati EI, Anaisise EJ. Fusarium : a significant emerging pathogen in patients with haemorrhagic malignancy : ten years experience at a cancer centre and implication for management. *Blood* 1997, 90: 999 – 1008.

Boysen ME, Jacobsson KG, Schnurer J. Molecular identification of species from the *Penicillium roqueforti* group associated with spoiled animal feed. *Appl Environ Microbiol* 2000,66:1523–1526

Brandt ME, Gaunt D, Iqbal N, McClinton S, Hambleton S, Sigler L. False-positive *Histoplasma capsulatum* Gen-Probe chemiluminescent test result caused

by a *Chrysosporium* species. *J Clin Microbiol* 2005, 43:1456-1458.

Bretagne S, Costa JM, Khuong AM, Poron F, Cordonnier C, Vidaud M, Feith F, Detection of Aspergillus species DNA in bronchoalveolar lavage samples by competitive PCR. *J Clin Microbiol* 1995, 33 : 1164 -1168.

Brilhante RSN, Ra Cordeiro, DJA Medrano, MFG Rocha, AJ Monteiro, CSP Cavalcante, TEF Meireles, Sidrim JJC. Onychomycosis in Ceará (Northeast Brazil): epidemiological and laboratory aspects. Mem. Inst. Oswaldo Cruz vol.100 no.2 Rio de Janeiro Apr. 2005

Buchman, TG, Rossier M, Mertz WG, Charache P. Detection of surgical pathogens by in vitro DNA amplification. Part I. Rapid identification of *Candida albicans* by *in vitro* amplification of a fungus specific gene. *Surgery* 1990,108: 338–347.

Buffington J, Reporter R, Lasker BA, McNeil MM, Lanson JM,Ross LA, Mascola L, Jarvis WR. Investigation of an epidemic of an invasive Aspergillosis : utility of molecular typing with the use of random amplified polymorphic DNA probes. *Paediatric Infectious diseases* 1994, 13 : 386 – 393

Burda CD, Fisher E. The use of cortisone in establishing experimental fungal keratitis in rats*. Am J Ophthalmol*. 1991, 48:330-335.

Burgess LW General ecology of the fusaria. *J Clin Microbiol* 1981,225-235.

Burt A,Carter DA, White TJ, Taylor JW. DNA sequencing with arbitrary primer pairs. *Mol Ecol* 1994, 3:523–525.

Burt A, Carter DA, Koenig GL, White TJ, Taylor JW. Molecular markers reveal cryptic sex in the human pathogen *Coccidioides immitis*. *Proc Natl Acad Sci* USA 1996, 93:770–773

Buzina WD, Loidolt LH, Freudenschuss BK, Stammberger H.Development of molecular methods for identification of *Schizophyllum commune* from clinical samples. *J Clin Microbiol* 2001,39:2391-2396.

Callanan D, Scott IU, Murray TG, Oxford KW, Bowman CB, Flynn HW Jr. Early onset endophthalmitis caused by Aspergillus species following cataract surgery.*Am J Ophthalmol* 2006, 142:509-11.

Callister ME, Griffiths MJ, Nicholson AG, Leonard A, Johnson EM, Polkey MI, Kerr JR. A fatal case of disseminated aspergillosis caused by a nonsporulating strain of *Aspergillus fumigatus J Clin Pathol* 2004, 57:991–992.

Carroll NM., Jaeger EE, Choudhury S, Dunlop AA, Matheson MM, Adamson, P, Okhravi N, Lightman S. Detection of and discrimination between gram-positive and gram-negative bacteria in intraocular samples by using npCR. *J Clin Microbiol* 2000,38:1753–1757.

Carter DA, Burt A, Taylor JW, Koenig JL, Dechairio B, White. A set of electrophoretic molecular markers for strain typing and population genetic studies of *Histoplasma capsulatum*. *Electrophoresis* 1997, 18:1047–1053.

Chang PK, Bhatnagar D, Cleveland TE,Bennett JW. Sequence variability in homologs of the aflatoxin pathway gene aflR distinguishesspecies in Aspergillus section Flavi *Appl Environ Microbiol* 1995, 61:40–43.

Chang DC, Grant GB, O'Donnell K, Wannemuehler KA, Noble-Wang J, Rao CY, Jacobson LM, Crowell CS, Sneed RS, Lewis FM, Schaffzin JK, Kainer MA, Genese CA, Alfonso EC, Jones DB, Srinivasan A, Fridkin SK, Park BJ Multistate outbreak of Fusarium keratitis associated with use of a contact lens solution *JAMA,* 2006, 296 : 953-63.

Chen X,Romaine CP, Tan Q, Schlagnhaufer B, Ospina-Giraldo MD,Royse DJ, Huff DR.. PCR-based genotyping of epidemic and preepidemic *Trichoderma* isolates associated with green mold of *Agaricus bisporus*. *Appl Environ Microbiol* 1999, 65:2674–2678.

Chen YC, Eisner JD, Kattar MM, Rassoulian-Barrett SL, LaFe K, Yarfitz SL, Limaye AP, Cookson BT.. Identification of medically important yeasts using PCR-based detection of DNA sequence polymorphisms in the internal transcribed spacer 2 region of the rRNA genes. *J Clin Microbiol* 2000, 38:2302–2310.

Chen YC, Eisner JD, Kattar MM, Rassoulian-Barrett SL, LaFe K, Yarfitz SL, Limaye AP, Cookson BT.Polymorphic internal transcribed spacer region 1 DNA sequences identify medically important yeasts.*J Clin Microbiol* 2001,39:4042– 51

Chi CC, Wang SH, Chou MC. The causative pathogens of onychomycosis in southern Taiwan. *Mycoses* 2005, 48 :413-20.

Chryssanthou E, Andersson B, Petrini B, Lofdahl S, Tollemar J. Detection of *C. albicans* DNA in serum by PCR. *Scand J Infect Dis* 1994, 26:479–485.

Clark WL, Kaiser PK, Flynn HW, Belfort A, Miller D, Meisler DM. Treatment strategies and visual acuity outcomes in chronic postoperative *Propionibacterium acnes* endophthalmitis. *Ophthalmology* 1999, 106:1665–1670.

Clayton YM, Hay RJ**.** Epidemiology of fungal skin and nail disease:roundtable discussion held at Dermatology 2000, Vienna, 17 1993. *Br J Dermatol*. 1993 130(Suppl. 4):9–11.

Clellan, Billson FA. Suppurative corneal ulceration in Bangladesh. A study of 142 cases examining the microbiological diagnosis, clinical and epidemiological feature of bacterial and fungal keratitis. 1994, *Aust N Z J Ophthalmol*. 22:105–110.

Clinch TE,Robinson MJ, Barron BA, Insler MS, Liang K, Kaufman.HE Fungal keratitis from nylon line lawn trimmers. *Am J Ophthalmol* 1992,114 : 437–440

Cohen JL, Scher RK, Pappert AS. 1992. The nail and fungus infections, 106–122. In B. Elewski (ed.), Cutaneous fungal infections.

Collee JG, Miles RS, Watt B. Tests for identification of bacteria. In: Collee JG, Fraser AG, Marimon BP, Simmons A (editors.) Mackie and McCartney's Practical Medical Microbiology. 14th ed. Churchill Livingstone: Edinburgh; 1996. p. 131-50. 11.

Cowan CL, Madden WM, Hatem GF, Merritt JC. Endogenous Bacillus cereus panophthalmitis. *Ann Ophthalmol* 1987, 19:65–68.

Crampin AC, Mathews RC. 1993. Application of PCR to the diagnosis of Candidiasis by amplification of hsp 90 gene fragment. J. of Med. Microbol. 39: 233-8.

Crous PW . Taxonomy and pathology of Cylindrocladium (Calonectria) and allied genera. APS Press, Minnesota, U.S.A.2002

Crous PW, Slippers B, Wingfield MJ, Rheeder J, Marasas WFO, Philips AJL, Alves A, Burgess T, Barber P, Groenewald JZ. Phylogenetic lineages in the Botryosphaeriaceae *Studies in mycology* 2006, 55: 235–253.

Cuero RG. Ecological distribution of *Fusarium solani* and its opportunistic action related to mycotic keratitis in Cali, Colombia. *J Clin Microbiol* 1980,12:455–461

Davey KG, Holmes AD, Johnson EM, Szekeley A, Warnock DW. Comparative evaluation of broth micro dilution methods for antifungal drug susceptibility testing of *Candida* species and *Cryptococcus neoformans*. *J Clin Microbiol* 1998;36:926-30

Davis JI Species concepts and phylogenetic analysis. Introduction. *Sys. Bot* 1995, 20:555–559.

De Baere T, Claeys G, Swinne D, Verschraegen G, Muylaert A, Massonet C, Vaneechoutte M. Identification of cultured isolates of clinically important yeast species using fluorescent fragment length analysis of the amplified internally transcribed rRNA spacer 2 region (ITS2). *BMC Microbiol* 2002, 2:21.

De Hoog, GS, Guarro J, Gene J, Figueras MJ. Atlas of clinical fungi, 2000, 2nd ed. Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands 112 -120

Denning DW, Radford SA, Oakley KL, Hall L, Johnson EM, Warnock DW. Correlation between in-vitro susceptibility testing to itraconazole and in-vivo outcome of *Aspergillus fumigatu*s infection. *J Antimicrob Chemother* 1997, 40:401–414

Deshpande SD, Koppikar GV. A study of mycotic keratitis in Mumbai. *Ind J Pathol Microbiol*, 1999, 42:81–7.

Diaz-Guerra, TM, Martinez-Suarez JV, Laguna F, Rodriguez-Tudela JL.. Comparison of four molecular typing methods for evaluating genetic diversity among *C. albicans* isolates from human immunodeficiency virus-positive patients with oral candidiasis. *J Clin Microbiol* 1997, 35:856–861

D'Mellow G, Hirst LW, Whitby M, Nimmo G, Stallard K. Intralenticular infections. *Ophthalmology* 1991, 98:1376–1378.

Donahue SP, Greven CM, Zuravleff JJ, Eller AW, Nguyen MH, Peacock JE, Wagener, MW, Yu VL. Intraocular candidiasis in patients with candidemia. Clinical implications derived from a prospective multicenter study. *Ophthalmology* 1994, 101:1302–1309.

Dubach MM, Lacroix V, Chauvin M, Gall IL, Guidecelli C, Lorenzo F, Derouin F.Rapid discrimination among dermatophytes, *Scytalidium* spp., and other fungi with a PCR restriction fragment length polymorphism ribotyping method*. J Clin Microbiol*. 2001, 39: 685-90.

Dunlop AA, Wright ED, Howlader SA, Nasrul I, Husain R, McClellan K, Billson FA. Suppurative corneal ulceration in Bangladesh: a study of 142 cases, examining the microbiological diagnosis, clinical and epidemiological features of bacterial and fungal keratitis. *Aust N Z J Ophthalmol* 1994, 22:105–110.

Dyavaiah M, Ramani R, Chu DS, Ritterband DC, Shah MK, Samsonoff WA, Chaturvedi S, Chaturvedi V. Molecular characterization, biofilm analysis and experimental biofouling study of *Fusarium* isolates from recent cases of fungal keratitis in New York State. *BMC Ophthalmol*. 2007 ,30:1.

Einsele H, Hebart H, Roller G, Loffler J, Rothenhofer I, Muller CA,Bowden RA, van Burik JA, Engelhard D, Kanz L, Schumacher U.Detection and identification of fungal pathogens in blood by usingmolecular probes. *J Clin Microbiol* 1997, 35:1353–1360

Elewski BE. Clinical pearl: diagnosis of onychomycosis. *J Am Acad Dermatol* 1995, 32:500–501.

Elewski BE. Large scale epidemiological study of the causal agents of onychomycosis: mycological findings from the multicenter onychomycosis study of terbinafine. *Arch Dermatol*. 1997,133:1317–1318

Elewski BE. Onychomycosis: Pathogenesis, Diagnosis,and Management *Arch Dermatol* 1998,11 ;415-429

Ellis DH., Watson AB,. Marley JE, Williams TG. Nondermatophytes in onychomycosis of the toenails. *Br J Dermatol* 1997, 136:490–493.

Ellis D. Amphotericin B: spectrum and resistance. *J Antimicrob Chemother* 2002, 49(Suppl. 1):7–10Endophthalmitis Vitrectomy Study. A randomized trial of immediate vitrectomy and of intravenous antibiotics for the treatment of postoperative bacterial endophthalmitis. *Arch Ophthalmol* 1995, 113:1479–1496.

Faggi E, Pini G, Campisi E, Bertillini C, Fanzo ED, Mancianti F. Application of PCR to distinguish common species of dermatophytes. *J Clin Microbiol* 2001, 39: 3382-85

Ferencz JR, Assia EI, Diamantstein L, Rubinstein E. Vancomycin concentration in the vitreous after intravenous and intravitreal administration for postoperative endophthalmitis. *Arch Ophthalmol* 1999, 117: 1023–1027.

Ferrer C, Colom F, Frases S, Mulet E, Abad JL, Alio JL. Detection and identification of fungal pathogens by PCR and by ITS2 and 5.8S ribosomal DNA typing in ocular infections. *J Clin Microbiol*. 2001,39:2873– 2879.

Ferrer C, Munoz G, Alio JL, Abad JL, Colom F. Polymerase chain reaction diagnosis in fungal keratitis caused by *Alternaria alternata*. *Am J Ophthalmol* 2002,133:398–399.

Ferro JF, de-Pablos M, Logrono MJ, Guisasola L, Aizpuru F.Postoperative contamination after using vancomycin and gentamicin during phacoemulsification. *Arch Ophthalmol* 1997,115:165–170.

Field D, Eggert L, Metzger D, Rose R, Wills C. Use of polymorphic short and clustered coding-region microsatellite to distinguish strains of *C. albicans*. FEMS *Immunol Med Microbiol* 1996, 15:73–79.

Fiscella RG, Nguyen TK, Cwik TK, Phillpotts BA, Friedlander SM, Alter DC, Shapiro MJ, Blair NP, ieser JP. Aqueous and vitreous penetration of levofloxacin after oral administration. *Ophthalmology* 1999,106:2286–2290.

Flahaut M, Sangalard D, Mond M, Bille J, Rossier M. Rapid detection of *Candida albicans* in clinical specimens by DNA amplification of common region from *C. albicans* secreting aspartic Proteinase genes . 1998, *J Clin Microbiol*, 36: 395-401.

Foster RK, Rebell G. Animal model of *Fusarium solani* keratitis. Am J *Ophthalmol* 1975, 79:510-515.

Foster CS. Fungal keratitis. *Infect Dis Clin N Am*1992; 6:851–7

Foster RE, Martinez JA, Murray TG, Rubsamen PE, Flynn HW, Forster RK. Useful visual outcomes after treatment of *Bacillus cereus* endophthalmitis. *Ophthalmology* 1999,103:390–397.

Foroozan R, Eagle RC, Cohen EJ. Fungal keratitis in a soft contact lens wearer *CLAO J* 2000; 26: 166 – 168

Forbes B, Baron EJ Bailey and Scott's diagnostic microbiology 10th edition Basic laboratory methods in mycology 850-875. Published by Mosby, Missouri.

Fraaije B, Lovll DJ, Rohel EA, Hollomon DW. Rapid detection and diagnosis of Septoria tritci epidemics in wheat using a polymerase chain reaction. *J Appl Microbiol* 1999, 86: 701- 708

Freidank H. Hyalohyphomycoses due to *Fusarium* species – 2 case reports and review of Literature . *Mycoses* 1995;38 : 69 -74.

Fromtling RA. 1995. Mycology, p. 697–855 In Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH (ed.), Manual of clinical microbiology, 6th ed. ASM Press, Washington, D.C.

Fujita SI, Lasker BA, Lott TJ, Reiss E, Morrison CJ. Microtitration plate enzyme immunoassay to detect PCR-amplified DNA from Candida species in blood. *J Clin Microbiol* 1995, 33:962–967.

Fujita SI, Senda Y, Nakaguchi S, Hashimoto T. Multiplex PCR using internal transcribed spacer 1 and 2 regions for rapid detection and identification of yeast strains *J Clin Microbiol* 2001, 39: 3617-3622

Gamis A, Gudnason T, Giebink G, Ramsay N. Disseminated infection with *Fusarium* in recipients of bone marrow transplants. *Rev Infect Dis* 1991,13: 1077–1088

Garg P, Gopinathan U, Choudhary K, Rao GN. Keratomycosis - clinical and microbiologic experience with dematiaceous fungi. *Ophthalmology* 2000, 107:574–580.

Gaudio PA, Gopinathan U, Sangwan V, Hughes TE. PCR based detection of fungi in infected corneas. *Br J of Ophthalmol*. 2002, 86: 755-760.

Geiser DM, Timberlake WE, Arnold ML. Loss of meiosis in Aspergillus. *Mol Biol Evol* 1996,13:809–817.

Geralch N, Nirenberg H. The genus *Fusarium* – a pictorial atlas Mitt BiolBunderant, Land - Forstw, Berlin Dehlem 1982; 209 : 1-406

Ghannoum MA, Rex JH, Galgiani JN. Susceptibility testing of fungi: Current status of correlation *in vitro* data with clinical outcome. J Clin Microbiol 1996;34:489-95.

Giese MJ, Berliner JA, Riesner A, Wagar EA, Mondino BJ. A comparison of the early inflammatory effects of an agr*/sar*versus a wild type strain of *Staphylococcus aureus* in a rat model of endophthalmitis. *Curr Eye Res* 1999,18:177–185.

Gonzales CA, Srinivasan M, Whitcher JP, et al. Incidence of corneal ulceration in Madurai District, south India. *Ophthalmic Epidemiol* 1996;3:159–66.

Gopinathan U, Garg P, Fernandes M, Sharma S, Athmanathan S, Rao GN. The epidemiological features and laboratory results of fungal keratitis. A 10-year review at a referral eye care center in South India. *Cornea* 2002, 21:555–559

Gordon YJ. Rapid diagnostic tests for infectious ocular disease. *Int Ophthalmol Clin* 1993;33:153–61.

Greenwald MJ ,Wohl LG, Sell CH. Metastatic bacterial endophthalmitis: a comtemporary reappraisal. *Surv Ophthalmol* 1986, 31:81–101

Griffiths RL, Whiteley AS, O'Donnell AG, Bailey MJ. Rapid method fo co extraction of DNA and RNA from natural environments for analysis of ribosomal DNA nd RNA based microbial community composition. *Appl Environ Microbiol* 2000; 66: 5488-5491

Grueb M, Rohrbach JM, Zierhut M. Amphotericin B in the therapy of Candida glabrata endophthalmitis after penetrating keratoplasty *Cornea* 2006, 25:1243-4.

Gryzenhout M, Myburg H, Hodges CS, Wingfield BD, Wingfield MJ Microthia and Holocryphia, two new genera of fungi from Eucalyptus representing the species previously known as Cryphonectria havanensis and Cryphonectria eucalypti. Studies in *Mycology* 2006, 55: 35–52.
Guarro J, Vroey CD, Gene J. Concerning the implication of *Arthrobotrys oligospora* in a case of keratitis. *J Med Vet Mycol* 1991,29**:**349–352.

Guarro J, Gene J. Opportunistic Fusarial infections in humans *Eur J Clin Microbiol Infect Dis* 1995; 14: 747 -54.

Guarro J, Gene J, Stchigel AM. Developments in fungal taxonomy. *Clin Microbiol Rev* 1999 a, 12:454-500.

Guarro J, Gene J, Stchigel AM,. Developments in fungal taxonomy. *Clin Microbiol Rev*, 12 : 454 – 500 Jones, B. R, D. B. Jones, and Y. Clayton. 1969. Corneal and intraocular infection due to *Fusarium solani*. *Trans Ophthalmol Soc* 1999 b, 89:757-779.

Guarro J, Akiti T, Horta RA, Filho LAMFL, Gene J´ ,Gomes SF, Aguilar C, Ortoneda M. Mycotic keratitis due to *Curvularia senegalensis* and in vitro antifungal usceptibilities of Curvularia spp. *J Clin Microbiol* 1999c, 37:4170–4173.

Guarro JA, Lima LH, Gene J, Freitas DD, Godoy P, Zorat-Yu ML, Zaror L, Fischman O**.** Corneal ulcer caused by the new fungal species *Sarcopodium oculorum*. *J Clin Microbiol* 2002.,40**:** 3071– 3075

Gugani HC, Talwar RS, Njoku-Obi AN, et al. Mycotic keratitis in Nigeria. A study of 21 cases. *Br J Ophthalmol* 1976;60:607–13.

Gupta AK., Kohli Y, Summerbell RC. Exploratory study of single copy genes and ribosomal intergeic spacers for distinction of dermatophytes. *Studies in Mycol* 2002, 47:87-96.

Hagan ME. Wright M, Newman, Dolin P, Johnson G. Causes of suppurative keratitis in Ghana. *Br J Ophthalmol*. 1995, 79:1024–1028

Hall L, Wohlfiel S, Roberts GD. Experience with the MicroSeq D2 large-subunit ribosomal DNA sequencing kit for identification of commonly encountered, clinically important yeast species. *J Clin Microbiol* 2003, 41:5099–5102.

Hall L, Wohlfiel S, Roberts GD.Experience with the MicroSeq D2 large-subunit ribosomal DNA sequencing kit for identification of filamentous fungi encountered in the clinical laboratory. *J Clin Microbiol* 2004,42:622–626.

Halliday C, Wu QX, James G, Sorrell T . Development of a nested qualitative Real – Time PCR assay to detect *Aspergillus* DNA in clinical specimens *J of Clin Microbiol* 2005, 43 : 5366-5368

Han DP, Wisniewski SR, Wilson LA, Barza M, Vine AK, Doft BH, Kelsey SF . Spectrum and susceptibilities of microbiologic isolates in the Endophthalmitis Vitrectomy Study. *Am J Ophthalmol* 1996,122:1–17.

Hanazawa R, Latge JP, Lortholary J, Makimura K, Morrison CJ, Murayama SY, Naoe S, Paris S, Sarfati J, Shibuya K, Sullivan D, Uchida K, Yamaguchi H. Molecular diagnosis and epidemiology of fungal infections. *Med Mycol* 1998, 36(Suppl. 1):249–257.

Hawser S, Douglas LJ**.** Resistance of *C. albicans* biofilms to antifungal agents in vitro. *Antimicrob Agents Chemother* 1995, 39**:**2128–2131.

Hayashi K. PCR-SSCP: a simple and sensitive method or detection of mutations in the genomic DNA . *PCR Methods Appl*. 1991, 1: 34-38

Haynes KA, Westerneng TJ, Fell JW, Moens W. Rapid detection and identification of pathogenic fungi by PCR amplification of large subunit ribosomal DNA*. J Med Vet Mycol* 1995, 33:319–325.

Healy M, Reece K, Walton D, Huong J, Shah K, Kontoyiannis DP.Identification of the species level and differentiation between strains of *Aspergillus* species clinical isolates by automated repetitive – sequence based PCR. *J Clin Microbiol* 2004, 42 : 4016 – 4024.

Heaven CJ, Mann PJ, Boase DL. Endophthalmitis following extracapsular cataract surgery: a review of 32 cases. *Br J Ophthalmol*.1992;76:419–423.

Hendolin PH, Kahkola PK , Anttila VJ, Malmberg H, Richardson M, Ylikoski T panfungal PCR and multiplex liquid hybridization for detection of fungi in tissue specimens *J Clin Microbiol* 2000,38: 4186 - 4192

Hennequin C, Abacin E, Symoens F . Identification of *Fusarium* species involved in human infections by 28SrRNA gene sequencing. *J Clin Microbiol*. 1999, 37 : 3586 - 3589.

Hennequin C. Lavarde J, Poirot L, Rabodonirina M, Datry A, .Aractingi S, Dupouy CJ, Caillot D, Grange F, Kures L, Morin O, Lebeau B, Bretagne S, Guigen C, Basset D, Grillot R. InvasiveFusarium infections: a retrospective survey of 31 cases. The French Grouped'Etudes des Mycoses Opportunistes.*J Med Vet Mycol*. 1997, 35:107–114.

Henry T, Iwen PC, Hinriche H. Identification of *Aspergillus* species using ITS regions 1 and 2*. J Clin Microbiol*. 2000, 38: 1510 – 1515.

Hidalgo JA. Alangaden GJ, Eliott D, Akins RA, Puklin J, Abrams G,Vazquez JA. Fungal endophthalmitis diagnosis by detection of *C. albicans* DNA in intraocular fluid by use of a species-specific PCR assay.*J Infect Dis* 2000, 181:1198–1201

Hinukson HP, Hurst SF,Lott TJ, Warnock DN, Morrison CJ. Assessment of ribosomal large subunit D1 – D2, ITS 1 and ITS 2 regions as targets for molecular identification of medically important *Aspergillus* species. *J Clin Microbiol* 2005, 43: 2092 – 2103

Hopfer RL, Walden P, Setterquist S, Highsmith WE.. Detection and identification of fungi in clinical specimens using polymerase chain reaction amplification and restriction enzyme analysis. *J Med Vet Mycol* 1993, 31: 65-75

Horne J, Hollomon DW**.** Molecular mechanisms of azole resistance in fungi. *FEMS Microbiol Lett* 1997, 149:141–149.

Hsu MC, Chen KW, Lo HJ, Chen YC, Liao MH, Lin YH, Li SY. Species identification of medically important fungi by use of real time light cycler PCR *J of Med Microbiol*. 2003, 52: 1071-76.

Hudson RR. How can the low levels of DNA sequence variation in regions of the Drosophila genome with low recombination rates be explained? *Proc. Natl. Acad*. *Sci*. USA 1994, 91:6815–6818

Hue FX, Huerre M, Bievre**.** Specific detection of *Fusarium* species in blood and tissues by a PCR technique *J Clin Microbiol* 1999, 37: 2434 – 2438

Hykin PG, Tobal K, McIntyre G, Matheson MM, Towler HM, Lightman SL. The diagnosis of delayed postoperative endophthalmitis by PCR of bacterial DNA in vitreous samples. *J Med Microbiol* 1994;40:408-15

Ilkit M. Onychomycosis in Adana, Turkey: a 5-year study. *Int J Dermatol* 2005, 44: 851-4.

Ingroff AVE, Pfaller MA .In Chapter 124: Susceptibility testing methods for yeasts and filamentous fungi 1880-1894 In P. R. Murray, E. J. Baron,M. A. Pfaller, J. H. Jorgensen, and R. H. Yolken (ed.), Manual of clinical microbiology, 8th ed. American Society for Microbiology, Washington, D.C. 2003, p 1726–1760

Ishibashi Y, Matsumoto Y, Takei K. The effects of intravenous miconazole on fungal keratitis. *Am J Ophthalmol* 1984, 98:433–437.

Iwastu TM, Miyaji M, Taguchi H, Okamoto S. 1982. Evaluation of skin test for chromoblastomycosis using antigen prepared from cultural filtrates of *Fonsecaea pedrosoi, Phialophora verrucosa*, *Wangiella dermatitidis* and *Exophiala jeanselmei. Mycopathologia* 77:59–64

Iwen PC, Hinriche H, Rupp ME. Utilization of ITS regions as molecular targets to detect and identify fungal pathogens. *Med Mycology* 2001, 40 : 87 – 109.

Iyer RS, Pal RB, Patel RB, Banker DD. PCR based diagnosis of systemic fungal infections and sensitivity testing of fungal isolates. *Indian J Med Microbiol* 2002, 20:160-2.

Jackson CJ, Barton RC, Evans GV. Species identification and strain differentiation of dermatophyte fungi by analysis of ribosomal DNA intergenic spacer regions. *J Clin Microbiol* 1999, 37: 931-36.

Jaeger EM, Carroll NM, Choudhury S, Dunlop AA, Towler HM, Matheson MM Rapid detection and identification of *Candida, Aspergillus* and *Fusarium* species in ocular samples using nested PCR. *J Clin Microbiol* 2001, 38:2902-8

Jett BD, Atkuri RV, Gilmore MS. *Enterococcus faecalis* localization in experimental endophthalmitis: role of plasmid-encoded aggregation substance. *Infect Immun* 1998, 66:843–848.

Johnson MW, Doft BH, Kelsey SF, Barza M, Wilson LA, Barr CC,Wisniewski SR, and the Endophthalmitis Vitrectomy Sudy Group. The Endophthalmitis Vitrectomy Study: relationship between clinical presentation and microbiologic spectrum. *Ophthalmology* 1997,104:261–272

Johnson GJ, Minassian DC, Weale R. The epidemiology of eye disease.1st ed. New York: Chapman and Hall Medical, 1998:143.

Jonas JB, Knorr HL, Budde WM. Prognostic factors in ocular injuries caused byintraocular or retrobulbar foreign bodies*. Ophthalmology* 2000, 107:823–828.

Jones BR, Jones DB, Clayton Y. Corneal and intraocular infection due to *Fusarium solani*. *Trans Ophthalmol Soc* 1969, U. K. 89:757-779.

Jones BR. Principles in the management of oculomycosis. *Am J Ophthalmol* 1975, 79:719–751.

Jones ME, Fox AJ, Barnes AJ, Oppenheim BA, Balagopal P, Morgenstern GR, Scarffe. JH. PCR-ELISA for the early diagnosis of invasive pulmonary *Aspergillus* infection in neutropenic patients*. J Clin Pathol*, 1998, 51:652–656

Jordan JA. PCR identification of four medically important *Candida* species by using a single primer pair. *J Clin Microbiol*. 1994,32:2962–2967

Jousson O, Lechenne B, Bantonnes O, Capaccia S, Mignon B, Barblan J, Quadroni M, Monod M. Multiplication of an ancestral gene encoding secreted fungalysin preceded species differentiation in the dermatophytes, *Trichophyton* and *Microsporum*. *Microbiology*. 2004, 150: 301-310.

Kairuz B, Zuber JP, Jaunin P, Buchman TG, Bille J, Rossier M. Rapid detection and identification of *C. albicans* and Torulopsis (Candida) glabrata in clinical specimens by species-specific npCR amplification of a cytochrome P-450 lanosterol-alpha-demethylase (L1A1) gene fragment. *J Clin Microbiol*. 1994, 32:1902–1907.

Kalicharan D, Jongebloed WL, Los LI, Worst JGF. (An)aerobic bacteria found in the secondary-cataract material. A SEM/TEM study. Doc Ophthalmol. 1992, 82:125–133.

Kanbe T, Yamaki K, Kikuchi A. Identification of the pathogenic *Aspergillus* species by nested PCR using a mixture of specific primers to DNA topoisomerase II gene. *Microbiol Immunol* 2002,46:841–848.

Kano RY, Watari NT, Watanabes S, Takahashi H Tsujimoto H, Hosegawa A. Molecular analysis of chitin synthase gene sequences of *Trichpohyton mentagrophytes* complex and *T.rubrum. Curr Microbiol* 1998, 37: 236-39.

Karl SA, Avise JC. Balancing selection at allozyme loci in oysters: implications from nuclear RFLPs. *Science* 1992, 256:100–102

Kaspar MD, Zoulek HG, Paredes ME,Alborno RE, Medina D, Morinigo M., Ortiz de Fresco, Aguero F. Mycotic keratitis in Paraguay. *Mycoses* 1991, 34:251–254.

Kattan HM, Flynn HW, Pflugfelder SD, Robertson C, Forster RK. Nosocomial endophthalmitis survey: current incidence of infection after intraocular surgery. *Ophthalmology* 1991, 98:227–238.

Keya De, Ramamurthy T, Ghose AC, Islam MS, Takeda Y, Nair GB. Modification of the multiplex PCR for unambiguous differentiation of the ElTor and classical biotypes of *Vibrio cholerae* O *Indian J Med Res*. 2001;114:77-82.

Khan S, Singhal S, Mathur T, Upadhyay DJ, Rattan A. Antifungal susceptibility testing method for resource constrained laboratories . *Indian J Med Microbiol* 2006 24:171-6.

Khairallah SH, Byrne KA, Tabbara KF. Fungal keratitis in Saudi Arabia. *Doc*. *Ophthalmol*. 1992, 79:269–276.

Khanal B, Kaini KR, Deb M Microbial keratitis in eastern Nepal. *Tropical Doctor* 2001; 31:168–9

Khor WB, Aung T, Saw SM,Wong TY, Tambyah ,Tan AL,Beuerman R.An out-break of *Fusarium* Keratitis Associated With Contact Lens Wear in Singapore *JAMA.* 2006;295:2867-2873.

King D, Rhine-Chalberg J, Pfaller MA, Moser SA, Merz WG. Comparison of four DNA-based methods for strain deliniation of *Candida lusitaniae*. *J Clin Microbiol* 1995,33:1467–1470

Kiryu H. 1991. Invasion and survival of *Fusarium solani* in the dexamethasone-treated cornea of rabbits. *J Med Vet Mycol* 29:395-406.

Klich MA. Identification of common *Aspergillus* species. Central bureau voor Schimmelcultures, Utrecht, The Netherlands 2002.194-202

Klimyuk VI, Carroll BJ, Thomas CM, Jones JDG Alkali treatment for rapid preparation of plant material for reliable PCR analysis. *Plant journal* 1993, 3: 493-494.

Klotz SA, Penn CC, Negvasky GJ, Butrus SI**.** Fungal and parasitic infections of the eye. *Clin Microbiol Rev*2000, 13: 662-85

Knox CM, Cevallos V, Margolis TP, Dean D. Identification of bacterial pathogens in patients with endophthalmitis by 16S ribosomal DNA typing. *Am J Ophthalmol* 1999, 128:511–512*.*

Koufoanou V, Burt A, Taylor JW.Concordance of gene genealogies reveals reproductive isolation in the pathogenic fungus *Coccidioides immitis*. *Proc Natl Acad Sci* USA 1997, 94:5478–5482

Kresloff MS, Castellarin AA, Zarbin MA.Endophthalmitis *Surv Ophthalmol*. 1998,43:193–224.

Kunimoto, DY, Das T, Sharma S, Jalali, A, Majji B, Gopinathan U, Athmanathan S, Rao TN. Microbiologic spectrum and susceptibility of isolates: part 1 postoperative endophthalmitis. Endophthalmitis Research Group. *Am J Ophthalmol ,* 1999,128:240–242

Kurtzman CP, Robnett CJ. Identification of clinically important Ascomycetous yeasts based on nucleotide divergence in the 5' end of the large-subunit (26S) ribosomal DNA gene. *J Clin Microbiol* 1997, 35:1216–1223

Lau A, Chen S, Sorrell T, Carter D, Malik R, Martin P, Halliday C. Development and clinical application of a panfungal PCR assay to detect and identify fungal DNA in tissue specimens*. J Clin Microbiol* 2007,45:380-5.

Lawry MA, Haneke E, Strobeck K, Martin S, Zimmer B, Romano PS. Methods for Diagnosing Onychomycosis A Comparative Study and Review of the Literature *Arch Dermatol.* 2000 ; 136: 1112-1116.

Leck AK, Thomas PA, Hagan M, Kaliamurthy J, Ackuaku E, John M, Newman MJ, Codjoe FS, Opintan JA, Kalavathy CM, Essuman V, Jesudasan CAN, Johnson GJ. Aetiology of suppurative corneal ulcers in Ghana and south India, and epidemiology of fungal keratitis. *Br J Ophthalmol* 2002, 86:1211–1215.

Lees ND, Skaggs B, Kirsch DR, Bard M. Cloning of the late genes in the ergosterol biosynthetic pathway of *Saccharomyces cerevisiae*—a review. *Lipids* 1995,30:221–226.

Levin LA , Avery R, Shore JW, Woog JJ, Baker AS The spectrum of orbital aspergillosis: a clinicopathological review. *Surv Ophthalmol* 1996**,** 41:142–154

Liesegang TJ, Forster RK. Spectrum of microbial keratitis in South Florida. *Am J Ophthalmol*. 1980, 90:38–47.

Lin SH, Lin CP, Wang HZ, Tsai RK, Ho CK. Fungal corneal ulcers of onion harvesters in southern Taiwan. *Occup Environ Med* 1999, 56:423–425.

Lindsley MD,Hurst SF, Iqbal NJ, Morrison CJ. Rapid identification of dimorphic and yeast like fungal pathogens using specific DNA probes. *J Clin Microbiol* 2001 , 39 : 3505-3511

Lischewski A, Amann RI, Harmsen D, Merkert H, Hacker J.Morschhauser J. Specific detection of *C. albicans* and *Candida tropicalis* by fluorescent in situ hybridization with an 18S rRNA-targeted oligonucleotide probe. *Microbiology* 1996, 142:2731–2740.

Liu ZL, Domier, Sinclair JB. Polymorphism of Genes Coding for Nuclear 18S rRNA Indicates Genetic Distinctiveness of Anastomosis Group 10 from Other Groups in the Rhizoctonia solani Species Complex. *Applied and environ Microbial* 1995, 61: 2659–2664

Liu D, Coloe S, Pederson J, Baird R**.** Use of arbitrarily primed PCR to differentiate *Trichophyton* dermatophytes*. FEMS Microbiol. Lett*. 1996,136:147-150.

Loeffler J, Henke N, Hebart. Quantification of fungal DNA by using fluorescence resonance energy transfer and the Light cycler system *J Clin Microbiol* 2000, 38: 2902-2908

Lohmann CP, Linde HJ, Reischl U. Improved detection of microorganisms by PCR in delayed endophthalmitis after cataract surgery. *Ophthalmology* 2000, 107:1047–1051.

Lohmann CP, Heeb M, Linde H-J, Gabel VP, Reischl U. Diagnosis of infectious endophthalmitis after cataract surgery by polymerase chain reaction. *J Cataract Refract Surg* 1998, 24:821–826

Loudon KW, Burnie JP, Coke AP,Matthews RC.Application of polymerase chain reaction to fingerprinting *Aspergillus fumigatus* by random amplification of Polymorphic DNA. *J Clin Microbiol* 1993, 31:1117–1121

Lowe T, Baba F, Shulman M et al. Endogenous endophthalmitis due to *Fusarium* case report and review. *Clin Infect Dis* 1994, 18: 585 - 588.

Luo G,Mitchell TG. Rapid identification of pathogenic fungi directly from cultures by using multiplex PCR *J Clin Microbiol* 2002, 40: 2860–2865

Lutsar I, Marr KA, Lionakis MS, Torres HA, Jafri H, Walsh TJ. Infections due to *Aspergillus terreus:* a multicenter retrospective analysis of 83 cases. *Clin Infect Dis*. 2004, 39:192–198.

Lyons CN, White TC. Transcriptional analysis of antifungal drug resistance in *C. albicans . Antimicrob agents and chemother* 2000,144 : 2296-2303.

Maiwald M, Kappe R, Sonntag G. Rapid presumptive identification of medically relevant yeasts to the species level by polymerase chain reaction and restriction fragment enzyme analysis. *J Med Vet Mycol* 1994 , 32: 115- 122

Makimura K, Murayama SY, Yamaguchi H.. Detection of a wide range of medically important fungi by the PCR. *J Med Microbiol*. 1994 , 40:358–364

Makimura K, Tamura Y, Ochizaki TM, Haregawa A, Tajiri Y, Hanazawa R, Uchida, K, Saito H, Yamagochi**.** Phylogenetic classification and species identification of dermatophyte strains based on DNA sequence of nuclear ribosomal ITS1 regions. *J Clin Microbiol*,1999, 37: 1685-90

Makimura K, Suzuki T, Tamura T, Ikedo M, Hanazawa R,Takahashi Y. Comparative evaluation of standard dilution method and commercial kit for frozen plate antifungal susceptibility testing of yeasts using 200 clinical isolates. *Microbiol Immunol* 2004, 48:747-53..

Marchisio VF, Gallo MG, Tullio V, Nepote S, Piscozzi A, Cassinelli C. Dermatophytes from cases of skin disease in cats and dogs in Turin, Italy. *Mycoses* 1995,38:239-44.

Margolis M , Whitcher C. *Fusarium*--A new culprit in the contact lens case.*J Am Med Association* 2006;296:985-987

Maria D. Bernal, Nisha R. Acharya, Thomas M. Lietman, Erich C. Strauss, Stephen D. McLeod, Hwang DG *Arch Ophthalmol* 2006 124:1051-1053.

Maskin SL, Fetchick RJ, Leone CR, Sharkey PK, Rinaldi MG.. Bipolaris hawaiiensis—caused phaeohyphomycotic orbitopathy. A devastating fungal sinusitis in an apparently immunocompetent host. *Ophthalmology* 1989, 96:175– 179

Massry G, Hornblass GA, Harrison W. Itraconazole in the treatment of orbital aspergillosis. *Ophthalmology* 1996, 103:1467–1470.

Mattson MP, Culmsee C, Fu Z, Camandola S. Roles of nuclear factor alpha B in neuronal survival and plasticity. *J Neurochem* 1998, 74: 443–456.

McClellan, Billson FA. Suppurative corneal ulceration in Bangladesh: a study of 142 cases, examining the microbiological diagnosis, clinical and epidemiological features of bacterial and fungal keratitis. *Aust NZ J Ophthalmol* 1994, 22:105–110.

McGinis MR, Chandler FW. Pythiosis insidioi. P 1081-1084. In D.H.Connor, FW.Chandler, DA. Schwartz, H.J.Manz and E.E.Lack. Pathology of infectious diseases. Appleton and Lange Standford, Conn.

McGinnis MR**.** Laboratory handbook of medical mycology. Academic Press, Inc., New York, N.Y. 1980**,** 41:142–154

Mcilhatton BP, Keating C, Curran MD, Mcmullin MC,. Barr JG, Madrigal A, Middleton D. Identification of medically important pathogenic fungi by reference strand-mediated conformational analysis (RSCA), *J Med Microbiol* 2002, 51: 468–478

McNeil MM, Nash SL, Hajjeh RA, Phelan MA, Conn LA, Plikaytis BD, Warnock DW. Trends in mortality due to invasive mycotic diseases in the United States, 1980-1997. *Clin Infect Dis* 2001, 33:641–647

Mendoza L, Ajello L, McGinnis MR. Infections caused by the oomycetous pathogen *Pythium insisdiosum J Clin Microbiol* 1995, 31: 2967-83

Meredith TA. Posttraumatic endophthalmitis. *Arch Ophthalmol* 1999, 117:520–521.

Merz WG, Connelly C, Hieter P.. Variation of electrophoretic karyotype among clinical isolates of *C. albicans*. *J Clin Microbiol* 1988, 26:842–845

Miyakawa Y, Mabuchi T, Kagaya K, Fukazawa Y. Isolation and characterization of a species specific DNA fragment for detection of *C. albicans* by PCR*. J Clin Microbiol*. 1992, 30:894– 900.

Mohanty JC, Mohanty SK, Sahoo RC, Sahoo A, Prahara Diagnosis of superficial mycoses by direct microscopy - A statistical evaluation *Ind J Dermatol*. *Venereology and Leprology* 1999, 65 : 72-74

Morace G, Sangueineth M,Posteraro B, Carcio GL, Fadda G. Identification of various medically important Candida species in clinical specimens by PCR -RFLP *J Clin Microbiol* 1997, 35: 667-672

Moriarty AP, Crawford GJ, McAllister IL, Constable IJ. Severe corneoscleral infection. A complication of beta irradiation scleralnecrosis following pterygium excision. *Arch. Ophthalmol*. 1993, 111:947–951.

Motoyoma AB, Venancio EJ, Brandao GO**.** Molecular identification of *P.brasiliensis* by PCR amplification of ribosomal DNA *J Clin Microbiol*. 2000, 38 : 3106 - 3109

Mselle J. Fungal keratitis as an indicator of HIV infection in Africa.*Tropical Doctor* 1999, 29:133–5.

Mügge C, Haustein UF, Nenoff P. Causative agents of onychomycosis — a retrospective study *Journal der Deutschen Dermatologischen Gesellschaft* 2006,218

Murdoch D, Parr D. *Pythium insidiosum* keratitis.*Aust NZ J Ophthalmol* 1997;25:177–9.

.

Nascimento AM, Martinez NM**.**. 18SrDNA sequencing, enzyme patterns and morphological characterisation of *Trichophyton* isolates. *Braz J Microbiol* 2001 , 32: 516 -520

Nitulescu C. Fungal keratitis : diagnostic and therapeutic approach. *Oftalmologia* 2006, 50 : 33-38

National Committee for Clinical Laboratory Standards. 1997. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard M27A. NCCLS, Wayne, Pa. keratitis in Paraguay. Mycoses 34:251–254.

Ng JQ, Morlet N, Pearman JW, Constable IJ, McAllister IL, Kennedy CJ. Management and outcomes of postoperative endophthalmitis since the endophthalmitis vitrectomy study: The Endophthalmitis Population Study of Western Australia (EPSWA)'s fifth report. *Ophthalmol* 2005 ,112:1199206.

In Nelson PE, Toussoun TA, Cook RJ(ed.), *Fusarium*:diseases, biology, and taxonomy. Pennsylvania State UniversityPress, University Park.Toussoun, TA., and Nelson PE. 1975. Variation and speciationin the fusaria. *Annu Rev Phytopathol* 13:71-82.

Nelson PE, Dignani MC, Anaissie EJ. Taxonomy, biology and clinical aspects of Fusarium species. *Clin Microbiol Rev* 1994, 7 :479–504.

Nelson PE, Toussoun TA, Marasas WF. *Fusarium* species: an illustrated manual for identification. Pennsylvania State University Press, University Park. 1983.

Ninet B, Jan I, Bontems O, Lechenne B, Jousson O, Panizzon R, Lew D, Monod M. Identification of dermatophyte species by 28S ribosomal DNA sequencing with a commercial kit. *J Clin Microbiol* 2003, 41:826– 830

Nolting S, Brautigam M, Weidinger G.Terbinafine in onychomycosis with involvement by non-dermatophytic fungi. *Br J Dermatol Mycoses* 1994, 32:609–619.

O'Brien TP, Choi S. Trauma-related ocular infections. *Int Ophthalmol Clin N Am* 1995, 8:667–679.

O'Day DM, Akrabawi PL, Richmond LD, Jones BR, Clayton Y. An animal model of *Fusarium solani* endophthalmitis.*Br J Ophthalmol* 1979,63:277-280.

O'Day DM. Selection of appropriate antifungal therapy. *Cornea*1987;6:238–45.

O'Donnell K, Cigelnik E, Nirenberg HI. Molecular systematics

and phylogeography of the Gibberella fujikoroi species complex. *Mycologia* 1998, 90:465–493.

O'Donnell K.Molecular phylogeny of the Nectria haematococca-*Fusarium solani* species complex. *Mycologia* 2000, 92:919–938.

Odds FC, van Gerven F, Ingroff AE, Bartlett MS,Ghannoum MA, Lancaster MV, Pfaller, MA,Rex JH, Rinaldi MG, Walsh TJ. Evaluation of possible correlations between antifungal susceptibilities of filamentous fungi in vitro and antifungal treatment outcomes in animal infection models. *Antimicrob Agents Chemother* 1998, 42:282–288.

Okhravi N, Towler HMA, Hykin P, Matheson M, Lightman S. Assessment of a standard treatment protocol on visual outcome following presumed bacterial endophthalmitis.*Br J Ophthalmol* 1997, 81:719 – 725.

Okhravi N, Adamson P, Mant R, Matheson MM, Midgley G, Towler HM, Lightman. S. PCR and restriction fragment length polymorphism mediated detection and speciation of *Candida* spp. causing intraocular infection. *Invest Ophthalmol Vis Sci* 1998,39:859–866.

Okhravi N, Adamson P, Matheson MM, Towler HMA, Lightman S.PCR/RFLP based detection and speciation of bacteria causing endophthalmitis.*Invest Ophthalmol Vis Sci* 2000, 41:1438–1447

Ormerod LD, Hertzmark E, Gomez DS, et al. Epidemiology of microbial keratitis in southern California. A multivariate analysis. *Ophthalmology* 1987, 94:1322–33.

Orita M., Iwahana H, Kanazawa H, Hayashi K, Sekiya T. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc. Natl. Acad. Sci.* USA, 1989 86:2766–2770.

Orti G, Pearse DE, Avise JC. Phylogenetic assessment of length variation at a microsatellite locus. *Proc Natl Acad Sci* (USA) 1997, 20:10745–10749.

Pan S, Cole GT. Electrophoretic karyotypes of clinical isolates of *C.immitis*. *Infect Immun* 1992, 60:4872–4880.

Panda A, Sharma N, Das G. Mycotic keratitis in children: epidemiologic and microbiologic evaluation. *Cornea* 1997;16:295–9.

Park CS, Kim J, Montone KT. Detection of Aspergillus ribosomal RNA using biotinylated oligonucleotide probes. *Diagn Mol Pathol*, 1997, 6:255–260.

Paz NR, Strahilevitz, Shapiro M, Keller N. Clinical and epidemiological aspects of infections caused by *Fusarium* species a collaborative study from Isreal *J Clin Microbiol* 2004, 42: 3456 -61.

Peterson SW. Phylogenetic relationships in Aspergillus based on rDNA sequence analysis, 2000.p. 323–355. In R. A. Samson and J. I. Pitt (ed.),Integration of modern taxonomic methods for Penicillium and Aspergillus classification.

Harwood Academic Publishers, Amsterdam, The Netherlands.

Pfaller MA, Diekema DJ. Unusual fungal and pseudofungal infections of humans *J Clin Microbiol* 2005, 43 :1495–1504

Pfaller MA. NCCLS Reference method for broth dilution antifungal susceptibility testing of conidium forming filamentous fungi; proposed standard 1997;18:16-9.

Pffugfelder SC, Flynn Jr., Zwickey TA,Forster A, Tsiligiani, Culbertson Mandelbaum**.**Exogenous fungal endophthalmitis.*Ophthalmology*, 2000**,** 95; 19-30

Pfeifer JD,Grand MG, Thomas MA, Berger AR, Lucarelli MJ, Smith ME. 1991. Endogenous Pseudallescheria boydii endophthalmitis.Clinicopathologic findings in two cases. Arch. Ophthalmol. 109:1714–1717.

Pitt JI, Samson RA, Frisvad JC.. List of accepted species and their synonyms in the family Trichocomaceae, p. 9–49. In R. A. Samson and J. I. Pitt (ed.), Integration of modern taxonomic methods for Penicillium and Aspergillus classification. 2000,Harwood Academic Publishers, Amsterdam, TheNetherlands.

Pogson, GH, Mesa KA, Boutilier RG. Genetic population structure and gene flow in the Atlantic Cod Gadus morhua: a comparison of allozyme and nuclear RFLP loci. *Genetics* 1995, 139:375–385.

Polanco AM, Rodriguez JL , Tudela, Suarez JVM.Detection of pathogenic fungi in human blood by the PCR. *Eur J Clin Microbiol Infect Dis* 1995, 14:618–621.

Poulat HS, Metin DY, Inci R, Dereli T, Kilinc I, Tumbay E**.** Non-dermatophytic molds as agents of onychomycosis in Izmir, Turkey - a prospective study. *Mycopathologia*. 2005,160:125-8.

Pounder JI, Simmon KE, Barton CA, Hohmann SL, Brandt ME, Petti CA. Discovering Potential Pathogens Among Fungi Identified as “Nonsporulating Molds *J Clin Microbiol* 2007, 45: 568-571.

Pounder J, Williams S, Hanson D, Healy M, Reece K, Woods GL**.** Rep-PCR based DNA finger printing using Diversilab system for identification of commonly encountered dermatophytes. *J Clin Microbiol* 2005,42: 2141-47.

Prariyachatigul C, Chaiprasert A, Meevootisom V, Pattanakitsakul S. Assessment of a PCR technique for the detection and identi-fication of *Cryptococcus neoformans*. *J Med Vet Mycol* 1996, 34:251–258

Priya K, Madhavan HN, Malathi J. Use of uniplex polymerase chain reaction and evaluation of multiplex PCR in the rapid diagnosis of viral retinitis. *Indian J Med Res* 2003, 117:205-10.

Radford SA, .Johnson EM, Leeming JP, Cornish MR, Foot ABM, Warnock DW.. Molecular epidemiological study of *Aspergillus fumigatus* in a bone marrow transplantation unit by PCR amplification of ribosomal intergenic spacer sequences. *J Clin Microbiol* 1998, 36:1294-99.

Rajeev B, Biswas J . Molecular biologic techniques in ophthalmic pathology. *Indian J Ophthalmol* 1998, 46: 3–13.

Rakeman JL , Bui U, La Fe K, Chen YC , Honeycutt RJ, Cookson BT. Multilocus DNA sequence comparisons rapidly identify pathogenic molds. *J Clin Microbiol* 2005, 43:3324-3333.

Ralph ED, Hussain Z. Chronic meningitis caused by *C. albicans* in a liver transplant recipient: usefulness of the PCR for diagnosis and for monitoring treatment. *Clin Infect Dis* 1996, 23:191–192

Ramani R, Ramani A, Susan J. Wong Rapid Flow Cytometric Susceptibility Testing of *C. albicans.J Clin Microbiol*, 1997, 35 : 2320–2324

Ramani R, Chaturvedi V. Proficiency testing program for clinical laboratories performing antifungal susceptibility testing of pathogenic yeast species *J Clin Microbiol* 2003;41:1143-6.

Reddy LV, Kumar A, Kurup VP. Specific amplification of *Aspergillus fumigatus* DNA by PCR. *Mol Cell Probes*, 1993, 7:121–126.

Reischl U, Lohmann CP. [Polymerase chain reaction (PCR) and its possible applications in diagnosis of infection in ophthalmology.] Klin Monatsbl Augenheilkd 1997;211:227–34.

Reiss E, Tanaka K, Bruker G, Chazalet V, Coleman D, Debeaupuis JP, Richardson MD, Kokki MH. New perspectives in the diagnosis of systemic fungal infection. *Ann Med* 1999, 31:327–335

Rex JH, Pfaller MA, Walsh TJ, Chaturvedi V, Espinel-Ingroff A, Ghannoum MA Antifungal susceptibility testing: Practical aspects and current challenges. *Clin Microbiol Rev* 2001, 14:643-58.

Rieseberg LH. Homology among RAPD fragments in interspecific comparisons. *Mol Ecol* 1996, 5:99–105

Rodriguez E, De Meeus T, Mallie M, Renaud F, Symoens F, Mondon P, Piens MA, Lebeau B, Viviani MA, Grillot RA, Nolard N, Chapuis F,Tortorano AM, Bastide JM. Multicentric epidemiologicalstudy of Aspergillus fumigatus isolated by multilocus enzyme electrophoresis. *J Clin Microbiol* 1996, 34:2559–2568.

Romero CF, Rai MK, Lowder CY, Adal KA. Endogenous endophthalmitis: case report and brief review. *Am Fam Phys* 1999, 60:510–514.

Rosa RH, Miller D, Alfonso EC. The changing spectrum of fungal keratitis in South florida.Ophthalmology 1994, 101: 1005-1013

Rossman AY, Samuels GJ.Towards a single scientific name for species of fungi. *Inoculum* 2005, 56: 3–6.

Samson CM, Foster CS. Chronic postoperative endophthalmitis.*Int Ophthalmol Clin* 2000, 40:57–67.

Sanche, S., A. Fothergill, and M. Rinaldi. 1997. Interspecies variation of the susceptibility of Fusarium species to Schering 56592 in vitro, abstr. E66, p.125. In Program and abstracts of the 37th Interscience Conference on AntimicrobialAgents and Chemotherapy. American Society for Microbiology,Washington, D.C.

Sandhu GS, Kline BC, Stockman L, Roberts GD. Molecular probes for diagnosis of fungal infections. *J Clin Microbiol* 1995,33:2913–2919

Sayed FE, Ammoury A, Feghaly R ,Haybe, Dhaybi R**.** Onychomycosis in Lebanon: a mycological survey of 772 patients *Mycoses* 2006, 49: 216 -223

Scherer WP, Scherer BA. Comparison of Results from Two Mycology Laboratories for the Diagnosis of Onychomycosis A Study of 85 Cases in a Geriatric Population Journal of the American Podiatric Medical Association. Volume 94 Number 6 528-534 2004.

Schmitz SH, Dick B, Krummenaur F, Pfeiffer N. Endophthalmitis in cataract surgery: results of a German survey. *Ophthalmology* 1999, 106:1869–1877

Schwarz P,Bretagne S, Gantier C, Garcia-Hermoso D, Lortholary O, Dromer F, Dannaoui E. Molecular identification of zygomycetes from culture and 10 experimentally infected tissues. *J Clin Microbiol* 2006, 44:340-349.

Scott IU, Flynn HW, Feuer W, Pflugfelder SC, Alfonso EC, Forster RK, Miller D.Endophthalmitis associated with microbial keratitis. *Ophthalmology* 1996, 103:1864–1870.

Segal BH, Walsh TJ, Liu JM Invasive infection with *Fusarium chlamydosporum* in a patient with aplastic anaemia. *J Clin Microbiol* 1998, 36 : 1772 - 1776

Selvapandiyan A, Stabler K, Ansari NA, Kerhy S, Riemenschneider J, Salotra P, A novel semiquantitative fluorescence based multiplex polymerase chain reaction for rapid simultaneous detection of bacterial and parasitic pathogens from blood. *J Mol Diagn* 2005, 7:268-75

Sen P, Gopal L, Sen PR. Intravitreal voriconazole for drug-resistant fungal endophthalmitis: case series *Retina* 2006, 26:935-9

Seo K, Akiyoshi H, Ohnishi Y.. Alteration of cell wall composition leads to amphotericin B resistance in *Aspergillus flavus*. *Microbiol Immunol* 1999, 43:1017–1025.

Shamsuddin D, Tuazon CU, Levy C, Curtin J. *Bacillus cereus* panophthalmitis: source of the organism. *Rev. Infect. Dis* 1996, 4:97–103.

Sharmin S, Haritani K, Tanaka R, Abliz P, Takizawa K, Sano A,Fukushima K, Nishimura K, Miyaji M. The first isolation of *Hortaea werneckii* from a household guinea pig. Jpn J Med Mycol 2002, 43:175–180

Shenep JL, English BK, Kaufman L,Successful medical therapy for deeply Invasive facial infection due to *Pythium insidiosum* in a child. *Clin Infect Dis* 1998, 27:1388–93.

Shin JH, Nolte FS, Morrison CJ. Rapid identification of Candida species in blood cultures by a clinically useful PCR method. *J of Clin Microbiol* 1999,35: 1454-1459.

Sigler L, Verweij PE. *Aspergillus, Fusarium*, and other opportunistic moniliaceous fungi,. In P. R. Murray, E. J. Baron,M. A. Pfaller, J. H. Jorgensen, and R. H. Yolken (ed.), Manual of clinical microbiology, 8th ed. American Society for Microbiology, Washington, D.C. 2003, p 1726–1760

Snyder WC, Hansen HN. Variation and speciationin the genus *Fusarium*. *Ann N Y Acad Sci* 1954, 60:16-23.

Soltau JB, Rothman RF, Budenz DL, Greenfield DS, Feuer W, Liebmann JM, Ritch R. Risk factors for glaucoma filtering bleb infections. *Arch Ophthalmol* 2000, 118:338–342.

Spatafora J, Blackwell M. Molecular systematics of unitunicate perithecial Ascomycetes. The Clavicipitales-Hypocreales connection. *Mycologia* 1993, 85:912–922

Speaker MG, Milch FA, Shah MK, Eisner W, Kreiswirth BN. Role of external bacterial flora in the pathogenesis of acute postoperative endophthalmitis. *Ophthalmology* 1991, 98:639–650.

Spiess B, Buchheidt D, Baust C, Skladny H, W. Seifarth, U. Zeilfelder, C.Leib-Mosch, H. Morz, and R. Hehlmann. Development of a LightCyclerPCR assay for detection and quantification of Aspergillus fumigatus DNAin clinical samples from neutropenic patients. *J Clin Microbiol* 2003, 41:1811–1818.

Spreadbury C, Holden D, Brown AA, Bainbridge B, Cohen J. Detection of *Aspergillus fumigatus* by PCR. *J Clin Microbiol* 1993, 31:615–621.

Srinivasan R, Kanungo R, Goyal JL**.** Spectrum of oculomycosis in South India. *Acta Ophthalmol* 1991,69:744–749

Srinivasan M, Gonzales CA, George C, Cevallos V, Marcarenhus JM, Asokau B Epidemiology and aetiological diagnosis of corneal ulceration in Madurai, South India. *Br J Ophthalmol* 1997; 81:965-71

Steele PE, Carle GF, Kobayashi GS, Medoff G. Electrophoretic analysis of Histoplasma capsulatum chromosomal DNA*. Mol Cell Biol* 1989, 9:983– 987.

Stern GA, Engel HM, Dreibe WT. The treatment of post-operative endophthalmitis: results of differing approaches to treatment. *Ophthalmology* 1989, 96:62–67.

Streilein JW. Immunoregulatory mechanisms of the eye. *Prog Retinal Eye Res* 1999, 18:357–370.

Stevens SX, Fouraker BD, Jensen HG. Intraocular safety of ciprofloxacin. *Arch Ophthalmol* 1991, 109:1737–1743

Stevens DA. Diagnosis of fungal infections: current status. *J Antimicrob*. *Chemother*. 2002,. 49(Suppl. S1):11–19.

Sturtevant J. Applications of Differential display Reverse transcriptase PCR to molecular pathogenesis and Medical Mycology. *Clin Microbiol Rev* 2002 ,13 : 408-427

Summerbell RC.. Epidemiology and ecology of onychomycosis. *Dermatology* 1997, 194 (Suppl. 1):32–36

Summerbell RC. *Trichophyton, Microsporum, Epidermophyton* species- Agents of superficial mycoses . In P. R. Murray, E. J. Baron,M. A. Pfaller, J. H. Jorgensen, and R. H. Yolken (ed.), Manual of clinical microbiology, 8th ed. American Society for Microbiology, Washington, D.C. 2003, 1798–1819

Sutton DA, Sanche SE, Revankar SG, Fothergill AW, Rinaldi MG**.** *In vitro* amphotericin B resistance in clinical isolates of *Aspergillus terreus* with head to head comparison of voriconazole. *J Clin Microbiol* 1999, 37: 2343 –2345

Tang CM, Holden DW, Brown AA, Cohen J.. The detection of *Aspergillus* spp. by the PCR and its evaluation in bronchoalveolar lavage fluid. *Am Rev Respir Dis*. 1993, 148:1313–1317.

Tanure MA, Cohen EJ, Grewal S, Rapuano CJ, Laibson PR.Spectrum of fungal keratitis at Wills Eye Hospital, Philadelphia, Pennsylvania. *Cornea* 2000, 19:307–312.

Tarai B, Gupta A, Ray P, Shivaprakash MR, Chakrabarti A. Polymerase chain reaction for early diagnosis of post-operative fungal endophthalmitis. *Indian J Med Res* 2006,123:671-8

Taravella, MJ, Johnson DW, Petty JG, Keyser RB, Foster CS, Lundberg BE. 1997. Infectious posterior scleritis caused by *Pseudallescheria boydii*. Clinicopathologic findings. Ophthalmology 104:1312–1316.

Taylor JW, Geiser DM, Burt A, Koufopanou P. The Evolutionary Biology and Population Genetics Underlying Fungal Strain Typing. *Clin Microbiol Rev*, 1999, 12 : 126-146

Teresa M, Gwerra D, Mellado E, Estrella MC, Gaztelurrutia L, Navarro JV, Tudela J Genetic similarity among one Aspergillus flavus strain isolated from a patient who underwent heart surgery and two environmental strains obtained from the operating room. *J Clin Microbiol* 2000, 38, 2419 – 2422.

Therese KL, Anand AR, Madhavan HN. Polymerase chain reaction in the diagnosis of endophthalmitis. *Br J Ophthalmol* 1998, 82:1078-82.

Thianprasit M, Chaiprasert A, Imwidthaya P. Human pythiosis. *Curr Top Med Mycol* 1996, 7: 43–54.

Thomas PA, Kalavathy CM, Abraham DJ, Rajasekaran J. Oral ketoconazole in keratomycosis. *Ind J Ophthalmol* 1987; 35:197-207.

Thomas PA, Abraham DJ, Kalavathy CM, Rajasekaran J. Oral itraconazole therapy for mycotic keratitis. Mycoses 1988;31:271-9.

Thomas PA. Fungi in keratitis: detection, susceptibility patterns and pathogenic mechanisms. Ph.D. thesis. Bharathidasan University, Tiruchirappalli,India,1990

.Thomas PA. Mycotic keratitis—an underestimated mycosis. *J Med Vet Mycol* 1994; 32:235–56.

Thomas PA, Kalavathy, Devanandan P. *Lasiodiplodia theobromae* keratitis—a clinical profile. *J Tamilnadu Ophthalmol Assoc* 1998, 39:31–32

Thomas, PA. 2003a. Fungal infection of the cornea. Eye 17:852–862

Thomas PA. Current perspectives on ophthalmic mycoses*. Clin Micro Rev* 2003 b, 16:730-797

Thompson ST, Parver LM, Enger CL, Meiler WF, Liggett PE. Infectious endophthalmitis after penetrating injuries with retained intraocular foreign bodies. *Ophthalmology* 1993, 100:1468–1474.

Ti SE, Scott JA, Janardhanan P, Tan DT. Therapeutic keratoplasty for advanced suppurative keratitis . *Am J Ophthalmol,* 2007, Feb 27; [Epub ahead of print]

Triscott JA, Weedon D, Cabana E. Human subcutaneous pythiosis. *J Cutan Pathol* 1993, 20: 267–71.

Tsang CY, Liu PU, Shi ZY, Lau YJ, Hu BS, Shyr JM, Tsai WS, Lin YH.

Endogenous endophthalmitis due to Escherichia coli:case report and review. *Clin Infect Dis* 1996, 22:1107–1108.

Tullari G, Mangone C, Freyle J, Shirazian D, Lehmen H, Wise GL. PCR used to detect candidemia in patients with candiduria. *Urology* 1998, 51:501–505.

Turenne CY, Sanche SE, Hoban DJ, Karlowsky JA, Kabani AM.. Rapid identification of fungi by using the ITS2 genetic region and an automated fluorescent capillary electrophoresis system. *J Clin Microbiol* 1999,37:1846–1851.

Turner DM, Poles A, Brown J, Arguello JR, Madrigal JA, Navarrette CV. HLA-A typing by reference strand-mediated conformation analysis (RSCA) using a capillary-based semiautomated genetic analyser. Tissue Antigens 1999, 54:400–404.

Upadhyay MP, Karmacharya PCD, Koirala S, Tuladhar NR,Bryan LE, Smolin G, Whitcher JP. Epidemiological characteristics, predisposing factors and aetiological diagnosis of corneal ulceration in Nepal. *Am J Ophthalmol*. 1991, 111:92–99.

Vajpayee RB, Angra SK, Sandramouli S, Honavar SG, Chhabra VK. Laboratory diagnosis of keratomycosis: comparative evaluation of direct microscopy and culture results. *Ann Ophthalmol* 1993, 25:68–71.

Van Burik J, Schreckhise RH, White TC, Bowden RA. Comparison of six extraction techniques for isolation of DNA from filamentous fungi. *Med Mycol*. 1998,36:299–303

Van Burik JA, Myerson D, Schreckhise RW, Bowden RA. Panfungal PCR assay for detection of fungal infection in human blood specimens. *J Clin Microbiol*, 1998, 36: 1169-75

Vazquez J. Use of the PCR for the diagnosis of invasive Candida infection. *Serodiagn Immunother Infect Dis* 1994, 6:173–178

Veer P, Patwardhan NS, Damle AS. Study of onychomycosis: Prevailing fungi and pattern of infection. Indian J Med Microbiol 2007,25:53-6.

Verbraeken H, Rysselaere M. Bacteriological study of 92 cases of proven infectious endophthalmitis treated with pars plana vitrectomy. *Ophthalmologica*. 1991, 203:17–23

Venugopal PL, Venugopal TL, Gomathi A, Ramakrishna ES, Ilavarasi S. Mycotic keratitis in Madras .*Ind J Pathol Microbiol*. 1989, 32:190-197

Vincent JL, Anaisiie E, Bruining H, Demajo W, El-Ebiary M, Haber J, Hiramatsu Y, Nitenberg G, Nystrom PO, Pittet D, Rogers T, Sandven P, Sganga G, Shaller, MD, Solomkin J**.** Epidemiology, diagnosis and treatment of systemic *Candida* infection in surgical patients under intensive care. *Intensive Care Med*. 1998, 24:206–216.

Virgile R, Perry HD, Pardanani B. Human infectious corneal ulcer caused by *Pythium insidiosum. Cornea* 1993; 12:81–3

Vismer HF, Marasai WF, Rheeder JP . *F. dimerum* as a cause of human eye Infections. *Med Mycol* 2002, 40: 399 – 406

Waheed S, Ritterband DC, Greenfield DS, Liebmann JM, Seedor JA, Ritch R. New patterns of infecting organisms in late bleb-related endophthalmitis: a ten year review. *Eye* 1998, 12:910–915.

Wahl JC, Katz HR, Abrams DA. Infectious keratitis in Baltimore. *Ann Ophthalmol* 1991;23:234–7.

Waite BH ,Stover RH. Studies on *Fusarium* wilt of bananas. VI. Variability and the cultivar concept in *Fusarium oxysporum f. cubense*. *Can J Bot* 1960, 38:985-994.

Walsh T. Kasai J, Francesconi A, Landsman D, Chanock SJ. New evidence that *C. albicans* possesses additional ATP-bindingcassette MDR-like genes: implications for antifungal azole resistance. *J Med Vet Mycol* 1997, 35:133–139.

Wanger A, Mills K, Nelson PW, Rex JH. Comparison of E test and National Committee for Clinical Laboratory standards brothmacro dilution method for antifungal susceptibility testing:Enhanced ability to detect amphotericin B resistant *Candida* isolates. *Antimicrob agents Chemother* 1995;39:2520-2.

Warnock DW, Johnson EM, Rogers TRF. 1998. Multi-centre evaluation of the Etest method for antifungal drug susceptibility testing of *Candida* spp. and *Cryptococcus neoformans. J Antimicrob Chemother* 42:321–331

Weinberg JM, Koestenblatt EK, Tutrone WD, Tishler HR, Najarian L. Comparison of diagnostic methods in the evaluation of onychomycosis. *J Am Acad Dermatol.* 2003 49:193-7.

Weissgold D, Orlin SE, Sulewski ME et al. Delayed onset fungal keratitis after endophthalmitis. *Ophthalmol* 1998; 105: 258 - 262

Weitzman I, Summerbell RC. The Dermatophytes. *Clin Microbiol Rev* 1995, 8: 240-259

Whitcher JP, Srinivasan M. Corneal ulceration in the developing world–a silent epidemic. *Br J Ophthalmol* 1997;81:622–3.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p. 315–322. InM. A. Innis, D. H. Gefland, J. J. Sninsky, and T. J. White (ed.), PCR protocols: a guide to

methods and applications. Academic Press, Inc., NewYork, N.Y.

Wickes BL, Moore TDE, Kwon-Chung KJ. Comparison of the electrophoretic karyotypes and chromosomal location of ten genes in the two varieties of *Cryptococcus neoformans*. *Microbiology* 1994, 140:543–550.

Wiedbrauk DL, Werner JC, Drevon AM. Inhibition of PCR by aqueous and vitreous fluids. *J Clin Microbiol* 1995, 33:2643–2646

Wiechens B, Neumann D, Grammer JB, Pleyer U, Hedderich J, Duncker GI. Retinal toxicity of liposome-incorporated and free ofloxacin after intravitreal injection in rabbit eyes. *Int Ophthalmol* 1998–1999 22:133–143

Wilhelmus KR, Jones DB.. Curvularia keratitis. *Trans Am Ophthalmol Soc* 2001 99:111–132

Williams, G., F. Billson, R. Husain, S. A. Howlader, N. Islam, andK. McClellan.. Microbiological diagnosis of suppurativekeratitis Bangladesh. *Br. J. Ophthalmol.* 1987, 71:315-321.

Williams J.Kubelik KR, Livak KJ, Rafalski JA, Tingey SV. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res*. 1990, 18:6531–6535

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res* 1990, 18:6531–6535.

Willinger B, Obradovic A, Selitsch B, Beck-Mannagetta J, Buzina W, Braun H, Apfalter P, Hirschl AM, Makristathis A, Rotter M. Detection and identification of fungi from fungus balls of the maxillary sinusby molecular techniques. *J Clin Microbiol* 2003, 41:581–585.

Wilson LA, Ajello L. Agents of oculomycosis: fungal infections of the eye, p. 525–567. In L. Collier, A. Balows, and A. Sussman (ed.), Topley and Wilson's microbiology and microbial infections, 1998, 9th ed., vol. 4. Arnold, London, England.

Wollenweber HW. Studies on the Fusarium problem. *Phytopathology* 1989, 3:24-29.

Wong TY, Ng TP, Fong KS, Tan DTH. Risk factors and clinical outcome between fungal and bacterial keratitis. A comparative study. *CLAO J*. 1997, 23:275–281.

Wong B.,Perfect JR, Beggs S, Wright KA. Production of the hexitol D-mannitol by Cryptococcus neoformans in vitro and in rabbits with experimental meningitis. *Infect Immun* 1990, 58:1664–1670.

Wu Z, Tsumura Y, Blomquist G, Wang X. 18S rRNA gene variation among common airborne fungi, and development of specific oligonucleotide probes for

the detection of fungal isolate. *Appl Environ Microbiol* 1996, 69:5389–5397.
Xie L., Dong X, Shi W.. Treatment of fungal keratitis by penetrating keratoplasty. *Br J Ophthalmol* 2001,85:1070–1074

Yamakami Y, Hashimoto A, Tokimatsu I, Nasu M. PCR detection of DNA specific for *Aspergillus* species in serum of patients with invasive aspergillosis. *J Clin Microbiol* 1996, 34:2464–2468.

Yamakami Y, Hoshimoto A, Yamagata E, Kamberi P, Karashina R, Nagai H, Nasu M, Evaluation of PCR for detection of DNA specific for Aspergillus species in sera of patients with various forms of pulmonary aspergillosis . *J Clin Microbiol* 1998, 36: 3619-3623

Yeo SF, Wong B . Current Status of Nonculture Methods for Diagnosis of Invasive Fungal Infections *Clin Microbiol Reviews*, 2002; 465 – 484

Yildiran ST, Mutlu FM, Saracli MA, Uysal Y, Gonlum A, Sobaci G, Sutton DA. Fungal endophthalmitis caused by *Aspergillus ust*us in a patient following cataract surgery**.** *Med Mycol.* 2006,44:665-9.

Yoshida T, Jono K, Okonogi K. Modified agar dilution susceptibility testing method for determining in vitro activities of antifungal agents, including azole compounds. *Antimicrob Agents Chemother* 1997, 41:1349-51

Zarzoso B, Belloch C, Uruburu F, Querol A. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. *Int J Syst*. *Bacteriol* 1999, 49:329–337.

Zhao J, Kong F, Li R, Wang X, Wan Z,Wang D. Identification of *Aspergillus fumigatus* and related species by nested PCR targeting ribosomal DNA internal transcribed spacer regions. *J Clin Microbiol* 2001, 39:2261–2266.

Zhong Z, Li D, Wang R.. Typing of common dermatophytes by random application of polymorphic DNA. *J Med Mycol* 1997, 38:239-246

Zipfel RD, Beer ZW de, Jacobs K, Wingfield BD, Wingfield MJ Multi-gene phylogenies define Ceratocystiopsis and Grosmannia distinct from Ophiostoma. *Studies in Mycology* 2006, 55: 75–97.

Zolan ME. Chromosome length polymorphisms in fungi. *Microbiol Rev* 1995, 59: 686–698

14 APPENDICES

APPENDIX I

1 Collection of corneal scrapings :

- A drop of local anaesthetic without preservative is instilled , and with the help of the slit lamp or operating microscope , the edge of the ulcer is firmly scraped using Bard Parker blade No. 15' after removal of debris or discharge in the vicinity. (Figure a)
- Several scrapings are collected and used in a sequence to inoculate culture media in the form of 'C' curves and smears were made

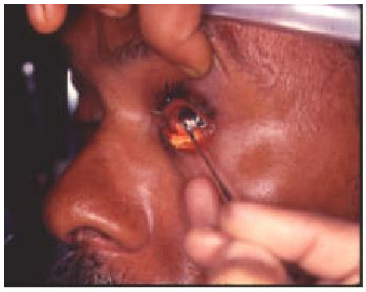


Figure a:
Collection of corneal scraping using Kimura's spatula

2 Collection of corneal button :

The corneal button removed by surgery is sent to Microbiology Laboratory in a sterile container immediately.

3 Collection and transport of intraocular specimens :

Uncontaminated VF is aspirated by a syringe connected to the suction port of vitreous cutter at the beginning of vitrectomy. A sterile disposable needle is fixed to the syringe, the air in it expelled carefully without causing aerosls and the needle is capped with a sterile rubber bung and sent to the laboratory immediately(Figure b).

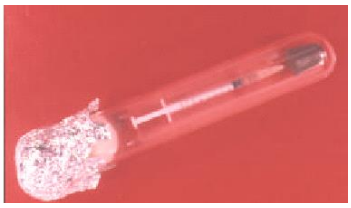


Figure b:
Transport of intraocular specimens to the laboratory

AH samples (150-200ml) are collected aseptically in a tuberculin syringe with a 30G needle. Vitreous aspirate transport tube with a rubbercork inside. After the air in the syringe is expelled to prever inactivation of anaerobic bacteria, the needle is fixed onto a sterile rubber bung and placed in a sterile large test tube container, which is immediately transported to the laboratory. Specimens should be ideally processed within 15 – 30 minutes after collection. Transport media of the conventional kind have no place in the transport of these intraocular specimens.

Figure c: Corneal scraping in the form of 'C' curves

4 Processing of ocular specimens

Corneal scrapings were collected by the ophthalmologist after application of local anesthetic. The culture plates were inoculated in the form of 'C' curves (Figure c) by the ophthalmologist and the smears were processed for detection of fungal etiology in the microbiology laboratory.

Figure d: Processing of corneal button

The corneal button, corneal biopsy and other ocular tissues are cut into small bits in a small sterile petridish (Figure d) following all aseptic precautions,and inoculated onto various culture media (Figure e) and crushed smearsare also made to be used for various staining methods

Figure e: Inoculation of corneal button on BHIB

About 50 -150µl of aqueous humor and 100 - 200 µl of vitreous humor was received in the laboratory for microbiological investigations. On receipt, the specimen was divided into two equal portions. One half was used for DNA extraction and the other half was subjected to conventional methods of direct smear and culture. The specimens (AH and VF, biopsy, corneal scrapings) were processed within 30 minutes after collection for direct microscopy and culture of bacteria and fungi. Direct microbiological investigations were carried out on cytospinned smears using the accessories shown in Figure f, g (Cytospin 2 Shandon, Southern Products Limited, Cheshire, England) of intraocular fluids and stained by KOH-Calcofluor white for detection of fungus and Gram's stain for detection of bacteria .

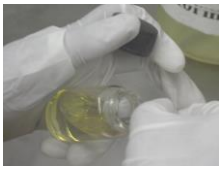


Figure f: Cytospin machine

Figure g : Cytospin accessories

Inoculation of media for culture of bacteria and fungi is to be carried out first, because the number of organisms are likely to be low. The AH and VF being normally sterile fluids, do not require selective media for culture. For culture, a variety of media are included for the favorable growth of aerobic and anaerobic bacteria and fungi. However, the number of media included depends on the volume of the sample available. For inoculation onto solid media, 1-2 drops of the

specimen is expressed through the needle onto the agar plate. The material is not spread with a loop to reduce the possibility of contamination. Liquid media

are also inoculated with 2 - 3 drops. The culture media used for isolation of bacteria and fungi were blood agar and MacConkey agar (incubated aerobically at 37 $^{\circ}$C),Chocolate agar (10% Carbon -di-oxide), Brucella blood agar (BBA, 37° C at anerobic work station), Sabouraud's dextrose agar (SDA) incubated at 25° C in a cooling incubator and liquid media brain heart infusion broth (BHIB), Thioglycollate broth incubated at 37° C. Culture plates and specially the liquid medium BHIB were incubated for a period of one month to isolate fungus. The fungal isolates were identified using standard mycological methods (Forbes BA, 1989).

5 Preparation of Lactophenol Cotton blue (LPCB)

Phenol crystals	20 g
Lactic acid	20 ml
Glycerol	40 ml
Distilled water	20 ml
Cotton blue	0.75 g

Used for identification of filamentous fungi. Stored in brown bottle and can be kept at room temperature.

6 a Preparation of Calcofluor white and potassium hydroxide

Stock solution A

Calcofluor white (fluorescent Brightner)	1.9 g
Distilled water	100 ml

Stock solution B

Evans Blue (Sigma)	0.05 g
Distilled water	100 ml

Working solution

Solution A	1 ml
Solution B	9 ml

Preparation of Potassium hydroxide

10 grams of potassium hydroxide dissolved in 100 ml of distilled water and stored at room temperature. Stored in brown bottle and can be kept at room temperature.

Application: Used for detection of fungi in clinical specimens. Yeasts and filamentous fungi exhibit bluish white fluorescence when examined under fluorescene microscope.

b Preparation of Gram's stain

Crystal violet: 1 g of crystal violet in 100 ml of distilled water

Gram's iodine: 15 g of Iodine dissolved in 100 ml of potassium iodide

Gram's decolouriser : Equal volumes of acetone and ethanol

Dilute carbol fuchsin: 1 g of basic fuchsin in 100 ml of distilled water.

Procedure: The smear is covered with crystal violet and left for 1 minute.

After washing with distilled water, the slide was covered with Gram's iodine and left for 1 minute.After washing with distilled water,the slide was decolourized quickly with decolourizer until no further violet colour comes out.Then the slide was counter stained with dilute carbol fuchsin for 1minute, washed with distilled water and observed the stained smear under the oil immersion objective.

Interpretation of results:

Gram positive bacteria appear violet or purplish blue and Gram-negative bacteria appear red or pink.Fungi appear Gram negative or unstained.

Preparation of culture media

7 Sabouraud's dextrose agar

Glucose	20 g
Peptone	10 g
Agar	15 g
Distilled water	1000 ml

Steam to dissolve and adjust pH to 5.4 . Autoclave at 121°C for 15 minutes at 15 lbs pressure and dispense in petridish following aseptic precautions.

8 Potato dextrose agar

Potato	200 g
Dextrose	20 g
Agar	20 g
Distilled water	1000 ml

Dissolve the ingredients and adjust pH to 5.4 . Autoclave at 121°C for 15 minutes at 15 lbs pressure and dispense in petridish following aseptic precautions.

9 Corn meal agar

Corn meal	400 g
Agar	15 g
Distilled water	1000 ml

Dissolve the ingredients and adjust pH to 5.4 . Autoclave at 121°C for 15 minutes at 15 lbs pressure and dispense in petridish following aseptic precautions

NOTE:

All the specimens for detection and isolation of fungi needs to be processed in Biological Safety Cabinet class II following all universal precautions. Care must be taken to wear gloves, safety goggles ,cap and face mask while processing the specimens.

APPENDIX –II

PCR, Agarose gel electrophoresis, Cycle sequencing and purification of amplified products

1 Quantification of DNA

1µl of the extracted DNA was dissolved in 999µl of water and quantified spectrophotometrically at 260nm and 280nm. The reading at 260nm gives the nucleic acid concentration. The ratio of readings at OD 260nm/ 280nm gives the purity of the nucleic acid.

2 sn PCR targeting ITS region for detection of panfungal genome

For a 50µl reaction, 8 µl of dNTPs (200 µmoles), 5 µl of 1 X PCR buffer (1.5 mM $MgCl_2$,50 mM KCl, 10 mM Tris Cl,0.001% gelatin), 6 µl of 25 mM $MgCl_2$ (1 in 10 diluted to get 1.5mM) , 10 picomoles of forward primer, ITS_1 - 5' tcc gta ggt gaa cct gcg g 3'and reverse primer ITS_4 - 5' tcc tcc gct tat tat gc 3'targeting ITS region and 10 µl of template DNA was used.(Ferrer et al, 2001). Amplification was carried out in Perkin Elmer thermal cycler (Model 2700). The first round of amplification yielded 520 – 611 bp product according to different fungal species for ITS region. Thermal cycling consisted of initial denaturation at 95˚C for 5 minutes followed by 35 cycles of denaturation at 95˚C for 30 seconds, annealing at 55˚C for 60 seconds and extension at 72˚C for 60 seconds followed by final extension at 72˚C for 6 minutes. Semi nested amplification was carried out using same PCR conditions as that of the first round with forward primer ITS_4 and reverse primer ITS_{86} - 5' gtg aat cat cga atc ttt gaa c 3'. Five µl of amplified product was transferred from the first round to the second round and amplification was carried out in initial denaturation at 95˚C for 5 minutes followed by 35 cycles of denaturation at 95˚C for 30 seconds, annealing at 55˚C for 30 seconds and extension at 72˚C for 30 seconds followed by final extension at 72˚C for 5 minutes. The amplified products were detected using 2% agarose incorporated with 0.5µg/ ml of ethidium bromide. The electrophoresis was carried out at 100 volts and documented using Gel documentation system (Vilber Lourmat)

3 PCR targeting 28SrRNA region for detection of panfungal genome

For a 50µl reaction , 8 µl of 200 µm dNTPs,5 µl of 10 X PCR buffer (15 mM

$MgCl_2$, 500 mM KCl, 100 mM Tris Cl,0.01% gelatin), 10 picomoles of forward primer, FU_1 and reverse primer FU_2 targeting 28SrRNA region was used and amplification was carried out according to the method of Anand et al, 2000.10 µl of template DNA was used.Amplification was carried out in Perkin Elmer thermal cycler (Model 2700). The amplification yielded 260 bp product . Thermal cycling consisted of initial denaturation at 94˚C for 5 minutes followed by 30 cycles of denaturation at 94˚C for 30 seconds, annealing at 60˚C for 60 seconds and extension at 72˚C for 120 seconds followed by final extension at 72˚C for 7 minutes.

4 PCR targeting 18SrRNA region for detection of panfungal genome

For a 50µl reaction , 8 µl of 200 µm dNTPs,5 µl of 10 X PCR buffer (5 mM $MgCl_2$,500 mM KCl, 100 mM Tris Cl,0.01% gelatin), 6 µl of 25 mM $MgCl_2$ (1 IN 10 diluted),10 picomoles of forward primer, Pffor and reverse primer Pfrev 2 given in Table 3a of Chapter 3 and 1.25U of *Taq polymerase* was used. These generated PCR products of 728, 743, 744 bp in *C. albicans, A. fumigatus, F. solani* respectively. The nested PCR was carried out using species specific *C. albicans, A. fumigatus, F. solani primers* (Table 3a of Chapter 3). The nested amplification was carried out as described by Jaegger et al, 2001

5 DNA extracts of microorganisms used in determining the analytical specificity of PCR

Specificity of primers were verified by using DNA extracts of *C. albicans* (ATCC 90028), *C. tropicalis* (ATCC 750), *C. parapsilosis* (ATCC 90018), *C. krusei* (ATCC 6258) *A.flavus* ATCC 204304, *A. fumigatus* ATCC 10894, *A. niger* ATCC 16404, *F. solani* ATCC 36031, laboratory isolates of *A. terreus*, *Curvularia* and *Alternaria* species, *S. aureus* (ATCC 12228),*Pseudomonas aeruginosa* (ATCC 27853), *M.tuberculosis* H37Rv, *M. fortuitum* ATCC 1529, *M.chelonae* ATCC 1524 and laboratory isolates of *Nocardia asteroides, Actinomyces species,* Herpes Simplex virus (HSV 1) ATCC 733 VR, *Acanthamoeba polyphaga* ATCC 30461 and human leukocyte DNA.

5 Gel Electrophoresis

Electrophoresis is a method by which the amplification of the gene sequence is confirmed after PCR. DNA, negatively charged moves to the cathode on subjected

to electric field and moves according to the molecular weight in a matrix like agarose or polyacrylamide gels. The gels are suitably stained to visualize the product (Fluorescent dyes for agarose gels and silver staining of poly acryl amide gels).

Agarose gel electrophoresis

Preparation of TBE buffer : 54.1 g of Tris, 27.8g of boric acid and 3.65 g EDTA were added in 500 ml water and pH adjusted to 8.0.The stock solution is diluted

1: 10 for further use.

Tracking dye- Bromophenol blue : 0.1gm BPB+100ml 1X TBE buffer in equal volumes of 40%sucrose solution.

Requirements

Molecular weight marker, Agarose,10 X TBE buffer,Ethidium bromide (2mg/ml), BPB

Preparation of agarose gel

The gel trough was cleaned with ethanol and the ends were sealed with cellophane tape with the combs placed in the respective positions to form wells. Two percentage of agarose gel was prepared by dissolving agarose in 1X TBE buffer and 8µl of ethidium bromide being finally added and mixed thoroughly and poured on to the trough followed by electrophoresis of the amplified prodcuts at 100 V for 30 to 45 minutes.The gel was then captured in the Vilber Lourmat and analysed using Imagemaster gel documentation system.

6 Sequencing in *ABI Prism 310/3100 AVANT* genetic analyzer

The sequence of the PCR amplified DNA is deduced with the help of the ABI Prism 310/3100 AVANT genetic analyzer that works based on the principle of Sanger dideoxy sequencing.

The fluroscent based detection by automated sequencer adopts the Sangers method and incorporates the fluorescent dyes into DNA extension products using 5'-dye

labeled primers or 3'-dye labeled ddNTPs (dye terminators called commercially as RRMIX). Each dye emits light at a different wavelength when extited by an argon ion laser. All four colours and therefore all four bases can be detected and distinguished in a single gel lane or capillary injection.

The amplified products with the dye at the terminated 3'end were subjected to capillary electrophoresis by an automated sample injection. The emitted flurorescence from the dye labels on crossing the laser area were collected in the rate of once per second by cooled, charge-coupled device (CCD) camera at particular wavelength bands (virtual filters) and stored as digital signals on the computer for processing that are analysed by software called as the sequence analysis softwares (Sequence Navigator in ABI 310 and seqscape manager in ABI 31000 AVANT machine). The ABI AVANT genetic analyser can be upgraded from 4 capillary to 16 capillary to that facilitates the electrophoresis of 16 samples at a given time.

Table 1: The four nucleotide bases with the respective acceptor dyes and colour emission.

Terminator	Acceptor dye	Colour of raw data on ABI PRISM310 electrophoretogram
A	dR6G	Green
C	dROX	Red
G	dR110	Blue
T	dTAMRA	Black

Table 2 (A) Protocol for cycle sequencing

Components	Volume (μl) **28SrRNA amplicons**	Volume (μl) **ITS amplicons**
Amplfiied products	1.0	1.0
Sequence buffer	3.0	2.5
Primer (2pmoles/μl)	2.0	2.0
RRMIX	1.0	1.5
Water	3.0	3.0

Table 2 (B) PCR conditions for cycle sequencing

PCR step	**Temperature (°C)**	**Time**
Initial denaturation	96	1 min
Denaturation	96	10sec
Annealing	50	5sec
Extension	60	4 min

The reaction was carried out for 25 cycles.

Purification of Extension Products The products were purified to remove the unincorporated dye terminators before sujecting the samples to capillary electrophoresis. ***Procedure*** 2 μl of125mM EDTA and 10 μl of 3M socium acetate (pH4.8) were mixed to the cycle sequenced prodcuts followed by the addition of 50μl of absolute ethanol and incubated at room temperture for 15 minutes follwed by centrifugation at 8000rpm for 20 minutes to precipitate the amplified product and remove the unutilized ddNTPs, primer (short length molecules) etc. The pellet was washed twice with 75% ethanol followed by air drying. The purified samples are suspended in fomamide and subjected for capillary electrophoresis in ABI PRISM

310/3100 genetic analyser. The sequences were then analyzed in Sequence Navigator software (version 1.0.1; ABI Prism 310) or Seq scape manager (version 2.1; ABI Prism 3100 AVANT)

APPENDIX III

GLOSSARY

- **Anamorph :** A somatic or reproductive structure that originates wiyhout nuclear recombination (asexual)

- **Ascocarp**, or **ascoma** (plural: **ascomata**): The fruiting body (sporocarp) of an ascomycete fungus. It consists of very tightly interwoven hyphae and may contain millions of **asci**, each of which contains typically eight **ascospores**. Ascocarps are most commonly bowl-shaped, but may take on a number of other designs.

- **Ascospore:** A haploid sexual spore that is formed by free-cell formation in an ascus following karyogamy and meiosis.

- **Ascus (pl. asci):** A saclike cell that gives rise to ascospore. Asci are characteristic of the Ascomycetes

- **Basidiospore:** A haploid sexual spore formed on a basidium following the process of karyogamy and meiosis.

- **Basidium (pl. basidia):** A specialized cell that gives rise to basidiospores. Basidia are characteristic of the Basidiomycetes.

- **Biseriate:** Having phialides arising from metulae on the vesicles of species of *Aspergillus.*

- **Bitunicate:** Having two walls

- **Catenulate:** In chains or end to end series

- **Chlamydoconidium (pl. chlamydoconidia):** A rounded, enlarged conidium that usually has a thickened cell wall and functions as a survival propagule.

- **Clavate:** Club shaped.

- **Cleistothecium (pl. cleistothecia):** An enclosed fruiting body that contains randomly dispersed asci.

- **Collarette:** The remnant of a cell wall present at the tip of the phialide. It is the result of the rupture of the tip of the conidiogenous cell during the release of the first phialoconidium

- **Conidiophore:** A specialized hypha upon which conidia develop.

- **Conidium (pl. conidia):** An asexual, non-motile, usually deciduous propagule that is not formed by cytoplasmic cleave, free-cell formation, or by conjugation.

- **Dematiaceous:** Having brown to black conidia or hyphae.

- **Dermatophyte:** A fungus in the genus *Epidermophyton, Microsporum, or Trichophyton* that infects hair, nail, or skin.

- **Fusiform:** Tapering at both ends; spindle shaped.

- **Hilum (pl. hila):** A scar at the base of a conidium

- **Hyaline:** Without color.

- **Hypha (pl. hyphae):** An individual filament of a fungus.

- **Mycelium :** Vegetative filament of a fungus

- **Macroconidium:** The larger of the conidia produced

- **Microconidium:** The smaller s of the conidia produced

- **Moniliform :** Having swellings that occur at regular intervals in a hypha

- **Monophialide:** A phialide which is cylindrical with asmall collarette, produced singly as a component of a complex branching system

- **Opportunistic fungus (or opportunistic pathogen):**

  This is a fungus that can produce invasive disease (disease in the tissue) only when the host defenses are weakened. That is, there must be breakdown of the immune system or the skin barrier before the fungus can produce disease. A classic example would be the fungus *Aspergillus*. This fungus is ubiquitous (found everywhere) in the environment, but cannot normally produce invasive disease in people unless the immune system is weakened (e.g., by taking high doses of steroids for prolonged periods of time).

  Special note regarding those opportunistic fungi often found in mould-contaminated buildings: The phrase "opportunistic" speaks to the ability to produce tissue invasion. Opportunistic fungi can also produce other less invasive diseases. For example, *Alternaria* can produce sinusitis or onychomycosis (nail infection) in otherwise apparently healthy individuals. However, there are often still subtle predisposing factors such as prior bacterial sinus infections. Also, the phrase "opportunistic" has nothing to do with the ability of a fungus to make toxins

- **Pectinate**: like the teeth or a comb

- **Polyphialide:** A phialide which is cylindrical with a small collarette, produced in many numbers as a component of a complex branching system

- **Sclerotium (pl. sclerotia):** An organized mass of hyphae that remains dormant during unfavorable conditions.

- **Spore:** A reproductive propagule that forms either following meiosis or asexually by a cleavage process

- **Sporodochium:** A cushion shaped stroma or mat of hyphae covered with conidiophores

- **Sterile Hyphae:** A mould that is growing only in its filamentous phase without produce conidia or other fruiting bodies. The identification of the moulds depends on seeing conidia, fruiting bodies, and other similar structures and the mould thus cannot be fully identified.

- **Synanomorph :** A second (or sometimes more) anamorph state of an anamorph fungus

- **Teleomorph:** A reproductive structure of a fungus that is the result of plasmogamy and nuclear recombination (sexual state)

- **Vesicle:** A swollen cell; the swollen apices of some conidiophores or sporangiophores.

- **Yeast :** A single fungal cell usually ovoid,that replicates by blastoconidia formation, planate division or reduced phialide or annellide

APPENDIX IV LIST OF CONSUMABLES

CONSUMABLES	COMPANY
Agarose	SRL, Mumbai, India
Amphotericin B	HiMedia, Mumbai, India
Brain Heart Infusion broth	Hi Media, Mumbai
Biogene kit	Imperial Biocorporals, India
Blood agar	HiMedia, Mumbai, India
Boric acid, Molecular grade	SRL, Mumbai, India
Bromophenol blue	HiMedia, Mumbai, India
Brucella blood agar	Hi Media, Mumbai, India
Calcofluor white	Hi Media, Mumbai, India
Chloramphenicol	Hi Media, Mumbai, India
Chloroform	Merck Limited, Mumbai, India
Chocolate agar	Hi Media, Mumbai, India
Corn meal agar	HiMedia, Mumbai, India
Cycloheximide	Sigma chemicals, Bangalore, India
100 bp DNA ladder, Phi X bacteriophage DNA	Bangalore Genei Pvt. Ltd., India
Hinf – I digest, *Hae* – III digest	Bangalore Genei Pvt. Ltd
dNTPs	
EDTA, Molecular grade	SRL, Mumbai, India
Ethanol, Absolute	S D fine chemicals Pvt Ltd, Mumbai,
Ethidium bromide	HiMedia, Mumbai, India
Fluconazole	Cipla Pharmaceuticals, Mumbai
Hae III	Bangalore Genei Pvt. Ltd., India
Hydroxyquinolone	Merck Limited, Mumbai, India
Isoamyl alcohol	Merck Limited, Mumbai, India
Ketoconazole	Cadila Pharmaceuticals, Ahmedabad
MacConkey agar	HiMedia, Mumbai, India
Parafilm	Axygen Inc, USA: Dialab Pvt Ltd, India
Potato dextrose agar	HiMedia, Mumbai, India
Phenol	SRL, Mumbai, India
Primers	Bangalore Genei Pvt. Ltd., India
Proteinase K	Bangalore Genei Pvt. Ltd., India
QIAamp DNA Blood Mini kit	QIAGEN, Germany: Genetix Pvt Ltd,
Sodium dodecyl sulphate	Merck Limited, Mumbai, India
Taq DNA Polymerase	Bangalore Genei Pvt. Ltd., India
Tris, Molecular grade	SRL, Mumbai, India
Yeast Nitrogen base	Hi Media, Mumbai, India

LIST OF PUBLICATIONS

- **Bagyalakshmi R,** Therese KL**,** Madhavan HN**.** Application of a semi nested polymerase chain reaction targeting Internal Transcribed Spacer (ITS) region for detection of panfungal genome in ocular specimens. Indian Journal of Ophthalmology 2007;55(4):261-5.

- Shankar S, Biswas J, Gopal L, **Bagyalakshmi R**, Therese KL, Borse NK. Anterior chamber exudative mass due to *Scedosporium apiospermum* in an immunocompetent individual Indian Journal of Ophthalmology 2007; 55(3):226-7.

- **Bagyalakshmi R,** Therese KL Madhavan HN. Nucleotide polymorphisms associated with Internal Transcribed Spacer (ITS) regions of ocular isolates of *Aspergillus flavus* Journal of Microbiological Methods 2007, 68 (1) : 1-10

- Therese KL, **Bagyalakshmi R**, Madhavan HN, Deepa P. *In –vitro* antifungal susceptibility testing by agar dilution method to determine the minimum inhibitory concentrations of Amphotericin B, fluconazole and ketoconazole against ocular fungal isolates.Indian Journal of Medical Microbiology 2006, 24(4): 273-9

- **Bagyalakshmi R,** Madhavan HN, Therese KL. Development and application of multiplex polymerase in reaction for the etiological diagnosis of infectious endophthalmitis. Journal of Postgraduate Medicine 2006, 52(3):179-82

- **Bagyalakshmi R**, Madhavan HN, Therese KL. Use of PCR in the diagnosis of endophthalmitis. Proceedings of the National Symposium on “Molecular biology: 50 years progress”,2003: p 90 -94

LIST OF PRESENTATIONS

- **National conference: "Molecular Biology 50 years progress" conducted in Ethiraj college, February 2002 :*Poster presentation*** on "Use of Polymerase Chain Reaction (PCR) in the diagnosis of endophthalmitis".

- **Indian Association of Medical Microbiologist (IAMM) Tamil Nadu Pondicherry Chapter meeting conducted in CMC Vellore, in March 2003:*Poster presentation*** on "Determination of Minimum inhibitory concentration of amphotericin B, fluconazole and ketoconazole on ocular fungal isolates by agar dilution method".

- **Indian eye Research group (IERG) National meeting held in L.V.Prasad Eye institute, Hyderabad 2003: *Oral presentation*** on 'Spectrum of fungi isolated from ocular specimens and *in vitro* antifungal sensitivity testing on fungal isolates". (Awarded Travel fellowship)

- **Indian Association of Medical Microbiologist (IAMM) National meet held in Mumbai in November 2003: *Oral presentation*** on "Application of PCR in detection of endophthalmitis and comparison with the conventional methods".

- **Indian eye Research group (IERG) National meeting held in Sankara Nethralaya, Chennai August 2004 : *Oral presentation*** on Application of PCR based RFLP for species level identification of fungi

- **Indian Association of Medical Microbiologist (IAMM) Tamil Nadu Pondicherry Chapter meeting conducted in Coimbatore, in October 2003:*Poster presentation*** on "PCR based RFLP for species level identification of fungi in ocular specimens:"

- **Indian Association of Medical Microbiologist (IAMM) National conference held in SRMC, Chennai in October 2005:*Oral presentation*** on "Development and application of semi nested PCR RFLP for detection of panfungal genome"

- **Society of Indian human and animal mycologists (SIHAM) conducted in Hyderabad, 2006*: Oral presentation*** on "Development and application of a novel duplex PCR for detection of panfungal genome".

- **Indian eye Research group (IERG) National level meeting held in L.V.Prasad Eye Institute held in Hyderabad, July 2006. *Oral presentation*** on "Phenotypic and genotypic characterization of *Fusarium* species"(Obtained Travel Fellowship)

Brief biography of the candidate

Ms. R. Bagyalakshmi obtained her B.Sc Microbiology degree from Valliammal College, University of Madras, Chennai in 1998. She obtained her M.S. (Medical Laboratory Technology) degree of Birla Institute of Technology and Science, Pilani in 2001 with course work at Medical Research Foundation, Chennai. Then she joined the Larsen & Toubro Microbiology Centre, Sankara Nethralaya as Junior Scientist and worked under the project funded by Cadila pharmaceuticals Private Limited. She registered for Ph. D in August 2003 in Birla Institute of technology & Science, Pilani She has made presentations in 9 national level conferences, comprising 6 oral presentations and 3 poster presentations. She has 6 publications out of which 5 are on her thesis work and published 10 abstracts presented in various national conferences.She has developed nucleic acid based amplification techniques for detection and identification of fungi from clinical specimens . She has received “Best performance award in Diagnostic Microbiology” (Sponsored by Hi Media,India) and Swarnalatha Punshi award (institutional) – for the best research worker in 2005. As an off-campus faculty of Birla Institute of Technology and Science, Pilani, at Medical Research Foundation, Chennai, she had taught microbiology and immunology for undergraduates and post-graduates. She has also conducted classes on PCR technology for the postgraduate students from other institutes. Her research interests are in the area of rapid diagnostic methods in the field of mycology.

Brief biography of the supervisor

Dr. K. Lily Therese is the Reader in Larsen & Toubro Microbiology Research Centre, Vision Research Foundation, Sankara Nethralaya, Chennai, India. She did MSc. Medical Microbiology in ALPGIBMS, University of Madras in 1986 securing university second rank. She, working as a Junior Research Fellow under ICMR Fellowship in Lady Harding College, Delhi University, New Delhi, India obtained her Ph.D degree in the year 1993 . She worked as Assistant Research Officer, in the research project, PL 480 – Project on Development of Streptococcal Vaccine in 1993 and joined Sankara Nethralaya as a post doctoral fellow and became a Reader in the year1998 in the Larsen & Toubro, Microbiology Research Centre, Vision Research Foundation, Sankara Nethralaya. She is a Life Member of Indian Association of Medical Microbiologists, Indian Virological Society and a Member of ARVO (Association for Research in Vision & Ophthalmology), SIHAM (Society for Indian Human and Animal Mycologists) and Member of American Society of Microbiology (ASM).She has received Swarnalatha Punshi award- best research scientist in 2002 and Alcon Fellowship award in 2006. She has undergone hands on training in DNA sequencing and ribotyping as a part of fulfillment of Alcon fellowship. She established PCR techniques for rapid detection of infectious agents in the Ocular Microbiology . She has successfully completed a ICMR project on detection and characterization of *Mycobacterium tuberculosis* and also co-investigator for 2 ICMR funded projects, one on *C. trachomatis* and the other on viral retinitis. Currently, she is the prinicipal investigator of 2 projects one funded by ICMR on *Toxoplasma gondii* and the other funded by DST on nucleic acid based amplification methods for detection and identification of fungi. Currently, she is the co-investigator for 2 ICMR funded projects, one on *M. fortuitum* and *M. chelonae* and the other on Rubella virus. She has 45 research publications in peer reviewed journals with publications in IOVS, British journal of ophthalmology, Journal of Microbiologial methods, Journal of Virological methods Ocular immunology and inflammation,Lancet and has filed 3 patencies. In academics she has 12 years experience in teaching microbiology and immunology to undergraduates and postgraduates. She is the Ph. D guide for 2 candidates. She along with her colleagues trained 19 Masters students who worked on Microbiology dissertation topics. She is a reviewer for the following journals: Indian Journal of Medical Microbiology, Indian Journal of Medical Research and Journal of Post graduate medicine.

Areas of Interest: Development of rapid molecular biological techniques for detection and species level identification of infective agents directly from clinical specimens