DOI: 10.7860/JCDR/2018/34188.11301

Original Article

Microbiology Section

Molecular Characterisation of Fungi from Mycotic Keratitis and Invasive Infections and Comparison with Conventional Methods

DEEPANKAR SRIGYAN[1], HIMANSHU SEKHAR BEHERA[2], GITA SATPATHY[3], NISHAT HUSSAIN AHMED[4]
NAMRATA SHARMA[5], RADHIKA TANDON[6], IMMACULATA XESS[7], JEEWAN SINGH TITIYAL[8]

ABSTRACT

Introduction: Fungi can cause systemic or localised infections in human beings, among which mycotic keratitis and Invasive Fungal Infections (IFIs) are very important, which can lead to severe complications and fatal consequences. In some instances, fungal culture isolates may not be identified accurately on the basis of morphology and microscopy. In those cases, molecular characterisation of isolates becomes more helpful in identification, which is essential for providing appropriate antifungal therapy and avoiding bad prognosis.

Aim: To identify the fungi collected from patients of mycotic keratitis and IFIs (diagnosed by conventional methods) using molecular methods and to compare the results with the results of conventional methods of identification.

Materials and Methods: In the present prospective study, fungal culture isolates were taken from 24 keratomycosis and seven IFIs patients (diagnosed by conventional methods) reporting to the OPD and wards of AIIMS Hospital, Delhi, India (March 2016 to March 2017). Isolates were further subcultured for DNA isolation and amplification for the variable ITS1 and ITS2 regions, and processed for nucleotide sequencing. The obtained sequences for ITS1 and ITS2 regions were searched for homology with NCBI-BLAST program. Results of molecular diagnosis at species level were compared with the conventional methods.

Results: Conventional methods could identify *Fusarium* spp., *Alternaria* spp., *Bipolaris* spp., *Cladosporium* spp., *Penicillium* spp., *Rhizopus* spp., up to genus level only, which could be further identified up to species level by molecular methods. Phenotypically identified isolates of *Acremonium* spp. was identified as *Simplicillium* spp., *Alternaria* spp. as *Chaetomium globosum*, *Mucor* spp. as *Rhizopus oryzae* by molecular methods.

Conclusion: As prognosis and therapy varies among different genera of fungi and in some cases from species to species, correct species level identification is important; hence, molecular methods may be complemented to traditional methods. Identification of rare fungal isolates is also important in clinical mycology, so that they will not be discarded as contaminants, which is possible using molecular techniques.

Keywords: Fungal keratitis, Invasive fungal infection, Keratomycosis, Nucleotide sequencing

INTRODUCTION

Fungal corneal infection or "mycotic keratitis" is a major cause of preventable blindness especially in tropical and sub tropical countries. In India, about 60,000 cases of mycotic keratitis and about 10,000 resultant corneal transplants occur each year [1,2]. The reported incidence of mycotic keratitis is 17-36% worldwide, whereas, it is about 44-47% in India [3-6]. Corneal trauma with plant or animal materials or dust particles due to either agricultural activity or animal husbandry, blocked nasolacrimal duct, poor hygienic practice of soft contact lens are the most common predisposing factors of keratomycosis [7,8]. Majority of the patients come from rural areas [7]. Fungal keratitis or keratomycosis, without early and prompt treatment, may lead to either loss of vision or severe visual impairment [2,3]. In contrast to bacterial keratitis, signs and symptoms of mycotic keratitis are mild and moderate in the early stage due to mild degree of inflammation; but, later on it leads to suppuration and ulceration [1].

Invasive fungal infections cause severe illness in the patients. IFIs are predominantly seen in immunocompromised individuals, but several cases were also reported in immunocompetent individuals [9,10]. Patients with IFIs show signs and symptoms of febrile illness that continue even after prolonged broad spectrum antibiotics treatment and lesions are visible in radiology report [9]. Systemic predisposing factors like diabetes, Human Immunodeficiency Virus (HIV) infection, cancer and treatment for a bacterial infection with antibiotics or steroids influence the occurrence of IFIs [10]. As the number of reported cases of IFIs are few and data is scanty, exact prevalence from India is hard to define. Early and accurate diagnosis with proper treatment is essential for preventing morbidity and mortality in these patients.

Fungal species make up for nearly 7% (611,000 species) of all eukaryotic species on earth and are widely distributed in plant debris, soil, dust and other organic substrates among which about 600 species are human pathogens [11,12]. Although, more than 105 species of fungi are known to cause eye infections, majority are caused by members of *Aspergillus* spp. and *Fusarium* spp. across India and South East Asia, among which, *Aspergillus* spp. was reported as the most predominate and prevalent one in Northern India [13,14]. The other commonly isolated filamentous fungi from fungal corneal infections are *Alternaria* spp., *Penicillium* spp. and *Curvularia* spp. [1]. Opportunistic yeasts like *Candida* specially *C. tropicalis* and *C. albicans* or filamentous fungi like *Aspergillus* spp. are the most predominant genera of fungus involved in IFIs in India and worldwide [15,16]. Other fungi such as *Fusarium*, *Trichosporon* and *Malassezia* spp., which were previously considered as non-pathogenic for humans or causing diseases sporadically are now considered as leading fungal pathogens for IFIs [15,16].

Keratomycosis and IFIs can be misdiagnosed leading to severe complications. In the early stage when symptoms are mild; accurate and rapid diagnosis is the key feature for the management of fungal keratitis and invasive infections. Previously, the laboratory diagnosis of fungal keratitis and IFIs were only made by direct microscopy of corneal scraping, tissues or body fluids with Potassium Hydroxide (KOH) mount and isolation in culture [17]. As the sensitivity of direct microscopy is low, the staining with calcofluor white and blankophor increases the sensitivity to some extent [18]. Culture remains the gold standard for diagnosis of fungal keratitis and IFIs [19]. In some cases, fungal culture isolates may not be identified accurately on the basis of morphology and microscopy. In case of keratomycosis or infections by highly invasive fungi like *Pythium* spp., the fungus can be misidentified as contaminant or *Aspergillus* spp. leading to incorrect therapy and severe morbidity, sometimes with fatal consequences. In those cases, molecular characterisation of isolates become more helpful in identification, which is essential for providing appropriate antifungal therapy and avoiding bad prognosis [20].

The present study aims to identify the fungi collected from the patients of fungal keratitis and IFIs (diagnosed by conventional methods) using Polymerase Chain Reaction (PCR) assay for both ITS regions of ribosomal RNA, nucleotide sequencing, analysis using National Center for Biotechnology Information-Basic Local Alignment Search Tool (NCBI-BLAST) against available data base in gene bank and to compare the results with the results of conventional methods of identification (direct microscopy and phenotypic identification from culture isolates).

MATERIALS AND METHODS

Fungal Culture Isolates

A prospective study conducted over a period of one year (March 2016 to March 2017) was carried out at the Department of Microbiology, All India Institute of Medical Science (AIIMS), Delhi, India. The fungal culture isolates on Sabouraud's Dextrose Agar (SDA) (only filamentous fungi) from corneal scrapings of 24 keratomycosis patients (diagnosed by conventional methods) attending the OPD/causality services of Dr. Rajendra Prasad Centre for Ophthalmic Sciences, AIIMS. Fungal isolates (only filamentous fungi) from seven IFI patients (diagnosed by conventional methods) admitted to different wards of the AIIMS hospital were included in this study. Patients of clinically suspected fungal keratitis and invasive infections by filamentous fungi were included in this study. Patients of either *Acanthamoeba* keratitis, bacterial keratitis or viral keratitis and IFI patients with yeast infections were excluded from this pilot study. The age of the patients of keratomycosis ranged from 6-79 years with the mean age of 39 years with predominance of male patients were 22 (92%). The age of the patients of IFIs varied from 24-52 years with the mean age of 39 years out of which 3 (43%) were males and 4 (57%) were females. All procedures performed in this study were in accordance with the ethical clearance of the institute. The fungal culture isolates were sub-cultured on SDA (HiMedia, India) with antibiotic gentamicin (0.02 mg/mL). Two tubes were taken per isolate, among which one was incubated at 25ºC and other at 37ºC, till the growth appeared [21,22].

Macroscopic and Microscopic Examination of the Culture

The culture slants were examined for morphological features, color, texture, diffusible pigments and spore features etc. Lactophenol Cotton Blue (LPCB) mount was prepared and visualised under a light microscope with 10X and 40X objectives for microscopic examination [23].

Molecular Methods

DNA isolation from fungal culture isolates: DNA was isolated from the 31 fungal culture isolates using the protocol described by Lee SB et al., with slight modifications [24]. Briefly, primary fungal culture isolates obtained from the patients were further subcultured on SDA plates, and kept in an incubator at 25ºC and 37ºC for at least 7-10 days until a full grown fungal mat was seen over the agar surface. Mycelial mat of approximately 0.3-0.5 gm was taken in a sterile mortar; liquid nitrogen was added and grounded quickly to prepare fine powder using a pestle. Fungal powder of about 0.2-0.3 gm was transferred to sterile 1.5 mL microcentrifuge tube and 600 µL of ATL lysis buffer (QIAGEN, Germany) was added. Microcentrifuge tubes were vortexed briefly and incubated at 65ºC for three hours. Lysis buffer AL (200 µL) (QIAGEN, Germany) and 20 µL of proteinase K was added to the tubes, vortexed and incubated at 56ºC for one hour followed by at 70ºC for 10 minutes. About 4 µL of RNase A (10 mg/mL) (QIAGEN, Germany) was added to the tubes; incubated at 37ºC for one hour to digest the contaminating RNA. Lysed solution was mixed with 200 µL of 100% ethanol and incubated for two minutes at room temperature. The solution was transferred to QIAamp mini-spin column (QIAGEN, Germany) and centrifuged at 8000 rpm for one minute. Tubes were washed with 500 µL of washing buffer AW1 followed by AW2 with centrifugation at 8000 rpm for one minute each. DNA was eluted with 100 µL of elution buffer AE (QIAGEN, Germany) and stored at -20ºC for PCR assay.

PCR assay for molecular diagnosis: PCR assay for amplification of the genus specific Internal Transcribed Spacer (ITS) regions ITS1 and ITS2 of ribosomal DNA (~280 bp) was standardised with published primers {ITS1 region: FP: (5'-TCC GTAGGTGAACCTGCGG-3') that hybridises at the end of 18S rDNA and RP: ITS86 (5'-GTTCAAAGATTCGATGATTCAC-3') hybridises with the 5.8S rDNA region} and {ITS2 region: FP: ITS86 (5'-GTGAATCATCGAATCTTTGAA C-3') that hybridises with the 5.8S rDNA region and RP: ITS4: (5'-TCCTCCGCTTATTGATATGC-3') which hybridizes at the beginning of 28S rDNA} by changing the concentration of $MgCl_2$ and annealing temperature until specific bands were visualised in 1.5% agarose gel [25-27]. Briefly, the protocol standardised for the PCR assay was as follows: a reaction mixture of 20 µL was prepared with 1X reaction buffer (Fermentas), 2.5 mm $MgCl_2$, 200 µm Deoxyribonucleotide Triphosphates (dNTPs) (Fermentas), 0.4 µm forward and reverse primers (IDT), 1.25 U Taq polymerase (Fermentas, USA) and milliQ water q.s. Final reaction volume of 25 µL was made with 5 µL of extracted fungal DNA (~0.5 ng). PCR assay was performed in a thermal cycler (Applied Bio system, USA) with the temperature profile: initial denaturation at 94˚C for 5 minutes, followed by denaturation at 94˚C for 30 seconds, primer annealing at 52˚C for 30 seconds (ITS1 region) and 54˚C (ITS2 region), strand elongation at 72˚C for 30 seconds for 35 cycles, with the final elongation at 72˚C for 10 minutes. Reaction mixture with 5 µL distilled water was used as a negative control and reaction mixture with DNA isolated from known isolates of *Aspergillus flavus* was used as a positive control in the PCR assay. Amplified PCR products were electrophoresed on 1.5% agarose gel and visualised under a gel documentation system (Syngene, USA).

Sequencing and sequence homology analysis: Amplified DNA bands for ITS1 and ITS2 regions were cut from the agarose gel and DNA was extracted using QIAquick Gel Extraction Kit (QIAGEN, USA) as per the manufacturer's instructions. Nucleotide sequences of the purified DNA were determined commercially (Biolink, India) using primers ITS1 and ITS4 (sequences described earlier). Nucleotide sequences of ITS1 and ITS2 regions were searched for homology analysis with available sequences found in the Gene bank with NCBI BLAST computer program (NCBI, USA). Nucleotide sequences of both ITS1 and ITS2 regions from 31 culture isolates were submitted in the NCBI databank (NCBI,

USA) (Nucleotide sequence accession numbers are given in results).

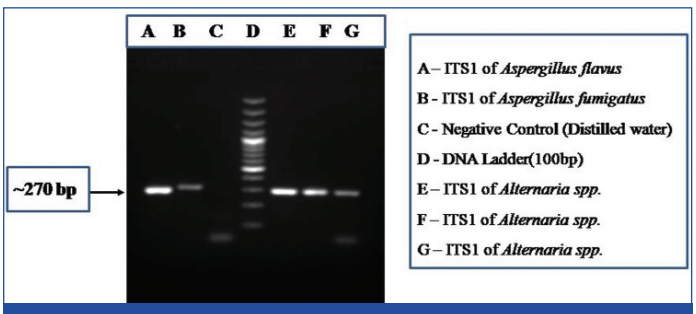
RESULTS

Of the 31 fungal culture isolates, visual and microscopic examinations could identify 22 isolates up to genus level and nine isolates up to species level [Table/Fig-1]. Amplified PCR products for the ITS1 and ITS2 regions when electrophoresed on 1.5% agarose gel shows bands of approximately 280 bp length when visualised with a gel documentation system [Table/Fig-2,3]. Nucleotide sequences submitted to the NCBI databank are now available with the accession numbers MF000891-MF000910 and MF033427-MF033435 for ITS1 region and MF000921-MF000940 and MF033438-MF033445) for ITS2 region [Table/Fig-1]. Of the 31 fungal culture isolates taken for sequencing to reach species level identification, eight were identified as *Aspergillus flavus*, one as *Aspergillus fumigatus*, four as *Cladosporium cladosporioides*, three as *Simplicillium* spp., three as *Fusarium solani*, one as *Fusarium equiseti*, three as *Alternaria tenuissima*, one as *Alternaria alternata*, two as *Penicillium chrysogenum*, one as *Penicillium citrinum*, one as *Rhizopus microsporus*, one as *Rhizopus oryzae*, one as *Bipolaris sorokiniana* and one as *Chaetomium globosum*. In five instances, fungal isolates identified by molecular methods were different from conventional methods [Table/Fig-1].

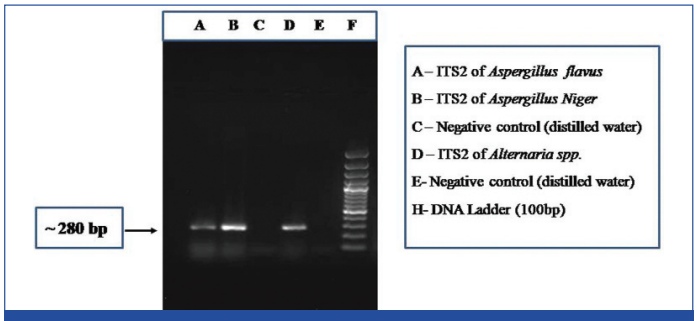
Nucleotide sequences of *Aspergillus flavus* (n=8) from conventional culture share 100%/99% identity with *Aspergillus flavus* by sequencing and NCBI-BLAST (accession numbers: LN482517, LN482514, LN482513, KR611594, JQ781721, FJ011545). Similarly, *Aspergillus fumigatus* (n=1) from conventional culture

S. No. of Patients	Nature of specimen	Fungus identified by conventional culture	Fungus identified by sequencing and NCBI-BLAST	Accession numbers obtained (NCBI) (ITS1 and ITS2)	Identity (%)	Accession numbers showing highest similarity
1	Corneal scraping	*Acremonium* spp.	*Simplicillium* spp.	MF000891 and MF000921	99%	KX020567, KX020563
2	Corneal scraping	*Aspergillus fumigatus*	*Aspergillus fumigatus*	MF000892 and MF000922	99%	KU743889, JN226978
3	Corneal scraping	*Acremonium* spp.	*Simplicillium* spp.	MF033427 and MF033438	99%	KT318874, KP184323
4	Corneal scraping	*Fusarium* spp.	*Fusarium solani*	MF000893 and MF000923	99%	GQ451337, GQ121291
5	Corneal scraping	*Bipolaris* spp.	*Bipolaris sorokiniana*	MF033428 (ITS1)	98%	KU870641, KT310049
6	Corneal scraping	*Aspergillus flavus*	*Aspergillus flavus*	MF000894 and MF000924	99%	LN482517, LN482514
7	Corneal scraping	*Alternaria* spp.	*Chaetomium globosum*	MF000895 and MF000925	99%	KU936228, KP281435
8	Corneal scraping	*Cladosporium* spp.	*Cladosporium cladosporioides*	MF000896 and MF000926	96%	MF000907, MF000906
9	Corneal scraping	*Aspergillus flavus*	*Aspergillus flavus*	MF033439 (ITS2)	99%	KR611594, JQ781721
10	Corneal scraping	*Fusarium* spp.	*Fusarium solani*	MF033440 (ITS2)	99%	KR527137, KY848498
11	Invasive (Tissue)	*Fusarium* spp.	*Fusarium equiseti*	MF000897 and MF000927	100%	KR812230, KJ677237
12	Corneal scraping	*Penicillium* spp.	*Penicillium chrysogenum*	MF000898 and MF000928	99%	MF000902, KX901289
13	Corneal scraping	*Alternaria* spp.	*Alternaria tenuissima*	MF033429 (ITS1)	100%	KR709011, KR912298
14	Corneal scraping	*Alternaria* spp.	*Alternaria tenuissima*	MF000899 and MF000929	100%	HQ647307, KU508797
15	Corneal scraping	*Alternaria* spp.	*Alternaria alternata*	MF033430 and MF033441	99%	MF040794, MF168401
16	Corneal scraping	*Aspergillus flavus*	*Aspergillus flavus*	MF000900 and MF000930	99%	KU561920, KU561919
17	Corneal scraping	*Acremonium* spp.	*Simplicillium* spp.	MF000901 and MF000931	99%	KX020567, KX020563
18	Invasive (Aspirate)	*Penicillium* spp.	*Penicillium chrysogenum*	MF000902 and MF000932	99%	KU982597, JF807949
19	Corneal scraping	*Aspergillus flavus*	*Aspergillus flavus*	MF000903 and MF000933	99%	KP296143, KX345284
20	Corneal scraping	*Alternaria* spp.	*Alternaria tenuissima*	MF000904 and MF000934	100%	LT799975, KX064997
21	Corneal scraping	*Fusarium* spp.	*Fusarium solani*	MF000905 and MF000935	99%	GQ451337, GQ121291
22	Corneal scraping	*Cladosporium* spp.	*Cladosporium cladosporioides*	MF000906 and MF000936	99%	JN227029, KX960912
23	Corneal scraping	*Cladosporium* spp.	*Cladosporium cladosporioides*	MF000907 and MF000937	99%	KP689250, KF876823
24	Invasive (Tissue)	*Aspergillus flavus*	*Aspergillus flavus*	MF033431 and MF033442	99%	MF163443, MF120213
25	Invasive (Tissue)	*Rhizopus* spp.	*Rhizopus microsporus*	MF033432 (ITS1)	99%	KJ408570, KM527225
26	Invasive (Tissue)	*Aspergillus flavus*	*Aspergillus flavus*	MF000908 and MF000938	99%	HM560052, HM560051
27	Corneal scraping	*Aspergillus flavus*	*Aspergillus flavus*	MF033433 and MF033443	100%	MF033444, MF033443
28	Invasive (BAL)	*Penicillium* spp.	*Penicillium citrinum*	MF000909 and MF000939	99%	KY754577, KX363454
29	Invasive (Tissue)	*Mucor* spp.	*Rhizopus oryzae*	MF000910 and MF000940	99%	KX685359, FJ433877
30	Corneal scraping	*Aspergillus flavus*	*Aspergillus flavus*	MF033434 and MF033444	100%	MF033444, MF033443
31	Corneal scraping	*Cladosporium* spp.	*Cladosporium cladosporioides*	MF033435 and MF033445	100%	KJ728689, KJ410037

[Table/Fig-1]: Details of 31 fungal isolates with accession numbers obtained from NCBI.



[Table/Fig-2]: PCR assay showing the amplification of ITS1 region (~270 bp) of fungus.



[Table/Fig-3]: PCR assay showing the amplification of ITS2 region (~280 bp) of fungus.

shares 99% identity with *Aspergillus fumigatus* (accession numbers: KU743889, JN226978, JX469423). All three *Cladosporium* spp. share 100%/99% identity with *Cladosporium cladosporioides* (KJ728689, KP689250, KX610162). All the three *Acremonium* spp. share 99% identity with *Simplicillium* spp. (KX020567, KX020563, KP184324). Of the four *Fusarium* spp., three share 99% identity with *Fusarium solani* (GQ451337, GQ121291, KX381129) and one shares 100% identity with *Fusarium equiseti* (KR812230, KJ677237, KJ677236). Of the five *Alternaria* spp., three share 100% identity with *Alternaria tenuissima* (HQ647307, KU508797, KX064997), one shares 99% identity with *Alternaria alternata* (MF040794, MF168401, MF167641) and one shares 99% identity with *Chaetomium globosum* (KU936228, KP281435, JX406510). Of the three *Penicillium* spp., two share 99% identity with *Penicillium chrysogenum* (MF000902, KX901289, KU743900) and one shares 99% identity with *Penicillium citrinum* (KY754577, KX363454, KX363446). *Rhizopus* spp. (n=1) shares 99% identity with *Rhizopus microsporus* (KJ408570, KM527225, KM527224). *Mucor* spp. (n=1) shares 99% identity with *Rhizopus oryzae* (KX685359, FJ433877, KY244030) and *Bipolaris* spp. (n=1) shares 98% identity with *Bipolaris sorokiniana* (KU870641, KT310049, KF922886).

DISCUSSION

Keratomycosis is one of the most frequently encountered ophthalmic infections in tropical and sub tropical countries like India, caused by a variety of fungal species including opportunistic fungal pathogens and in some instances by these, previously classified as contaminant fungi [28]. IFIs are the leading causes of morbidity and mortality in immunocompromised patients [9,10]. Accurate identification of fungi to the species level holds an importance in developing countries like India, where fungal infections are more common and correct choice of antimicrobial therapy requires fungal infections to be distinguished from other microbial aetiology. As identification of the causative fungi with conventional culture needs more than a week, rapid and high throughput molecular diagnostic tools like PCR assay and sequencing are very helpful for species level identification either from culture isolates or directly from clinical specimens for correct treatment [26]. In this pilot study, we have identified the pathogenic fungi to species level from culture positive clinical isolates to guide better therapy for the management of infections. Some fungal isolates could be fully identified by conventional methods such as visual and microscopic morphological examinations. Correct identification is important because treatment varies among different genus of fungi and in some cases from species to species, as relatively high Minimum Inhibitory Concentrations (MICs) of amphotericin B and itraconazole are needed for *F. solani* compared to *F. oxysporum* [29].

Fungal species identification is based on the variation in nucleotide sequences of the variable ITS1 and ITS2 regions of ribosomal DNA (rDNA). As 28S, 18S and 5.8S rRNA are the conserved regions of fungal ribosomal DNA, these are often used for designing forward and reverse primers to amplify the inner variable regions in PCR assay. ITS1 and ITS2 regions can be amplified jointly with forward primer ITS1 and reverse primer ITS4, which is about 560 bp in length, but it has some limitations due to “chimera formation”. To avoid such type of limitations, these targets (ITS1 and ITS2) can be amplified separately for species level identification [26,30,31].

Among *Aspergillus* species, *A. flavus* is the predominant fungi causing mycotic keratitis and second only to *A. fumigatus* as a cause of human invasive infections [32,33]. In this pilot study, it was also reported that, of the total seven *Aspergillus* spp. isolates from mycotic keratitis patients, six were *A. flavus* and only one was *A. fumigatus* which corroborates with the previous studies.

The species of *Simplicillium* is considered as coincidental opportunistic pathogen to humans and animals as it occurs in a wide range of ecological niches, such as soil, mushroom, diseased plant tissue, rust, nematode, dog tissue and human nails [34]. *Acremonium* spp. is morphologically similar to *Simplicillium* spp. where the isolates do not produce their distinctive macroconidia. Some of the species of *Simplicillium* such as *Simplicillium obclavatum*, originally described as *Acremonium obclavatum*, which makes it difficult to differentiate between them by conventional methods [35,36]. Therefore, genus level identification of both the fungus is difficult exclusively on morphological characteristics and there is always a possibility of misidentification. Hence, molecular identification may be performed as a complementary test to avoid such type of misdiagnosis. In this study, phenotypically identified all three isolates of *Acremonium* spp. was identified as *Simplicillium* spp. by molecular methods.

Cladosporium spp. was considered as a coincidental opportunistic pathogen, causing mycotic keratitis and some cutaneous and subcutaneous infections [37]. *Cladosporium cladosporioides* is a very rare pigmented fungi with few reported cases of keratomycosis [2]. In some case reports from Asian and African countries, corneal keratomycosis resulting from *Cladosporium cladosporioides* had been reported [38-40]. In the present study, we found four isolates of *Cladosporium cladosporioides* with PCR assay and sequencing from mycotic keratitis patients which were reported as *Cladosporium* spp. with traditional mycological diagnostic method.

A study from India reported the incidence of *Alternaria* spp. in mycotic keratitis patients as 3.3-10.4% [41]. Among all *Alternaria* spp., *A. alternata* and *A. tenuissima* are more common in ocular infections [41]. In the present study, we found one isolate of *A. alternata* and three isolates of *A. tenuissima*, which corroborates with the previous studies. *Alternaria* spp. and *Chaetomium* spp. both belong to dematiaceous moulds, so they can be misidentified by conventional methods. In the present study, one isolate which was identified as *Alternaria* spp. by conventional methods, later characterised as *Chaetomium globosum* by molecular methods. Association of *Chaetomium globosum* was already reported with keratomycosis [42].

Species level identification of Zygomycetes by standard mycological methods always remains a difficult and time-consuming task, which requires an expertise that is restricted to few reference laboratories. DNA typing to differentiate between the species among Zygomycetes is particularly important in those cases where these fungi differ in their susceptibility to antifungal drugs. The two most common pathogenic species of *Rhizopus* i.e., *R. oryzae* and *R. microsporus*, which are difficult to differentiate phenotypically, could be clearly differentiated from each other genotypically, as their sequences showed only 70% similarity [43]. In the present study, we found one *Rhizopus microsporus* from tissue specimen, which was earlier reported as *Rhizopus* spp. Another invasive fungal isolate which was misidentified as *Mucor* spp. by conventional methods was identified as *Rhizopus oryzae* with molecular diagnosis. These molecular typing results hold importance in differentiating between different species of Zygomycetes to start an appropriate antifungal therapy. Hence, molecular diagnosis is useful for species identification within Zygomycetes from culture isolates.

Studies from South India and central China reported *Fusarium* spp. as the most common cause of mycotic keratitis; among which *F. solani* is the most predominant one [28,44]. Few cases of *F. equiseti* infections were also reported in some parts of the world [45,46]. In the present study, we had reported three *F. solani* and one *F. equiseti* isolates from fungal infections by nucleotide sequencing, which suggests that, *F. solani* is most prevalent among all *Fusarium* spp. causing eye infections.

This study presented a standardised rapid and high throughput technique (PCR assay) for the detection and identification of fungi in ocular and invasive samples. It consists of a sensitive, precise, rapid but relatively expensive method of identification of fungi based on the amplification of ITS1 and ITS2 regions and sequencing. There are several species

within a genus that differ in their susceptibility to antifungal drugs, in those instances molecular typing by PCR assay can give guidance for better therapy to manage such infections. Molecular assays are neither been widely used nor widely available. There is a need for widespread use of molecular assays for correct identification, so that some fungal isolates will not be discarded as contaminants. Misidentification of rare isolates can be avoided by using molecular techniques.

LIMITATION

The present study included culture isolates of filamentous fungi only. IFIs due to *Candida* spp. could also have been included.

CONCLUSION

Molecular diagnosis with PCR assay can be an important complement to conventional culture methods (gold standard) and may constitute a rapid and high throughput means of fungal identification, which is becoming very important in clinical mycology. As the cost of PCR assay to diagnose infections generally exceeds that of conventional culture, which limits its widespread use, especially in low income countries like India, it should be implemented at least in reference centers for a definitive diagnosis and treatment.

ACKNOWLEDGEMENTS

Authors thank all the clinical faculty members and resident doctors of Dr Rajendra Prasad Centre for Ophthalmic Sciences and AIIMS for sending the clinical specimens for clinical investigations.

REFERENCES

[1] Thomas PA, Kaliamurthy J. Mycotic keratitis: epidemiology, diagnosis and management. Clin Microbiol Infect. 2013;19(3):210-20.
[2] Chowdhary A, Singh K. Spectrum of fungal keratitis in North India. Cornea. 2005;1:8-15.
[3] Rosa RH, Miller D, Alfonso EC. The changing spectrum of fungal keratitis in South Florida. Ophthalmol.1994;101(6):1005-13.
[4] Srinivasan M, Gonzales CA, George C, Cevallos V, Mascarenhas JM, Asokan B, et al. Epidemiology and aetiological diagnosis of corneal ulceration in Madurai, South India. Br J Ophthalmol. 1997;81:965-71.
[5] Dunlop AA, Wright ED, Howlader SA, Nazrul I, Husain R, McClellan K, et al. Suppurative corneal ulceration in Bangladesh. A study of 142 cases examining the microbiological diagnosis, clinical diagnosis, and epidemiological features of bacterial and fungal keratitis. Aust N Z J Ophthalmol. 1994;22:105-10.
[6] Upadhyay MP, Karmacharya PC, Koirala S, Tuladhar NR, Bryan LE, Smolin G, et al. Epidemiologic characteristics, predisposing factors, and aetiologic diagnosis of corneal ulceration in Nepal. Am J Ophthalmol. 1991;111:92-99.
[7] Tilak R, Singh A, Maurya OP, Chandra A, Tilak V, Gulati AK. Mycotic keratitis in India: a five-year retrospective study. J Infect Dev Ctries. 2010;4(3):171-74.
[8] Bharathi MJ, Ramakrishnan R, Vasu S, Meenakshi R, Palaniap pan R. Epidemiological characteristics and laboratory diagnosis of fungal keratitis: a three-year study. Indian J Ophthalmol. 2003;51(4):315-21.
[9] Ramana KV, Kandi S, Bharatkumar V, Sharada CH, Rao R, Mani R, et al. Invasive fungal infections: a comprehensive review. Am J Infect Dis Microbiol. 2013;1(4):64-69.
[10] Xie GH, Fang XM, Fang Q, Wu XM, Jin YH, Wang JL, et al. Impact of invasive fungal infection on outcomes of severe sepsis: a multicenter matched cohort study in critically ill surgical patients. Crit Care. 2008;12:R5.
[11] Mora C, Tittensor DP, Adl S, Simpson AG, Worm B. How many species are there on Earth and in the ocean? PLoS Biol. 2011;9(8):e1001127.
[12] Brown GD, Denning DW, Levitz SM. Tackling human fungal infections. Science. 2012;336:647.
[13] Rautaraya B, Sharma S, Kar S, Das S, Sahu SK. Diagnosis and treatment outcome of mycotic keratitis at a tertiary eye care center in Eastern India. BMC Ophthalmol. 2011;11:39.
[14] Chander J, Singla N, Agnihotri N, Arya SK, Deep A. Keratomycosis in and around Chandigarh: a five-year study from a north Indian tertiary care hospital. Indian J Pathol Microbiol. 2008;51(2):304-06.
[15] Jorda-Marcos R, Alvarez- Lerma F, Jurado M, Palomar M, Nolla-Salas J, Leon MA, et al. Risk factors for candidemia in critically ill patients: a prospective surveillance study. Mycoses. 2007;50:302-10.
[16] Singh T, Kashyap AK, Ahluwalia G, Chinna D, Sidhu SS. Epidemiology of fungal infections in critical care setting of atertiary care teaching hospital in North India: a prospective surveillance study. J Clin Sci Res. 2014;3:14-25.
[17] Vengayil S, Panda A, Satpathy G, Nayak N, Ghosh S, Patanaik D, et al. Polymerase chain reaction-guided diagnosis of mycotic keratitis: a prospective evaluation of its efficacy and limitations. Invest Ophthalmol Vis Sci. 2009;50:152-56.
[18] Chander J, Chakrabarti A, Sharma A, Saini JS, Panigarhi D. Evaluation of calcofluor staining in the diagnosis of fungal corneal ulcer. Mycoses. 1993;36(7-8):243-45.
[19] Byrne KA, Burd EM, Tabbara KF, Hyndiuk RA. Diagnostic microbiology and cytology of the eye. Boston, MA: Butterworth-Heinemann. 1995;94-104.
[20] Krajaejun T, Pracharktam R, Wongwaisayawan S, Rochanawutinon M, Kunakorn M, Kunavisarut S. Ocular pythiosis: is it under-diagnosed? Am J Ophthalmol. 2004;137(2):370-72.
[21] Das S, Sharma S, Kar S, Sahu SK, Samal B, Mallick A. Is inclusion of sabouraud dextrose agar essential for the laboratory diagnosis of fungal keratitis? Indian J Ophthalmol. 2010;58(4):281-85.
[22] Basu S, Bose C, Ojha N, Das N, Das J, Pal M, et al. Evolution of bacterial and fungal growth media. Bioinformation. 2015;11(4):182-84.
[23] Thomas PA, Kuriakose T, Kirupashanker MP, Maharajan VS. Use of lactophenol cotton blue mounts of corneal scrapings as an aid to the diagnosis of mycotic keratitis. Diagn Microbiol Infect Dis. 1991;14(3):219-24.
[24] Lee SB, Milgroom MG, Taylor JW. A rapid, high yield mini-prep method for isolation of total genomic DNA from fungi. Fungal Genetics Newsletter. 1988;35:23–24.
[25] Sugita C, Makimura K, Uchida K, Yamaguchi H, Nagai A. PCR identification system for the genus *Aspergillus* and three major pathogenic species: *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus* niger. Med Mycol. 2004;42(5):433-37.
[26] Ferrer C, Colom F, Frases S. 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(8):2873-79.
[27] Lott TF, Burns BM, Zancope-Oliveira R, Elie CM, Reiss E. Sequence analysis of the internal transcribed spacer 2 (ITS2) form yeast species within the genus *Candida*. Curr Microbiol. 1998;36:63-69.
[28] Garg P, Gopinath G, Choudhary K, Rao GN. Keratomycosis: clinical and microbiological experience with dematiaceous fungi. Ophthalmol. 2000;107(3):574-80.
[29] Nucci M, Anaissie E. *Fusarium* infections in immunocompromised patients. Clin Microbiol Rev. 2007;20(4):695-704.
[30] Ferrer C, Munoz G, Alio JL, Abad JL, Colon F. Polymerase chain reaction diagnosis in fungal keratitis caused by *Alternaria alternata*. Amer J Ophthalmol. 2002;33:398-99.
[31] Nilsson RH, Tedersoo L, Ryberg M, Kristiansson E, Hartmann M, Unterseher M, et al. A Comprehensive, automatically updated fungal ITS sequence dataset for reference-based chimera control in environmental sequencing efforts. Microbes and Environments. 2015;30(2):145-50.
[32] Denning DW. Invasive aspergillosis. Clin Infect Dis. 1998;26(4):781-803.
[33] Thomas PA. Fungal infections of the cornea. Eye (Lond). 2003;17(8):852-62.
[34] Zare R, Gams W. A revision of *Verticillium* section *Prostrata*. IV. The genera *Lecanicillium* and *Simplicillium* gen. Nova Hedwigia. 2001;73:1-50.
[35] Summerbell RC, Gueidan C, Schroers HJ, de Hoog GS, Starink M, Guarro J, et al. *Acremonium* phylogenetic overview and revision of *Gliomastix*, *Sarocladium*, and *Trichothecium*. Stud Mycol. 2011;68:139-62.
[36] Liu F, Cai L. Morphological and molecular characterization of a novel species of *Simplicillium*from China. Cryptogamie, Mycologie. 2012;33(2):1-13.
[37] Gugnani HC, Sood N, Singh B, Makkar R. Subcutaneous phaeohyphomycosis due to *Cladosporium cladosporioides*. Mycoses. 2000;43:85-87.
[38] Chew FL, Subrayan V, Chong PP, Goh MC, Ng KP. *Cladosporium cladosporioides* keratomycosis: a case report. Jpn J Ophthalmol. 2009;53(6):657-59.
[39] Gugnani HC, Gupta S, Talwar RS. Role of opportunistic fungi in ocular infections in Nigeria. Mycopathologia. 1978;65:155.
[40] Leck AK, Thomas PA, Hagan M, Kaliamurthy J, Ackuaku E, John M, et al. Aetiology of suppurative corneal ulcers in Ghana and south India, and epidemiology of fungal keratitis. Br J Ophthalmol. 2002;86:1211-15.
[41] Panda A, Sharma N, Gopal D, Kumar N, Satpathy G. Mycotic keratitis in children: epidemiologic and microbiologic evaluation. Cornea. 1997;16:295-99.
[42] Vanzzini Zago V, Alcantara Castro M, Naranjo Tackman R. Support of the laboratory in the diagnosis of fungal ocular infections.Int J Inflam. 2012; 643104:1-8.
[43] Schwarz P, Bretagne S, Gantier JC, Garcia-Hermoso D, Lortholary O, Dromer F, et al. Molecular identification of zygomycetes from culture and experimentally infected tissues. J Clin Microbiol. 2006;44(2):340-49.
[44] Wang L, Sun S, Jing Y, Han L, Zhang H, Yue J. Spectrum of fungal keratitis in central China. Clin Exp Ophthalmol. 2009;37(8):763-71.
[45] Kiyofumi M, Ikumi S, Hiroki M, Kiyofumi O, Kazuko N. Identification and sensitivity of two rare fungal species isolated from two patients with *Fusarium* keratomycosis. J Infect Chemother. 2012;18:939-44.
[46] Soler C, Daban JL, Mac Nab C, Appere G, Poirier H, St. Blancart P, et al. *Fusarium equiseti*: about the first case of human infection in a war casualty. *Aspergillus* & Aspergillosis website. 2012. (Accessed 26 June 2017).

PARTICULARS OF CONTRIBUTORS:
1. Junior Resident, Department of Microbiology, All India Institute of Medical Sciences, New Delhi, Delhi, India.
2. PhD Student, Department of Ocular Microbiology, Dr. Rajendra Prasad Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, New Delhi, Delhi, India.
3. Professor and Head, Department of Microbiology, All India Institute of Medical Sciences, New Delhi, Delhi, India.
4. Assistant Professor, Department of Microbiology, All India Institute of Medical Sciences, New Delhi, Delhi, India.
5. Professor, Department of Ophthalmology, Dr. Rajendra Prasad Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, New Delhi, Delhi, India.
6. Professor, Department of Ophthalmology, Dr. Rajendra Prasad Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, New Delhi, Delhi, India.
7. Professor, Department of Microbiology, All India Institute of Medical Sciences, New Delhi, Delhi, India.
8. Professor, Department of Ophthalmology, Dr. Rajendra Prasad Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, New Delhi, Delhi, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Gita Satpathy,
Professor and Head, Department of Microbiology and Professor Incharge, Ocular Microbiology, Dr. Rajendra Prasad Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, New Delhi-110029, Delhi, India.
E-mail: gita.satpathy@gmail.com

FINANCIAL OR OTHER COMPETING INTERESTS: As declared above.

Date of Submission: **Oct 08, 2017**
Date of Peer Review: **Nov 22, 2017**
Date of Acceptance: **Jan 25, 2018**
Date of Publishing: **Mar 01, 2018**