

Polymerase Chain Reaction Based Detection of Fungi in Suspected Infectious Keratitis Patients

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ABSTRACT

Background: Fungal infections of the eye are rampant in tropical regions and are responsible for significant ocular morbidity and blindness. Prompt and specific identification of fungi is important in order to define clinical treatment. However, in most of the cases conventional culture identification can be considered to be time consuming, of low sensitivity and not without errors. The aim of the study was to evaluate a polymerase chain reaction (PCR) based assay for fungal infections in the eye.

Methods: Polymerase chain reaction (PCR) for detection of fungal DNA was done with panfungal primers (ITS1 and ITS4) for diagnosis of fungal aetiology. Ocular samples (conjunctival swabs, corneal scrapings, corneal biopsies, vitreous aspirates and globe contents) from 30 patients with presumed fungal infection were evaluated using this assay, as well as by standard microbiological techniques, and the results were compared.

Results: Thirty clinical specimens were evaluated by PCR and by other conventional techniques (culture and staining). Of the 30 specimens analyzed, fungal keratitis was definitively diagnosed by culture in 18(60%). Total 17 (57%) of these 18 specimens were PCR positive. One specimen (3% of the 30 total) was fungal culture positive but PCR negative. Total 12 (40%) of 30 specimens were fungal culture negative, and 7(23%) of these 12 were also PCR negative. Five (17%) were PCR positive but fungal culture negative. Of the seven specimens negative by both PCR and fungal culture, all showed no growth. All 30 specimens were also examined by light microscopy with potassium hydroxide (KOH) and Gram's staining. KOH and/or Gram's staining was found to be positive in 8(27%) out of 30 cases. All positive smears revealed septate hyphae. 3 (38%) of these 8 specimens showed fungi on smear but were fungal culture negative, and two of these three specimens were PCR positive which were clinically to have fungal keratitis.

Conclusion: PCR not only proved to be an effective rapid method for the diagnosis of fungal infections in the eye, but was also more sensitive than staining and culture methods. Due to the promising results of the DNA extraction protocol and PCR assay in our laboratory, we were able to make early diagnosis. Hence, effective therapy was given, which had a major impact on the improvement in the prognosis of a subject with fungal infections.

Key-words: DNA extraction, Fungal keratitis, Lyticase, PCR assay, Polymerase chain reaction, Proteinase K

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INTRODUCTION

Fungal infections of the eye are on rise and are responsible for ocular morbidity and blindness.

Fungal infections of the cornea (mycotic or fungal keratitis, keratomycosis) are usually present as suppurative, usually ulcerative, lesions ^[1]. Fungal keratitis in particular occurs most frequently in individuals who work in agriculture ^[2,3]. This condition is also associated with diabetes mellitus ^[4] and the acquired immune deficiency syndrome (AIDS) ^[5]. The incidence of invasive fungal infections has increased dramatically in recent years. Early and specific diagnosis is vital, and the decision to treat a patient with invasive fungal infections is based mainly on clinical and mycological information. However, the traditional methods (culture and microscopy) used in routine practice for the diagnosis of

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invasive fungal infections may be insensitive and somewhat nonspecific [6]. This has focused attention on the rapid and accurate diagnosis of invasive fungal infections using molecular biological techniques. These methods have the potential to provide both high detection rates and identification of specific fungal pathogens, as the latter becomes increasingly important with the widespread use of antifungal therapy and the problem of antifungal resistance. The use of molecular diagnostic tools to detect fungal specific nucleic acid sequences has recently been reviewed [7-9], and many researchers have reported the usefulness of DNA-based methods for the diagnosis of invasive fungal infections. However, a major limitation of the molecular method in comparison to blood culture was the difficulty associated with problems in breaking fungal cell walls since the DNA extraction step is still a limiting factor, requiring more than half of a working day.

A novel pan ocular DNA extraction method was standardized without using commercial DNA extraction kit, which involved enzyme lyticase and proteinase K having greater activity to disrupt cell wall of moulds. Polymerase chain reaction (PCR) for detection of fungal 28S ribosomal DNA was done with panfungal primers (ITS1 and ITS4) was performed to detect the fungal genome in the ocular fluids of patients with fungal keratitis. Ocular samples (conjunctival swabs, corneal scraping, corneal biopsies, vitreous aspirate, foreign body and globe contents) from patients with presumed fungal infection were evaluated using this assay, as well as by standard microbiological techniques, and the results were compared. Hence, it was concluded that PCR not only proved to be an effective rapid method for the diagnosis of fungal infections in eye but was also more sensitive than staining and culture methods.

MATERIALS AND METHODS

Patient Selection and Sample Collection

The present study was conducted on the samples of suspected infectious keratitis patients attending the out (OPD) and indoor patient department (IPD) of ophthalmology at SRMS IMS, Bareilly, India from Nov 2015 to Mar 2016. The samples were processed at Central Research Laboratory, Department of Biochemistry at SRMS IMS, Bareilly. Ethical approval was not needed for the current study as all samples were received for the clinical diagnosis.

A common protocol for diagnosis was used in all cases included in the study. The conjunctival swab was taken on a sterile swab-stick passed across the lower fornix. Corneal scrapings were obtained from clinically suspected cases of fungal corneal ulcers by the corneal surgeon using a sterile surgical blade no. 15 mounted on a Bard-Parker handle under topical anaesthesia (0.5% proparacaine hydrochloride) and slit lamp magnification. Corneal biopsies were the excised corneal tissues from patients undergoing therapeutic, penetrating keratoplasty for fungal corneal ulcers. Vitreous aspirates from clinically and

sonologically proven cases of infective (presumably fungal) endophthalmitis following intraocular surgery or trauma were also included for the study. These were obtained from eyes having endophthalmitis before giving intravitreal injections and from eviscerated globe contents in case of panophthalmitis in the operation theatre. A total of 30 patients were included in the study.

Conventional Microbiological Investigations

Direct microscopic examination at x400 and x1000 magnification of the ocular samples was performed with 10% KOH wet mount and/or by Gram's staining for demonstration of fungal elements by a trained microbiologist. Another portion of the collected sample was inoculated directly on culture media such as blood agar (BA), chocolate agar (CA), Brain-heart infusion broth (BHIB) and Sabouraud's dextrose agar (SDA) without cycloheximide. This inoculation was done in a C pattern. A small portion of the blades used for scraping from the cornea, or the syringes in which vitreous aspirates were collected, was added to microfuge tubes to be processed for PCR analyses. BA, CA and BHIB were incubated for 1 week at 37°C and were examined daily and discarded after 7 days if no growth or turbidity was seen. The SDA was incubated at 25°C and 37°C for 4 weeks. Cultures were checked daily during the first week and twice a week for the subsequent 3 weeks. Any growth obtained was further identified by standard laboratory techniques.

The cultures were considered positive if at least one of the following criteria was fulfilled:

1. The growth of the same organism was demonstrated on one or more solid media and/or if there was confluent growth at the site of inoculation on at least one solid medium.
2. The growth on one medium was consistent with direct microscopic findings.
3. The same organism was grown from repeated corneal scrapings in suspected fungal keratitis.

DNA Extraction for PCR Optimization

DNA Extraction from Culture

To standardize the fungal DNA extraction for filamentous fungi, vitreous fluid that was collected from eyes given intravitreal injections of antibiotics but which showed no infective growth of bacterial or fungal culture, was artificially seeded with different concentrations of *A. flavus* (American Type Culture Collection, Rockville, Maryland, ATCC 90028) achieving 10^0 , 10^1 , 10^2 , 10^3 , 10^4 , 10^5 conidia/ml. DNA extraction from 300 µl volumes of spiked specimen (150 µl vitreous fluid and 150 µl conidial suspension in NET (0.15 M NaCl, 10 mM EDTA and 10 mM Tris pH 7.5) buffer was performed according to the method described by Skladny *et al.* [10] for BAL samples. However, in the first step the spiked sample was incubated for 90 min at 37°C with lyticase rather than 30 min. The following steps of DNA extraction were done without any alteration. DNA pellet was dissolved in 30 µl of Tris EDTA

(TE) buffer.

PCR Standardization

PCR was done with panfungal primers ITS1 (IDT, Belgium) (5'TCC GTA GGT GAA CCT GCG G'3), and ITS4 (IDT, Belgium) (5' TCC TCC GCT TAT TGA TAT GC 3') to amplify ITS1, 5.8 S and ITSII region of the rDNA. The 50 µl PCR mixture contained 10 µl of DNA template, 5 µl PCR buffer with 1.5 mM MgCl₂, 200 µM each deoxynucleoside triphosphate (100 µM Bangalore Genei, Bangalore), 25 pmol of each primer, and 1.5 U of Taq DNA polymerase (Himedia, Mumbai). Reaction involved 1 cycle at 95°C for 5 min, followed by 35 cycles with a denaturation step at 95°C for 30 sec, an annealing step at 55°C for 1 min, and an extension step at 72°C for 1 min, followed by 1 cycle of 72°C for 6 min (done in XP thermal cycler, Bioer, Japan). *Candida albicans* (ATCC 204303), *Aspergillus flavus* (ATCC 90028), *Fusarium solani* (clinical isolate), *Staphylococcus aureus* (ATCC 25923), *Escherchia coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 29212) and *Pseudomonas aeruginosa* (ATCC 27853) were included as control in the present study. Ten microliter of the PCR products, mixed with loading buffer was then electrophoresed 1.5% agarose gel with 0.5 µg/ml ethidium bromide. A 100 base pair (bp) DNA ladder (Himedia, Mumbai) was used for determining the size of the amplicons. The gels were visualized using ultraviolet illumination. Images were captured and stored by using a UVP Gel Documentation System (UVP Ltd, USA).

Extraction of DNA from Ocular Samples

Swabs from conjunctival sac, corneal scrapings, corneal biopsies and globe contents were added into a microfuge tube containing 300 µl of NET buffer. The same method of DNA extraction as follows for culture was performed without any alteration in the clinical samples. Ten microliter of DNA extracted was added to PCR mixture and the PCR reaction was performed following the standard protocol. Positive control (DNA of *A. flavus* ATCC 90028) and negative control (bacterial genomic DNA of *S. aureus* ATCC, *E. coli* ATCC, *E. faecalis*, *P. aeruginosa* and without any template DNA) were carried out simultaneously. Aliquots (10 µl) of the PCR product was analysed on a 1.5 % agarose gel as described above.

RESULTS

Standard Fungal Isolate

PCR Specificity- The primers used in this study (ITS1 and ITS4) successfully amplified DNA from all the standard fungal strains tested. A product of approximately 500-600 bp was obtained. No amplification products were detected by using ITS1 and ITS4 primer pair with *S. aureus*, *E. coli*, *E. faecalis*, and *P. aeruginosa*.

PCR Sensitivity- Using cell dilution, the PCR was positive with samples containing 10 conidia/ml of *A. flavus*

(Fig. 1). Detection of amplified product in these spiked samples indicated that no PCR inhibitors were present after DNA extraction.

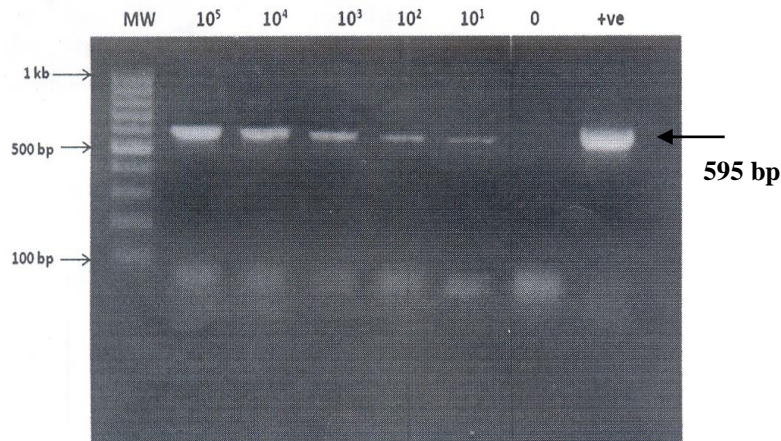


Fig. 1: Agarose gel electrophoresis of amplified fungal DNA extracted from spiked samples

Lane 1: 100bp DNA ladder

Lane 2-7: 10⁵ to 10⁰ conidia of *A. flavus*/ml

Lane 8: Positive control (*A. terreus* DNA)

Ocular samples

Thirty clinical specimens were evaluated by PCR and by other conventional techniques (culture and staining). The results are shown in Table 1. Of the 30 specimens analyzed, fungal keratitis was definitively diagnosed by culture in 18 (60%). 17 (57%) of these 18 specimens were PCR positive (Fig. 2). One specimen (3% of the 30 total) was fungal culture positive but PCR negative- an apparent "false negative" PCR result. Twelve (40%) of 30 specimens were fungal culture negative, and seven (23%) of these 12 were also PCR negative.

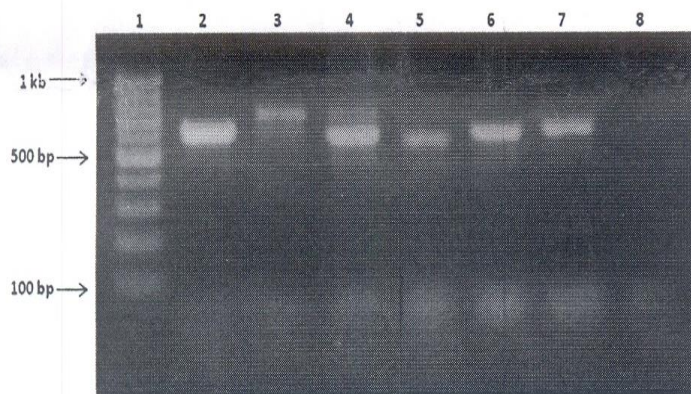


Fig. 2: Agarose gel electrophoresis of amplified fungal DNA extracted from Clinical samples

Lane 1: 100bp DNA ladder

Lane 2-6: Clinical samples

Lane 7: Positive control (*A. terreus* DNA)

Lane 8: Negative control

Five patients were PCR positive but fungal culture negative (Table 1); their clinical charts were reviewed. Based on the result of fungal staining and their response to antimicrobial treatment, 2 (7%) patients appeared clinically to have fungal keratitis despite negative fungal culture results. Total 2 (7%) patients were judged clinically to have bacterial infections, and one patient was lost to follow up with an uncertain clinical course. Among the 18 culture positive specimens (Table 1), six (20%) harboured *Fusarium* in culture, two (7%) had *Aspergillus*, and 10 (33%) culture isolates were not speciated. No specimen was found in culture to harbour yeast, and no specimen was positive with only *C. albicans* primers. Of the seven specimens negative by both PCR and fungal culture, all shown no growth are shown in Table 1.

Table 1: Results of culture and Polymerase Chain Reaction analysis of samples from patients with presumed infectious keratitis

N=30*	Culture positive for fungi	Culture negative for fungi
PCR positive for fungi	17 (57%) Culture results: Fusarium: 6 (20%) Aspergillus: 2 (7%) Unidentified: 10 (33%)±	5 (17%) Clinical findings: Fungal keratitis: 2 (7%) Bacterial keratitis: 2 (7%) Uncertain: 1 (3%)
PCR negative for fungi	1 (3%) Culture result: unidentified± Clinical finding: fungal keratitis	7 (23%) Culture results: No growth: 7 (23%)
Total	18 (60%)	12 (40%)

*Percentages of the total of 30 specimens are shown in parentheses ±These fungal culture isolates were examined microscopically and recorded as “hyaline” or “dematiaceous”. They were not speciated

All 30 specimens were examined by light microscopy with potassium hydroxide (KOH) and Gram’s staining. KOH and/or Gram’s staining was found to be positive in 8 (27%) of 30 cases. All positive smears revealed septate hyphae. Three (38%) of these 8 specimens showed fungi on smear but were fungal culture negative, and two of these three specimens were PCR positive which were clinically to have fungal keratitis.

DISCUSSION

In this report, we presented our experience in the detection of fungal pathogens in ocular samples to diagnose fungal keratitis. This study demonstrated that fungi can be detected in the infected corneas using PCR techniques. The advantage of PCR as shown is its rapidity. The PCR assay used in this study required 6 hours to generate results, significantly faster than the 2 days to 1 week required for any fungal culture technique in confirming the culture growth. On the other hand fungal smears also provide result

quite quickly, hence most clinicians and microbiologists resort to direct microscopic examination of the Gram’s smear and KOH wet mount for rapid diagnosis. But its effectiveness is variable and results are not definite. Another advantage of PCR based assay is its high sensitivity over conventional methods, ability to be performed on scanty samples and/or dead microorganisms present in the ocular samples.

A key parameter influencing the usefulness of a PCR assay in a clinical setting is a good DNA extraction method for fungi is essential before the amplification of DNA. Many earlier protocols used the enzyme Zymolyase (Novozyme 234, ICN Pharmaceuticals, Costa Mesa, CA, USA; or Mureinase, United States Biochemicals Corp. Cleveland, OH, USA) to disrupt the fungal cell wall. This results in the formation of fungal spheroplasts which have increased osmotic sensitivity [11]. Zymolyase efficiently release DNA from yeasts such as *Candida* sp. and *Cryptococcus* sp. [12], but it is ineffective in disrupting the cell walls of moulds, including *Aspergillus* spp. [13]. To overcome this obstacle, mechanical disruption with heat alkali treatment [14] or with a combination of glass beads and repeated freeze thawing using liquid nitrogen [15] have been used. However, these methods have some drawbacks like shearing of DNA, low sensitivity of the protocol due to inefficient fungal cell wall disruption and inefficient DNA release etc. However, another β-1, 3-glucanase, lyticase (Sigma) has been shown to effectively generate spheroplasts in moulds [16]. Lyticase has greater activity against the cell wall of moulds [17].

With the above parameters in mind, we required a DNA extraction protocol which should be sensitive enough to detect fungi in the scanty amount of samples from the eye and should cause minimal loss of shearing of DNA. Hence the enzymatic method (combination of lyticase and proteinase K) for DNA extraction was selected [10]. This protocol was evaluated in Bronchoalveolar lavage (BAL) and blood samples [10] and again in blood and tissues [18]. In our study, we have not only evaluated the efficacy of the DNA extraction protocol in spiked samples but also tried its applicability in various ocular samples without using commercial DNA extraction kit.

The patients included were clinically diagnosed as fungal ocular infections, 29 patients having corneal ulcers and 6 with endophthalmitis or panophthalmitis. Patients with corneal ulcer were clinically diagnosed to be having fungal infection on the basis of suggestive history like that of trauma with vegetative matter, long duration of symptoms, history of previous treatment, etc, and because of morphological features like marked ciliary congestion, presence of slough over the ulcer surface, dry appearance, feathery margins, satellite lesions and hypopyon. Of the other patients, 4 presented with delayed postoperative endophthalmitis, 1 with presumed endogenous endophthalmitis and 1 with post-traumatic panophthalmitis. Those with endophthalmitis were known diabetic patients with poor or borderline control and presented with endophthalmitis a

few weeks to months after intraocular surgery. One patient with endogenous endophthalmitis was suffering from septicaemia post septic abortion. One young male required evisceration due to panophthalmitis following penetrating ocular trauma that showed no intraocular foreign body on radiological screening.

In this study, the sensitivity and specificity of PCR were found to be 94% and 58%, respectively double to that of standard culture methods for detecting fungi in ocular samples.

CONCLUSIONS

We concluded in this study that ocular fungal infections are increasing and gaining attention in clinical practice. Therefore, newer research focused on improvement in diagnostic techniques with cost efficiency would be immensely helpful to the ophthalmologist in decreasing the morbidity associated with ocular mycosis. Hence, this method will be useful for fungal infections irrespective of ocular samples in developing countries like India where the diagnostic test should be cost and time effective. However, there is a need to evaluate this protocol in a larger number of patients with fungal infection in the eye.

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