Polymerase Chain Reaction–Guided Diagnosis of Mycotic Keratitis: A Prospective Evaluation of Its Efficacy and Limitations

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Purpose. To assess the utility of polymerase chain reaction (PCR) in diagnosing fungal keratitis and compare its sensitivity and specificity with those of the conventional microbiologic techniques used in the authors' laboratory.

Methods. A prospective nonrandomized investigation was undertaken at a tertiary-care ophthalmic facility to evaluate 40 eyes of 40 patients with presumed fungal keratitis, both fresh and treated. Besides routine bacterial culture and sensitivity, corneal scrapings were evaluated by fungal culture, potassium hydroxide (KOH) wet mount, Gram's stain, and PCR. The conventional PCR technique was followed with minor modifications to suit the setup, using primers targeted to *28S* rRNA sequence, which is common to all fungi that cause corneal infections in tropical climes.

Results. Of the 40 presumed cases of mycotic keratitis (30 untreated), PCR showed positivity in 50%, culture in 25%, hyphae in KOH in 40%, and Gram's staining in 35%. The sensitivities of PCR, KOH, and Gram's were 70%, 60%, and 40% and specificities 56.7%, 66.7%, and 66.7%, respectively. Among 10 of 40 eyes pretreated with antifungal agents, PCR was positive in 50%, but culture in 30%. The time taken for PCR assay was 4 to 8 hours, whereas positive fungal cultures took at least 5 to 7 days.

Conclusions. PCR not only proved an effective rapid method for the diagnosis of fungal keratitis, but was also more sensitive in our hands than KOH wetmount and Gram's smear. Barring the potential limitations, PCR remains a promising tool for faster diagnoses of fungal keratitis. (*Invest Ophthalmol Vis Sci.* 2009;50:152–156) DOI:10.1167/iovs.07-1283

Infective corneal ulcer is still a major cause of ocular morbidity in third-world countries like Ghana, India, Paraguay, and Nepal.[1–3] An estimated 840,000 people a year develop corneal ulcer in India, which is 30 times the number of corneal ulcers recorded in the United States.[4–6] The pernicious effect of this disabling disease and its sequelae, especially on the underprivileged class in their most productive years is alarming.

The fungal corneal ulcers are known to be more insidious, resistant to treatment, and hence more damaging with greater morbidity which demands early diagnosis and treatment. Among the various diagnostic techniques available for mycotic keratitis, culture has been considered to be the gold standard. Other commonly used techniques include direct microscopic examination methods such as KOH wet mount, Gram's stain, acridine orange, and Giemsa staining. Obtaining fungal growth is well known to be a difficult and time-consuming task, even with most efficient laboratory facilities available.

The utility of polymerase chain reaction (PCR) has been evaluated for the detection of many such difficult-to-grow organisms like *Mycobacteria*, *Microsporidia*, and *Acanthamoeba*.[7] But the role of this molecular technique in corneal infection may be still in its infancy. Reports are available in the literature that document the evidence that PCR is used to detect specific strains of fungi in keratitis and endophthalmitis.[8–10] PCR is known for its high false positivity and also for the cost factors involved in it. Hence, our goal in this study was to compare the routinely used microbiologic techniques with PCR and to study the sensitivity and specificity of each method in diagnosing mycotic keratitis.

Methods

Forty patients with presumed fungal corneal ulcers presenting to the outpatient department and specialty service of our tertiary eye care facility were included in this prospective study. The research method adhered to the tenets of the Declaration of Helsinki. The clinical presumption of fungal etiology was made both from the history and the morphology of the ulcers. The cases included patients with nonresponding ulcers of long-standing duration, a history of trauma with organic matter, use of steroid drops without proper notification of possible side effects, and a compromised immune system. Morphologically suggestive features included ulcers with dry surface, feathery and thickened margins, satellite lesions, deep infiltrates, pigmented ulcers, stromal abscess, endothelial plaques, and fixed hypopyon (Fig. 1).

All the patients were examined with a standard written protocol that included detailed history with regard to duration of symptoms, the precipitating factors, exact nature of the trauma, immediate treatment taken, delay in reporting to a medical practitioner and use of homemade medicines, diagnosis of dry eye, contact lens wear, and previous surgeries (intra- and extraocular). Ocular status before the onset of the ulcer and the systemic condition of the patient were noted with regard to immune status, with special attention to diabetes mellitus and chronic usage of systemic steroids.

Clinical Examination

Thorough examination of the involved and fellow eyes was performed as per the standard protocol decided on and approved by the institutional review board at the beginning of the study. The external ocular examination included lid and adnexal abnormalities such as entropion, trichiasis, skin lesions for vesicle or healed scars to rule out a herpetic cause, dry eye, and conditions predisposing to exposure-induced keratitis. Careful examination of the involved eye was performed on slit lamp biomicroscope. Pictorial documentation of the ulcer size, site, depth, extent of infiltration, abscess formation, perforation if any,

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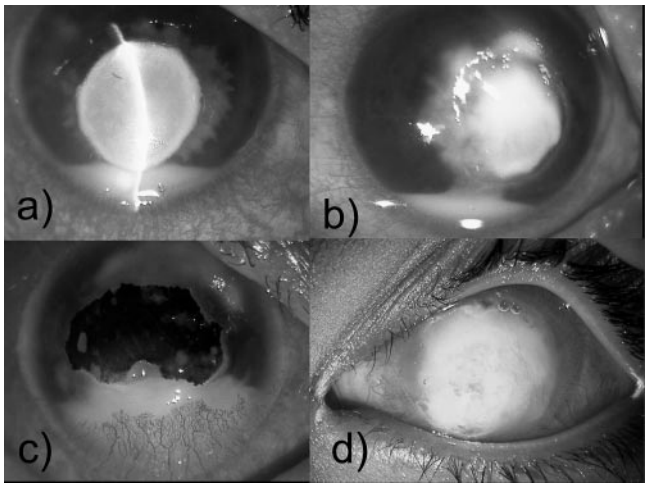

FIGURE 1. (**a**–**d**) Clinical slit-lamp biomicroscopic photographs of presumed cases of fungal corneal ulcers already being treated by antifungal therapy instituted elsewhere. (**a**) Dry surface; (**b**) feathery margins; (**c**) pigmented ulcer; (**d**) stromal abcess.

fluorescence (2%) staining, hypopyon if any, and scleral involvement were made. Visual acuity and digital intraocular tension were recorded. Ultrasonographic examination of the posterior segment was performed to rule out suspected endophthalmitis. Clinical photographs were recorded in each case and saved for documentation and further follow-up studies.

Diagnostic Studies

Corneal scrapings were collected in all cases, and the samples were subjected to KOH wet mount, Gram's stain, PCR, and bacterial culture/sensitivity, and fungal culture.

Smear Examination. After the procedure was properly explained and informed consent was obtained from the patients, the eye to be scraped was anesthetized with 0.5% proparacaine eye drops. All sterile surgical precautions were taken to avoid contamination during sample collection. A Barraquer wire speculum was applied, and the superficial debris and mucus strands were cleaned before the ulcer was scraped from the base and the leading edge with a blunt-tipped sterile iris repositor, with care being taken not to perforate the cornea. Potassium hydroxide (KOH) wet mount and Gram's staining of each smear was made and studied at our Department of Ocular Microbiology. The wet KOH mount was examined immediately under microscope for the presence of any hyphae. The Gram's-stained slide was also examined microscopically for bacteria and fungal hyphae.

Culture. The collected sample was transferred to a cotton-tipped applicator from the tip of the repositor and dipped into the bacterial culture tube and the Sabouraud's dextrose agar fungal culture media.

The fungal culture tubes were incubated at 25°C and examined every 48 hours for any growth. In case of growth, a lactophenol cotton blue (LCB) wet mount was prepared to study the detailed microscopic morphology of the isolate. If unidentified by LCB, then the slide culture technique was used.

Polymerase Chain Reaction. *Sample Collection and DNA Extraction.* The corneal scraping for PCR was collected in lysis buffer (50 mM Tris-Cl [pH 7.2], 50 mM EDTA, 3% SDS, and 1% β-mercaptoethanol in 400 μL). The specimens were kept at −20°C until further processing. The DNA extraction followed the protocol of Lee and Taylor[11] with minor modifications to suit our laboratory experimental conditions. In brief, the tubes were vortexed at moderate speed for 15 seconds, incubated at 65°C for 1 hour, followed by boiling for 10 minutes. Equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was then added followed by revortexing. The tubes were then centrifuged at 14,000 rpm for 15 minutes. To the aqueous phase, a 1:10 volume of 3 M sodium acetate (pH 5.2) and 0.6 volume of isopropanol were added. The tubes were then inverted and mixed gently, and DNA was precipitated by keeping the tubes at −20°C for 1 hour. The solution was then again centrifuged at 14,000 rpm for 15 minutes. The supernatant was discarded, and the pellet containing the DNA was washed twice with 70% alcohol. The pellet was dried (Speedvac) and resuspended in 30 μL of autoclaved double-distilled water.

PCR Assay. Primers [Forward primer sequence, 5′-GTG AAA TTG TTG AAA GGG AA-3′; and reverse primer sequence, 5′-GAC TCC TTG GTC CGT GTT-3] used in our study were specific for the *28S* rRNA gene, common to all medically important fungi.[12]

Primers were custom synthesized at Bangalore Genei Pvt., Ltd. (Bangalore, India). Amplification was performed in a 25-μL reaction mixture containing 10× PCR buffer without $MgCl_2$, 10 picomoles of primers, 1.5 U of *Taq* polymerase, 2 mM $MgCl_2$, 200 mM dNTPs, and 5 μL of template DNA. The amplification was performed in a thermal cycler for a total of 50 cycles (GeneAmp PCR System 9700; Applied Biosystems [ABI], Foster City, CA). After an initial denaturation at 95°C for 5 minutes, each cycle consisted of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute, and extension at 72°C for 5 minutes. The amplified product was then electrophoresed in 1% agarose gel with 0.5 μg/mL ethidium bromide and was visualized in an ultraviolet transilluminator.

Specificity of the primers was tested using DNA of various strains available in our microbiology laboratory. All the strains taken were laboratory isolates like *Escherichia coli, Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Alkaligenes*, *Proteus*, *Acinetobacter*, *Enterobacter*, *Klebsiella pneumoniae*, and *Pneumococcus*. The various fungal strains tested included *Aspergillus niger*,

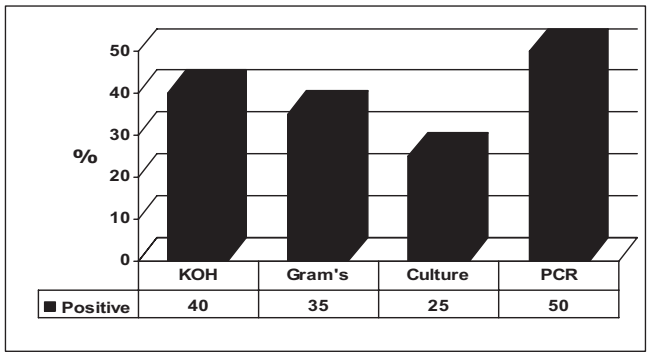

FIGURE 2. Percentage positivity of individual techniques in clinically presumed cases of fungal keratitis.

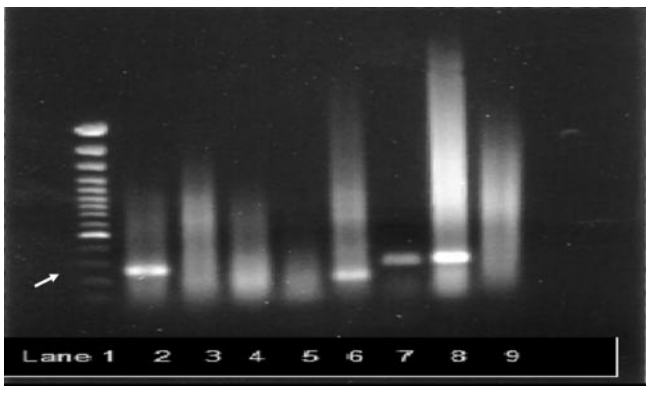

FIGURE 3. Agarose gel visualized in an ultraviolet transilluminator with molecular weight markers and amplified DNA fragments. *Lane 1*: molecular weight marker. *Lane 8*: positive control. *Lane 9*: negative control. *Lanes 3*, *4*, *5*: negative samples. *Arrow*: molecular weight marker corresponding to 260 bp, indicative of positive PCR results as seen in *lanes 2*, *6*, and 7.

Table 1. Fungal Species Isolated in PCR-Positive and -Negative Cases

Cases	*A. flavus*	*A. fumigatus*	*Fusarium*	*Curvularia*	Negative Culture	Total
PCR positive	3	—	4	—	13	20
PCR negative	1	1	—	1	17	20
Total	4	1	4	1	30	40

Data are the number of cases.

Aspergillus fumigatus, *Penicillium* spp., and *Candida albicans*. Sensitivity of the primers was estimated by testing a 10-fold dilution of *Aspergillus flavus* (0.2 ng of DNA).

Results

Culture Positivity

Of the 40 cases of presumed fungal corneal ulcers studied, 10 were culture positive (25%), among which one was from an untreated case. Of these 10 culture positive cases, *Aspergillus* spp. were the predominant isolates (50%) followed by *Fusarium* spp. (40%). One eye showed growth of *Curvularia*. Among the *Aspergillus* spp. *A. flavus* was more commonly isolated than *A. fumigatus* (4:1).

Smear Positivity: KOH and Gram's Staining

Potassium hydroxide (KOH) wet mount was found to be positive for fungal hyphae in 16 (40%) of 40 cases. Gram's smear showed hyphae in 14 (35%) of 40 cases (Fig. 2). Of the 10 culture positive cases, KOH picked up 6 (60%), whereas Gram's smear picked up only 4 (40%).

PCR Positivity

PCR results of representative positive and negative samples, along with molecular weight standards are shown in Figure 3. As depicted in Table 1, overall PCR was positive in 20 of 40 cases (50%). Of the 10 culture-positive cases, PCR was positive in 7 (70%). It is noteworthy that PCR showed positivity in 13 of 30 culture-negative samples, 7 of which were from those who had received antifungal therapy (Table 2). However, 12 (75%) of 16 KOH-positive cases and 10 (71.4%) of 14 Gram-positive cases were detected by our PCR technique alone (Tables 3, 4).

Sensitivity and Specificity

As depicted in Table 5 and Figure 4, the sensitivity of KOH smear was 60% and the specificity was 66.7%. The positive predictive value of KOH smear was 37.5%, whereas the negative predictive value was 83.3%.

The sensitivity and specificity of the Gram's smear was 40% and 66.7%, respectively. The positive predictive value of Gram's smear was 28.6%, and the negative predictive value 76.9%. The sensitivity of PCR was found to be 70% and the specificity 56.7%. The predictive value of the positive test was 35% and that of the negative test was 85%.

Pretreated Versus Untreated Cases

As denoted in Table 2, PCR was found positive in 5 of 10 patients who were pretreated with antifungal agents, whereas culture was positive only in 3.

Discussion

Diagnosis of fungal keratitis begins with clinical suspicion, and either culture or corneal biopsy confirms it. Although many characteristic morphologic features have been attached to fungal ulcer, none is pathognomonic. Up to 83% sensitivity has been advocated by Thomas et al.[13] in diagnosing fungal keratitis, by taking into consideration its main clinical features.

Clinicians often consider fungal keratitis only after a presumed bacterial keratitis worsens or if it remains refractive to antibiotic therapy, unless the history gives clear-cut evidence of trauma with organic matter. As in most other infections, the culture is considered the gold standard for diagnosing fungal keratitis.

However, the standard techniques for culture in fungal infections are complicated by many factors. Fungi, like a few other organisms, are often slow growing. The culture media should be freshly prepared and the chances of growth of contaminants are also high. Another major limitation is the time factor involved in confirming the culture growth.

Our study revealed 25% culture positivity, *Aspergillus* being the most common isolate. The culture positivity is known to vary widely from place to place and even within the same zone—for example, 8.3% in the north[14,15] and 46.3% in the south of the same country.[5,16] Our study showed 25%, but our sample size was small compared with some others. The relatively lower fungal isolation in our study may also be because one fourth of our patients had been previously treated.

Considering the shortcomings of culture, most clinicians and microbiologists thus resort to direct microscopic examination of the Gram's smear and KOH wet mount prepared from the corneal scraping for a rapid diagnosis. According to Sharma et al.,[17] KOH wet mounts carry a fairly high sensitivity and therefore is the fastest and most efficient screening test available. They reported a sensitivity of 61% and a specificity of 91% for the KOH wet mount. However, Chowdhary and Singh[14]

Table 2. Culture and PCR Positivity among the Treated and Untreated Groups of Cases

Cases	Culture Positive	Culture Negative	Total
Treated*	3†	7‡	10
Untreated	7§	23‖	30
Total	10	30	40

Data are the number of cases.
* Treated with antifungals.
† All 3 were PCR positive.
‡ 2 of 7 were PCR positive.
§ 4 of 7 were PCR positive.
‖ 11 of 23 were PCR positive.

Table 3. Comparison of PCR Results with That of Gram's Staining in Presumed Cases of Fungal Keratitis

Cases	Gram's Smear Positive for Hyphae	Gram's Smear Negative for Hyphae	Total
PCR positive	10	10	20
PCR negative	4	16	20
Total	14	26	40

Data are the number of cases.

TABLE 4. Comparison of PCR Results with That of KOH Wet Mount in Presumed Cases of Fungal Keratitis

Cases	KOH Positive	KOH Negative	Total
PCR positive	12	8	20
PCR negative	4	16	20
Total	16	24	40

Data are the number of cases.

reported KOH sensitivity of 62%, but a Gram's smear sensitivity of 60%, both showing specificity of approximately 97%. Panda et al.[18] and Vajpayee et al.,[19] in two separate studies from the same area, showed KOH smear sensitivities of 90% to 94%. However, a much lower sensitivity of 33% was reported from developed countries.[20] In our study, KOH wet mount was found to be positive for fungal hyphae in 40% of cases. Gram's smear showed hyphae in 35%. The seemingly lower sensitivity of KOH wet mount and Gram's smear in our study may be partly attributable to a small sample size and pretreatment in one fourth of the cases in contrast with other reported studies.

Contrary to these findings, inadequacy of sample material rarely has any effect on PCR results. Rather, PCR has reportedly been found to be of paramount value to the ophthalmologist, not only for the diagnosis of fungal keratitis but also delayed onset endophthalmitis, posterior uveitis, viral retinitis, and acanthamoeba keratitis, among others.[7] PCR results in our study seemed quite promising. However, the disparity between culture and PCR results in our study may be explained by the fact that the culture positivity requires viable organisms, whereas a PCR-based test can detect both viable and nonviable organisms. PCR test can theoretically be positive even if only a single copy of target DNA is present. The high positivity of PCR in already treated cases (5/10, Table 2) in comparison to culture, reiterates the difficulty in getting a positive culture from nonviable organisms in the sample.

Overall, our PCR assay had a sensitivity of 70% and a specificity of 56.7%. In contrast, Alexandrakis et al.[8] reported a sensitivity of 89% and specificity of 88% for their PCR technique used in an experimental model of *Fusarium* keratitis.

Gaudio et al.[21] while comparing PCR and culture in 30 presumed cases of fresh and untreated fungal corneal ulcers reported a positivity of 50%, a finding very much similar to ours. However 15 (94%) of 16 culture-positive cases in their study were PCR positive. The reason for the high sensitivity of PCR shown in their study may be twofold. First, the selected subjects in their study were all fresh and untreated, in contrast to ours wherein almost one fourth of the cases were pretreated. Second, they used a nested PCR assay that should have shown a higher yield of copy numbers. However, nested PCR assays have their own inherent drawbacks.

In yet another study, Ferrer et al.[9] highlighted the benefit of time factor in diagnosing fungal corneal ulcer. Although their PCR assay produced results in 8 hours, culture confirmation took almost 10 days.[9] Our study was thus very much comparable to theirs because the PCR method used by us yielded results in 4 to 8 hours, depending on the number of cycles

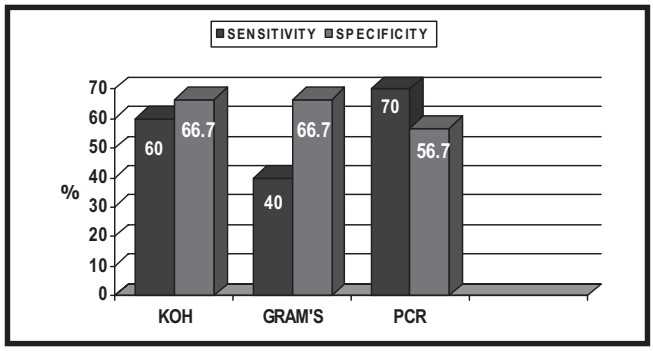

FIGURE 4. Comparison of sensitivity and specificity of three diagnostic techniques against the gold standard culture technique.

repeated. This is a major advantage of the technique, especially when compared to culture where it took at least 5 to 7 days for a positive growth in our setup. A very recent report[22] evaluated PCR-based ribosomal DNA sequencing technique for the diagnosis of mycotic keratitis. They adopted PCR and ITS2/5.8S rDNA sequencing to evaluate fungal infections. According to them, clinically significant pathogenic fungal strains in mycotic keratitis can be detected and differentiated rapidly, instituting thereby an early therapeutic intervention. Bagyalakshmi et al.[23] evaluated another ITS-targeted, nested PCR and found this technique to be quite rapid in detecting panfungal genome from ocular specimens.

Although various advantages have been attributed to PCR due to its rapidity and widespread applicability to bacteria, fungi, and viruses, the technique has various reported complexities and drawbacks, as evidenced from our study also. Some of the limitations are logistic and some technical. Among them is the difficulty in optimization, especially in case of fungi, apart from the difficulty in differentiating between active and latent infections, viable, and nonviable cells, and the inability to quantify the organismal load (as in qualitative PCR used by us). Moreover, the DNA sequence has to be known in advance, and the high sensitivity could lead to false-positive results. A high chance of false positivity can also be caused by laboratory contamination from reagents, intrasample contamination, and processing of positive control specimens.

In addition, high false negatives are also the areas of concern that need to be tackled to refine this procedure. Most important is the elimination of false negatives that could occur through several mechanisms. Specimens that contain EDTA can chelate divalent cations such as Mg^{2+} that are necessary for PCR. The presence of RNase or DNase (endonucleases) can also disrupt PCRs by degrading nucleic acid targets and/or primers. However, direct inhibition of the DNA polymerase is the best-known mechanism of PCR inhibition. Recent reports have shown that heme, heparin, phenol, polyamines, plant polysaccharides, and calcium alginate could all inhibit PCR in this manner.[24] Besides, the other important factors that need attention while performing a PCR include poor sample acquisition and preparation, loss of DNA during purification, and failure to release DNA from the sample due to technical errors.

TABLE 5. Statistical Analysis and Comparison of the Three Diagnostic Laboratory Techniques

	KOH (%)	GRAM (%)	PCR (%)
Sensitivity	60.0 (27.4–86.3)	40.0 (13.7–72.6)	70.0 (35.4–91.9)
Specificity	66.7 (47.1–82.1)	66.7 (47.1–82.1)	56.7 (37.7–74.0)
Positive predictive value	37.5 (16.3–64.1)	28.6 (9.6–58.0)	35 (16.3–59.1)
Negative predictive value	83.3 (61.8–94.5)	76.9 (55.9–90.2)	85.0 (61.1–96.0)

Data in parentheses indicate the 95% confidence interval.

In a well-developed modern laboratory, the gold standard of a bacterial culture should ideally be replaceable today with a reliable and reproducible PCR technique as the new gold standard. This is not to say that PCR negates the undeniable role of a bacterial culture—after all, the conventional as well as the rapid sensitivity testing, so essential for diagnosis and initiation of correct therapy (even after some time lag), are entirely dependent on culture, not on PCR. However, it is unfortunately well accepted that, even today fungal sensitivity testing has not been shown to be useful clinically. Therefore, diagnosis of fungal infections by PCR could effectively replace the fungal culture up to a point, as it is much faster in giving results compared with cultures, probably even more so when some specific therapy has already been instituted and, as is well known clinically, even when corneal scraping is not deep enough.

PCR remains a technically complex procedure involving skilled hands, expertise, and a fair degree of experience, in addition to the learning curve and standardization in relation to individual laboratories. The cost–benefit ratio of PCR should prove efficacious in the developed world. A cost effectivity factor would be arrived at differently by different personnel even in the same institution, but even that one patient saved would surely calculate it differently! What the future holds in store as the next viable gold standard remains to be seen.

Apart from these, the unavoidable cost of the investigation at least as of today limits its widespread use. An adequate molecular biology setup with the entire necessary infrastructure is essential, consuming maybe a disproportionate amount of space.

The cost incurred per patient (in our study) was calculated to be an equivalent of $12.5 USD and this remained a limitation in our widespread utility of the technique as a screening test.

Thus, our as well as other studies showed that the PCR is more sensitive than the currently used conventional techniques. This concurrence makes PCR a promising tool in early diagnosis of fungal keratitis, but the expertise required and the cost factor may render it inferior to the smear techniques. Another area that needs refinement is in curtailing the false positives and negatives. The current role of molecular diagnosis may be restricted to the resistant cases and the treated ones with reports negative with all other possible conventional techniques, but this powerful experimental (and research) tool could be made better available for clinical screening purposes with further refinements in the technique and its easier availability. PCR may be added to the diagnostic armamentarium for mycotic keratitis.

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