Original article

Utility of polymerase chain reaction for rapid diagnosis of paediatric fungal keratitis: A comparative study with conventional mycological work up

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A R T I C L E I N F O

A B S T R A C T

Background: Fungal keratitis (FK) is a serious infection with bad complications in paediatric population, thus, rapid diagnosis is necessary. Early diagnosis is a beneficial tool necessary for better management and prognosis of FK and also for prevention of subsequent complications such as endophthalmitis, amblyopia ,and loss of vision . In recent years, polymerase chain reaction (PCR) became the predominant diagnostic method for mycotic keratitis, being frequently employed for complementing microbiological approaches. The aim of this study to determine the risk factors of paediatric fungal keratitis and evaluate the utility of PCR in diagnosis by comparing its sensitivity and specificity with conventional fungal culture (the gold standard). **Methods:** Corneal scrapings obtained from clinical suspected patients with FK and subjected to direct microscopic examination with 10% KOH, Gram smear, fungal culture and conventional PCR using common probe to all fungi. **Results:** The most prominent predisposing factor for FK was ocular trauma (n=40, 57%) followed by vernal keratoconjunctivitis (n=14, 20%). Out of the 70 studied cases featuring presumed fungal keratitis, 35 were culture positive (50%), with Fusarium *spp*. (48.5%) and Aspergillus *spp.* (20%) were the predominant isolates. By comparing fungal culture (the gold standard) versus 10 % KOH examination, Gram smear, and PCR were 57.1%, 28.6%, and 97.1%, respectively. **Conclusion:** Ocular trauma and vernal keratoconjunctivitis are the most common risk factors for pediatrics FK with *Fusarium spp.* and *Aspergillus spp.* are the most common isolated fungi. Polymerase chain reaction is a sensitive technique for diagnosing paediatric patients with FK.

Introduction

Fungal keratitis (FK) is a dangerous type of keratitis with the worst visual prognosis, as it may result in blindness [1]. It accounts for over 40% of microbial keratitis cases found in numerous tropical as well as subtropical countries [2]. In paediatric patients (aged ≤ 16 years) it represents a significant proportion with incidence rates in United States as of 18%, China as of 48.7%, as well as South India as of 54.2%, respectively [3-5].

The risk factors of FK include trauma , a tropical climate, the rainy season, being an agriculture worker, and a rural area [3], whereas the other risk factors are related to urbanization, including contact lens wear, ocular surface disease, and immunocompromised status, such as diabetic

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mellitus and corticosteroid exposure. Trauma is the most prevalent predisposing factor, occurring in 40–60% of patients. Traumatizing agents either directly implant fungal conidia in the corneal stroma or abrade the epithelium, permitting fungal invasion [6,7].

Despite tropical climate is a major risk of FK; increased occurrences have been noticed in temperate climate regions [8]. In Egypt a statistically significant increase in the relative frequency of mycotic keratitis during the years 1997 to 2007 was noted; this rise was found to correlate significantly with rises in minimum temperature and the maximum atmospheric humidity in the greater Cairo area over the same period [9].

Two categories of FK are present, keratitis because of filamentous fungi and the other type is caused by yeast-like fungi [10]. *Fusarium*, *Curvularia*, *Aspergillus* and other *Paecilomyces* as well as *Scedosporium apiospermum* are the most common agents regarding filamentous fungi causing keratitis, however numerous other species showed implication [11].

Being an insidious infection, difficult in approach and resistant to treatment, thus more destructive to eyes, the fungal corneal ulcers requires early and accurate diagnosis in addition to treatment. Numerous diagnostic measures are existing, whereas culture has been regarded as the diagnosis gold standard. Other diagnostic tools are widely utilized in diagnosis as direct microscopic examination with KOH wet mount, Gram's stain, acridine orange, as well as Giemsa staining [10].

It is a challenging task to obtain fungal growth in rapid and accurate manner due to delay in fungal growth and more commonly due to contamination of the culture with other fungi that misleads the diagnosis even with most efficient laboratory facilities [10]. So PCR is a typical diagnostic technique towards mycotic keratitis as sample (corneal scrape or corneal biopsy material) with only a small quantity is sufficient to conduct the test, requiring short time to obtain the result; PCR assay takes 4–8 h when compared with ordinary fungal cultures that give optimum result in 2 to 7 days [5].

This study was conducted to determine the risk factors of paediatric FK and evaluate the utility of PCR in diagnosis by comparing its sensitivity and specificity with conventional fungal culture (the gold standard).

Materials and Methods

The current prospective cross-sectional study was performed in Outpatient Clinics of Ophthalmology Department and Microbiology department, Zagazig University Hospitals, Egypt, between February 2018 and January 2020 and got approval from the Research and Ethic Committee of Faculty of Medicine, Zagazig University.

Sample collection

Corneal scrapings were collected from 70 patients with presumptive diagnosis of FK (based on patients history and as well as ulcers' morphology) .After full history recording and ocular examination, corneal scrapings were collected by expert pathologist through scraping base as well as edges of ulcer using a tip of a sterile disposable 23-gauge needle, following topical anaesthetic medication instillation (0.5% tetracaine). Patients presumptively clinically diagnosed to have FK and able to give consent were included in this study. Exclusion criteria included uncooperative patients, or failure to obtain informed consent, in addition to improper or contaminated specimens, bilateral corneal ulcers, corneal ulcers originated from viral or parasitic source (as proposed by history as well as examination results), patients with endophthalmitis were also excluded.

Smear examination

For all samples, 10 % KOH wet mount in addition to Gram stained film were made and subjected to microscopic examination for screening of fungal elements (hyphae, pseudohyphea ,and yeast cells). Two to three drops of the KOH were kept on a grease-free, clean glass slide. The sample was placed in the KOH drops on the slide, and a clean cover slip was placed on the sample and pressed to prevent the formation of air bubbles. The sample was kept in KOH then examined microscopically to identify the existence of fungi according to the microscopic features of fungi The Gram stained smears were examined by light microscope at x400 and x1000 magnification.while the KOH were examined at ×200 and ×400 magnification [12].

Fungal culture and identification of isolate

The collected part of corneal scrapings for culture were inoculated on Sabouraud dextrose agar (SDA) with chloramphenicol (50 μg/mL) without cycloheximide, Blood agar (BA), and chocolate agar (CA). The samples were inoculated directly onto culture media by making a row of 'C' marks. Sabouraud dextrose agar plates were incubated aerobically at 27°C to enhance the growth of fungi,

BA and CA were incubated at 37°C aerobically. While, CA plates were incubated with 5% carbon dioxide. All Culture plates are checked every day during the 1st week and twice a week for the next 3 weeks. The growth occurring on the C streaks only is considered to be significant. Despite fungal growth usually occur within 1-week, negative culture media may imply incubation for up to 4 weeks. The Plates for bacterial culture were observed for 7 days before being considered as negative [13].

Positive cultures for filamentous fungi were identified by examining macroscopic and microscopic morphological characteristics of their colony. Texture, topography, rate of growth and pigmentation of the front and the reverse side of the culture were used for macroscopic identification. Microscopic identification of filamentous isolates was performed by placing pieces of a colony from SDA to clean grease free microscopic slide and staining with lactophenol cotton blue (LPCB). After placing a cover slip, the characteristics of conidia and mycelia of each isolate were studied microscopically. If unidentified by LPCB, then the slide culture technique was used [12]. For suspected positive yeast growth (smooth, and pasty colonies) were further identified by conventional methods (microscopic features by Gram stain, germ-tube test) [14].

DNA extraction and PCR assay

With a sterile cotton swab, corneal scrape was obtained from base and corneal ulcer leading edge. Subsequently, the acquired swab was positioned in a sterile micro-centrifuge tube, followed by recapping. Then, direct stirring of each specimen into sterile saline (200 μl) was done.

Extraction of fungal DNA was accomplished from scrapings by means of QIAamp DNA mini kit (QIAGEN GmbH, Hilden, Germany). An adapted protocol to extract DNA from fungal cells was followed. Briefly, pre-incubation of samples independently was realized in cellular lysis buffer (99 °C, 20 min) followed by processing guided by manufacturer instructions. An aliquot of each sample with a volume of 50 μl was stored at -20 °C.

The primers used in our study were forward; U1 [5′-GTG AAA TTG TTG AAA GGG AA-3′] and reverse; U2 [5′-GAC TCC TTG GTC CGT GTT-3′], which is specific for 28S rDNA which is universal for all medically significant fungi [15]. Primers used were supplied by (ThermoFisher Scientific, USA).

PCR amplifications were conducted by means of reaction master-mix beads (20 μl) (QIAGEN GmbH,

Hilden, Germany) utilizing a thermocycler (Bimetra®, Germany). Cycling conditions were set as follows: initial denaturation (95 °C, 10 min), then 49 denaturation cycles (95 °C, 1 min), annealing (50 °C, 1 min), followed by extension (72 °C, 2 min) and a final extension phase (72 °C, 10 min). Each PCR run comprised a positive control having purified *Aspergillus fumigatus* DNA in addition to two negative controls having blank reagents [16].

Separation of amplification products was conducted by means of electrophoresis using agarose gel (1%), followed by staining by means of ethidium bromide, in addition to analysis utilizing a gel electrophoresis. Interpretation of PCR products (260 bp length) was described as positive result, indicating the existence of fungal element [16].

Statistical analysis

Data were collected, tabulated, and analysed using SPSS version 16.0. The corneal smear (Gram stained, KOH wet mount) and PCR findings were compared to the culture results to analyse specificity, sensitivity, and predictive values of these technique.

Results

This study involved 70 corneal scrapings from 70 patients with unilateral corneal involvement. Table 1 showed the demographic characters of patients. The mean age reached 8.1 years (ranging from 1-16 years). A male preponderance was found with 1.3:1 male-to-female ratio. While, the median period between symptoms' onset and presentation time to hospital reached 5 days (ranging from 2–50 days) **(Table 1)**.

Presence of fungal causative agent was proven in 35 from 70 patients with a rate of (50%). By analysing the predisposing factors, the most prevalent factor was ocular trauma, related in 40 (57%) of cases. Followed by vernal keratoconjunctivitis in 14 cases (20 %) **(Table 2)**.

Mycological work up findings (smear examination and culture)

The KOH wet mount revealed fungal hyphae in 20 of 70 (28.5%) cases. Whereas Gram stained smear revealed fungal elements in 10 (14.2%) of 70 cases, 35 of 70 (50%) were culture positive **(Figure 4)**.

Of the 70 cases of presumed fungal keratitis studied, 35 cases were culture positive (50%), among which, *Fusarium spp.* were the main isolates (48.5%) and then *Aspergillus spp.* (20%) **(Table 3, Figure5)**.

PCR positivity

Of the 70 corneal scrappings samples 40 (70%) were PCR-positive. In addition, PCR produced high positive results of 15 from 35 cases (43%) in culture-negative samples **(Table 4)**.

By comparison between the three different techniques with culture ,which considered the gold standard for diagnosing of fungal keratitis , utilized to establish diagnosis we reported that   direct microscopic examination using 10% KOH possess sensitivity, specificity, a positive predictive and a negative predictive value as of 57.1%, 100%, 100%, respectively. Gram smear possess sensitivity, specificity, a positive predictive and a negative predictive value as of 28.6%, 100%, 100%, 58.3%, respectively. On the other hand, PCR possess sensitivity, specificity, a positive predictive and a negative predictive value as of 97.1%, 57.1%, 69.4%, 95.2%, respectively **(Table 5)**.

Figure 1. Microscopic appearance (X40) of KOH smear showing the   hyphae and macroconidial growth of *fusarium spp*.
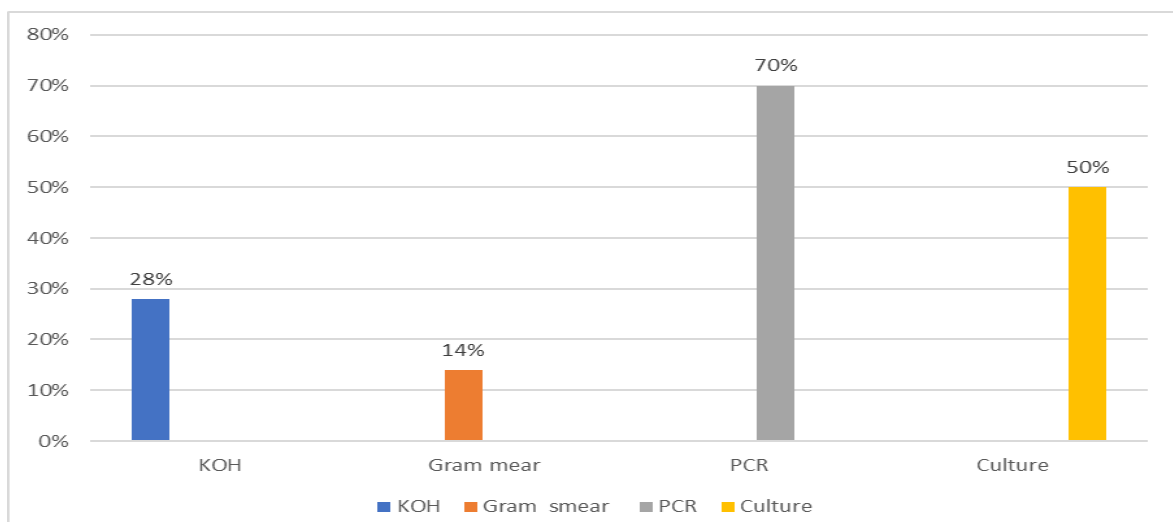

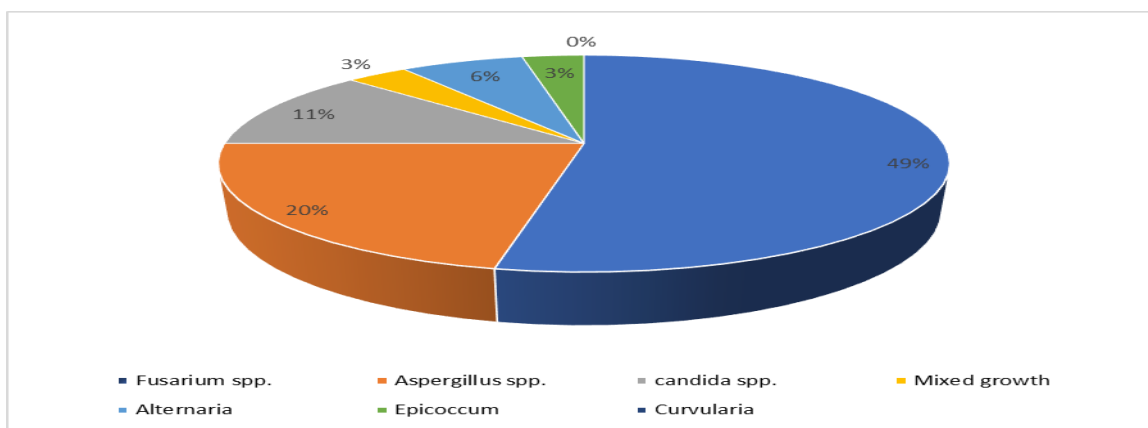**Figure 2.** KOH examination   of corneal scrapings showing hyphae.

**Figure 3.** SDA plate showing colonies of *Epicoccum spp*.

Figure 4. Percentage positivity of individual tests used in diagnosis of paediatric fungal keratitis patients n=70.

Figure 5. Frequency distribution of fungi isolated from positive culture.

Figure (6). Comparison of sensitivity and specificity of three diagnostic techniques versus t the gold standard; culture technique.
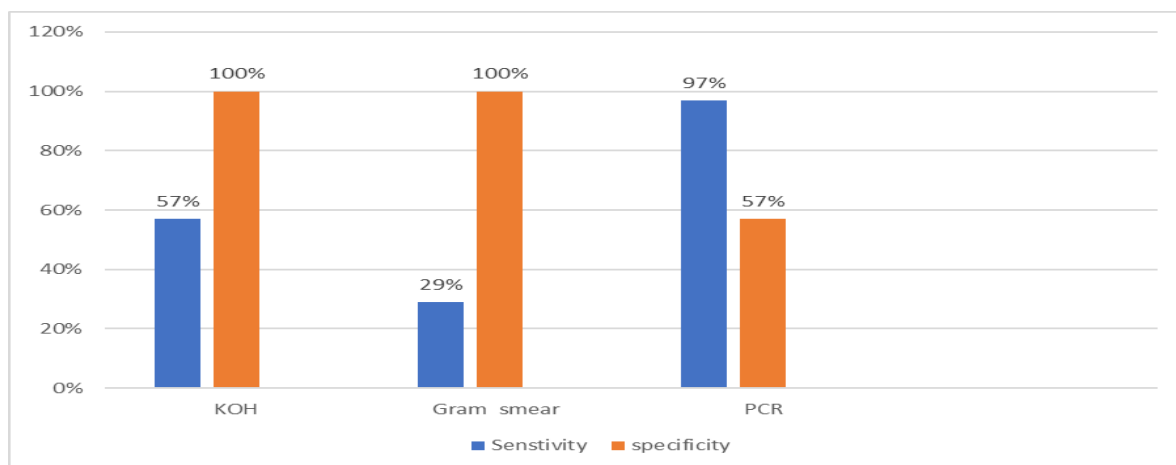
Table 1. Demographic details of paediatric patients with suspected fungal keratitis included in the study n=70.

Variable	n (%)
Age	
• 1-7 year	28(40%)
• 7-16 year	42(60%)
Gender	
Male	40(57%)
Time of presentation from the onset of symptoms	
▪ Within 48 h	▪ 5(7.1%)
▪ 2 day to week	▪ 50(71.4%)
▪ 1 week to month	▪ 10(14.2%)
▪ >1 month	▪ 5(7.1%)

Table 2. Predisposing factors in paediatric patients with suspected fungal keratitis included in the study n=70.

Predisposing factor	n (%)
Trauma	40 (57%)
Vernal keratoconjunctivitis	14 (20%)
Underlying systemic disease	9 (13%)
Underlying ocular disease	7(11%)
Previous ocular surgery	2(3%)

Table 3. Distribution frequency of isolated fungi from corneal scrapping positive for culture n (35).

Fungal isolate	n (%)
Fusarium spp	17(49%)
Aspergillus spp.	7(20%)
Yeast infection (*Candida* spp.)	4(11%)
Mixed infection of two hyphae (penicillium spp. and Absidia spp.)	1(3%)
Dematacious fungi	▪ Alternaria spp. 2 (6 %) ▪ Curvualria spp. 2(6 %) ▪ Epicoccum spp. 1(3%)

Table 4. Comparison between PCR positivity versus positivity fungal culture.

	Culture positive	Culture negative	Total
PCR positive	34	15	49
PCR negative	1	20	21
Total	35	35	70

Table 5. Comparison of sensitivity and specificity of three diagnostic techniques versus the gold standard culture technique.

	10 % KOH smear	Gram smear	PCR
Sensitivity	57.1%	28.6%	97.1%
Specificity	100%	100%	57.1%
Positive predictive value	100%	100%	69.4%
Negative predictive value	70 %	58.3%	95.2%

Discussion

Fungal keratitis is an insidious ocular infection, patients with mycotic keratitis are exposed to serious irreversible ocular sequelae as visual deprivation or amblyopia. In addition to the challenge in examination and sample collection because of lack of cooperation especially in paediatrics [17,18], there is another challenge at the diagnostic level which is the long time needed to obtain accurate results by ordinary mycological workup. So early diagnosis in combination with proper antifungal therapy are required towards best management of FK and inhibition of additional complications [19].

In this study the mean age of studied patient group was 8.1 years, while reported mean age changed in a variety of studies in the range of 8-14 years. Our study also showed a male predominance (n=40, 57%), which is similar to other previous studies [20-22], and this may be due to increased contact with ocular trauma as main predisposing element for FK.

Mycotic keratitis rarely occurs without previous predisposition. Corneal trauma (mainly with vegetative matter) which considered the mostly common element that predispose to that serious infection in many studies and accounts for 40%-60% of patients with FK [5,23-26]. Other studies stated that contact lens wearing displayed the mostly prevalent predisposing factor which is uncommon in our community in that age [22,27].

Herein, trauma as the mostly prevalent predisposing element representing 57% cases followed by vernal keratoconjunctivitis (20%) which is a common allergic problem in our community with a noticeable seasonal variation. In a preceding study done by **El Shabrawy et al.** on diverse age groups of patients with fungal keratitis [16] they stated that trauma (63.6.%) followed by chronic liver disease (30%) were the most common predisposing factors.

Simple, cheap and available detection of fungal element in mycotic keratitis can be achieved by direct microscopic examination for corneal scrapping material in 10% potassium hydroxide (KOH) [23].

In our work, fungal hyphae detected with 10% KOH mount in 28.5% of cases. While Gram's staining of corneal scrapings revealed hyphae in 14.2% of cases. The low sensitivity of KOH wet mount as well as Gram's smear, herein, may be attributed to a scanty sample size due to difficulty in obtaining the sample in pediatric patients.

In many cases, the diagnosis was based on direct microscopic examination of Gram's smear in addition to KOH wet mount produced from corneal samples to obtain fast results. Sharma and co-workers [28] stated that KOH wet mounts convey a higher sensitivity and consequently it is considered as the rapid and highly efficient available screening test. They stated a 61% sensitivity and a 91% specificity for KOH wet mount. Moreover, **Chowdhary** as well as **Singh** [29] stated that KOH sensitivity reached 62%, and sensitivity of Gram's smear is 60%, and both show 97% specificity.

Bharathi et al. [30] on their study on 3298 eyes investigating the diagnostic value of 10% KOH and Gram-stained smears for detection of fungal filaments. They concluded that 10% KOH

preparation represented an efficient diagnostic tool with 99.3% sensitivity, 99.1% specificity, 98.5% positive predictive value, and 99.6% negative predictive value. Also, Gram-stain was regarded as a valued technique with 89.2% sensitivity, 100% specificity, 100% positive predictive value, and 94% negative predictive value. According to the high diagnostic value of 10% KOH wet mount preparation, the authors recommended its application in all clinics for rapid diagnosis of infective keratitis including keratomycosis.

Fungal culture remains as the gold standard for the diagnosis of FK besides the microscopic examination .It has some advantages such as, it is simple, inexpensive and readily available, presumptive identification of common aetiologies to the genus and even to the species level , differentiating between mold and yeast infections,differentiating between dead and a live fungi. Isolation of fungi for further morphological and/or molecular studies, antifungal susceptibility testing.On the other hand it has some limitations like, it is time-consuming (needs days to weeks based on the fungus), false positive due to environmental contaminants, lack of precise identification to the species level in morphologically similar species .False negative due to the insufficient specimen, specimen from an inappropriate site [31].

In our study, the culture picked up 35 cases (50 %), and this is quite analogous to positive culture rate reported in previous researches [21,26,32-34].

Culture positivity is recognized to differ extensively in various places and even within same region. For instance, *fusarium* was regarded as the mostly prevalent (59.7%) fungal species originated from Indian children in 2015. This was in accordance with our results where *fusarium* was the mostly prevalent fungal cause of keratitis 17 cases (49%). While, in a previous study the culture positivity was reported to be 25% with *Aspergillus* spp were the most common isolated fungi from diverse age groups of patients with fungal keratitis [31].

Of the 70 corneal scrapings samples 49 (70%) samples were PCR-positive included 15(43%) samples from culture-negative samples. A study done by **Tananuvet et al.** [35] on 30 patients with suspected mycotic keratitis reported that PCR was positive in 93.3% of samples and culture- negative samples were PCR positive in 88.9 %.

PCR has figured out as a sensitive and specific technique for the diagnosis of FK. Different literatures had compared PCR with conventional diagnostic methods in patients with suspected fungal keratitis. PCR has the highest positive detection rate overall notably in cases with culture or smear-negative results [35-38].

The high positive rate in detection of fungi by PCR method could be explained by its ability to amplify even tiny amounts of pathogen DNA. Besides, DNA detection can be done from either dead or living organisms, while only living organisms can grow in ordinary culture plates which supports the growth of common fungal pathogens. The major limitation of PCR is that it is expensive and therefore not readily available [34].

In our study the PCR sensitivity, specificity were 97.1%, 57.1% respectively, with high detection rate in culture negative samples 15(43 %). In a previous prospective study done by **Kuo et al.** [38] on 50 patients with clinically suspected fungal keratitis they reported that PCR sensitivity and specificity were 100%, 96.7 % respectively .While, culture sensitivity and specificity were 50% ,100% respectively and concluded that PCR is more sensitive and rapid method than fungal culture

Conclusion

This study concluded that ocular trauma and vernal keratoconjunctivitis are the most common risk factors for pediatrics fungal keratitis with *Fusarium spp.* and *Aspergillus spp.* are the most common isolated fungi. Due to the possibility of dangerous complications from FK, it is crucial to identify the exact etiology of corneal ulcer to establish proper therapy in time to avoid such complications. PCR is sensitive technique for diagnosing of paediatric patients with FK. Despite PCR is capable of detecting fungal DNA in a high proportion of culture negative cases, its use as a routine diagnostic tool is hard in our low-income, low resources governmental hospitals .

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