

## PAPER DETAILS

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RESEARCH ARTICLE

## The incidence of fungal keratitis in Zagazig University Hospitals, Egypt and the value of direct microscopy and PCR technique in rapid diagnosis

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### ABSTRACT

**Objective:** To determine the frequency and risk factors of fungal keratitis (FK) and the value of direct microscopy and PCR techniques of corneal smears as appropriate diagnostic methods.

**Methods:** The keratitis cases in Ophthalmology Department of Zagazig University Hospitals, between January and June 2012 were enrolled. Corneal samples were examined by direct microscopic examination of wet mount preparation with KOH (10%), PCR technique and cultured simultaneously. The corneal smear and PCR findings were compared to the culture results to analyze specificity, sensitivity and predictive values of these techniques.

**Results:** A total of 350 patients diagnosed as keratitis and 60 of them were included in the study by a systemic random sampling method. The FK was proven in 33 (55%) cases with culture results. Ocular trauma (63.6%) was the most prevalent predisposing factor. The cultures revealed that the most frequent fungal pathogen were *Penicillium* spp. (24.2%) followed by *Aspergillus fumigatus* (21.2%). Direct microscopic examination had a sensitivity of 100%, specificity of 66.7%, a positive predictive value (PPV) of 78.6% and a negative predictive value (NPV) of 100%. PCR had a sensitivity of 100%, specificity of 88.9%, a PPV of 91.7% and a NPV of 100%.

**Conclusion:** Although, PCR is able to detect fungi in a high proportion of culture negative cases, it is difficult to be used as a routine diagnostic test due to the economic reasons. Therefore, we strongly recommend the use of direct microscopy of corneal smear as a rapid, economic and sensitive method for screening of FK. *J Microbiol Infect Dis* 2013;3(4): 186-191

**Key words:** Fungal keratitis, incidence, predisposing factors, rapid diagnose, direct microscopy, PCR

## Mısır, Zagazig Üniversitesi Hastaneleri'nde fungal keratit insidansı ve hızlı tanısında direkt mikroskopi ve PCR tekniğinin yeri

### ÖZET

**Amaç:** Fungal keratit (FK) sıklığının, risk faktörlerinin ve korneal örneklerin direkt mikroskopi ile incelenmesi ve PCR tekniğinin tanı değerinin araştırılması.

**Yöntemler:** Mısır'da, Zagazig Üniversitesi Hastaneleri Göz Hastalıkları Departmanı'nda Ocak-Haziran 2012 arasında keratit tanısı alan hastalar çalışmaya alındı. Korneal örnekler %10 KOH preparasyon yöntemi ile direkt mikroskopi ile incelendi, ayrıca örnekler PCR ile fungal DNA varlığı açısından test edildi ve kültür plaklarına ekim yapıldı. Korneal smear direkt mikroskopi ve PCR bulguları kültür sonuçları ile testlerin duyarlılığı, güvenilirliği, uygunluğu ve tahmin değerleri açısından karşılaştırıldı.

**Bulgular:** Çalışma süresince toplam 350 hasta keratit olarak değerlendirilerek bunlardan 60 tanesi rastgele örnekleme yöntemiyle çalışmaya dâhil edildi. Toplam 60 hastanın 33'ünde (% 55,0) kültürde fungus üremesi ile FK tanısı doğrulandı. Göz travması en sık predispozan faktör olarak değerlendirildi (% 63,6). En sık fungal ajan *Penicillium* spp. idi (% 24,2) ve bunu *Aspergillus fumigatus* (% 21,2) takip etti. Direkt mikroskopinin FK tanısında duyarlılığı % 100, özgüllüğü %66,7, pozitif tahmin değeri (PPV) % 78,6 ve negatif tahmin değeri (NPV) % 100 olarak hesaplandı. PCR tekniğinin ise duyarlılığı % 100, özgüllüğü % 88,9, PPV % 91,7 ve NPV % 100 olarak hesaplandı.

**Sonuçlar:** FK tanısında PCR, kültür negatif olgularda önemli oranda fungal etiyolojiyi gösterebilmesine rağmen, pahalı olması sebebiyle FK tanısında rutin tanısal test yöntemi olarak kullanılamayacağını, bunun yerine daha ekonomik, daha hızlı ve duyarlı bir yöntem olan korneal örneklerin direkt mikroskopik incelemesinin daha uygun olduğunu düşünüyoruz.

**Anahtar kelimeler:** Fungal keratit, insidans, predispozan faktörler, hızlı tanı, direkt mikroskopi, PCR

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## INTRODUCTION

Mycotic keratitis is a fungal infection of the cornea. This infection is difficult to treat and it can lead to severe visual impairment and even blindness. It is worldwide in distribution, but is more common in the tropics and subtropical regions. Trauma is the major predisposing factor, followed by ocular and systemic defects, contact lens wear, prior application of corticosteroids, and prolonged use of antibiotic eye-drops.<sup>1-3</sup> A recent report showed a steadily increase in the incidence of fungal keratitis in Cairo-Egypt correlating with the climatic changes (rises in minimum temperature and the maximum atmospheric humidity) in the region.<sup>4</sup>

Fungal keratitis can be classified into two forms: Keratitis due to filamentous fungi especially with *Fusarium* spp and *Aspergillus* spp, which commonly occurs in tropical and subtropical zones, and keratitis due to yeast-like and related fungi in areas with lower temperature.<sup>5</sup> In fungal keratitis, rapid diagnosis and early institution of antifungal therapy is necessary to prevent ocular morbidity and blindness.<sup>6</sup> Although culture helps in definite diagnosis and identification, direct microscopic detection of fungal structures in corneal scrapes or biopsies permits a rapid presumptive diagnosis.<sup>1</sup> A PCR technique using universal primer for fungi could be an effective rapid method for the diagnosis of fungal keratitis and could be added as the screening diagnosis test when an early mycotic keratitis is suspected.<sup>7</sup> In this study, we aimed to determine the frequency and risk factors of fungal keratitis and to compare direct smear with other diagnostic techniques including sample cultures and PCR as an appropriate diagnostic method for patients suffering from fungal keratitis admitted to ophthalmology department, Zagazig University Hospitals, Egypt.

## METHODS

### Study design and patient selection

This cross sectional study was carried out in ophthalmology department of Zagazig University Hospitals (regional tertiary care hospitals), Zagazig, Egypt, between January 2012 and June 2012. Patient inclusion criteria were the presence of a corneal ulcer with an epithelial defect of 1 mm at its greatest width, some portion of the infiltrate covering the central third of the cornea, and ability to provide appropriate consent. Exclusion criteria were bilateral corneal ulcers, corneal ulcers of viral or parasitic origin (as suggested by history and examination findings), presence of endophthalmitis, and inability to give consent. Approving for this study was taken by

the ethics committee and written informed consent was obtained from all participants.

All patients were examined by local experienced ophthalmologists to establish the diagnosis of ulcerative keratitis. All patients were then asked to stop antibiotic treatment for at least 72 hours before corneal scrapings were collected. Corneal scrapings were obtained by scraping the base and edges of the ulcer with a tip of a disposable 23-gauge needle, after instillation of topical anesthetic (0.5% tetracaine). Also a sterile Dacron swab was used to obtain a corneal scrape from the base and leading edge of the corneal ulcer for molecular analysis.<sup>7,8</sup>

### Direct microscopic examination of corneal samples

The presence of fungi in corneal scrapings was determined by direct smearing on glass microscopic slide for potassium hydroxide (KOH) 10% wet mount examination. These smears were examined and the presumptive identification of fungi was done according to the microscopic features of fungi.<sup>9</sup> The wet KOH mount was examined immediately under microscope for the presence of any hyphae.

### Sample cultures and identification of fungi

Three further scrapings were directly inoculated onto Sabouraud's glucose neopeptone agar (Emmon's modification), sheep blood agar and Sabouraud's dextrose brain heart infusion agar (SABHI) in the form of C-streaks. If the ulcer was very discrete or only a small amount of corneal material was available, a liquid phase medium (Sabouraud's broth) was also inoculated.<sup>5</sup> All media were obtained from market (Oxoid®, UK). Sabouraud's glucose neopeptone agar and Sabouraud's dextrose brain heart infusion agar media were supplemented with antibacterial agents (penicillin 20 U/ml and gentamicin 5 µg/ml).<sup>9</sup> All fungal media were incubated at 37°C and 25°C for a period of four weeks. Although fungal growth was usually seen within three to four days, a negative culture media required incubation for up to four weeks. Cultures were checked for fungal growth daily during the first week and twice a week for the next three weeks. Plates for bacterial culture were kept at 37°C and were observed for seven days before being considered as negative. A significant fungal growth was further re-cultivated on corn meal agar media. Molds growth was identified by the colony characters (growth rate, color, and morphology). Further identification of molds was done by transparency tape preparation from the corn meal agar for observation of their microscopic features (Hyphae character, fruiting bodies, and other special structure).<sup>9,10</sup> Positive yeast colo-

nies (smooth, and pasty) were further analyzed by standard tests (microscopic features, germ-tube test, sugar fermentation and assimilation tests) until a specific species was identified.<sup>11</sup>

### Molecular analysis of corneal scrapings

A sterile Dacron swab was used to obtain a corneal scrape from the base and leading edge of the corneal ulcer. The swab was then placed into a sterile micro centrifuge tube and capped. Each specimen was stirred directly into 200 µl of sterile saline and extracted using a QIAamp DNA mini kit (Qiagen®, Germany) using a protocol adapted for extraction of DNA from fungal cells. In brief, each sample was pre-incubated in cellular lysis buffer at 99°C for 20 min and then processed as suggested by the manufacturer. An aliquot of 50 µl was taken from each sample and stored at -20°C.

PCR procedure was performed for amplification of the 28S rDNA using universal primers. The primers were forward: U1 [5'-GTG AAA TTG TTG AAA GGG AA-3'] and reverse: U2 [5'-GAC TCC TTG GTC CGT GTT-3']. Primers were synthesized by MWG-Biotech (Ebersdorf®, Germany). PCR amplifications were carried out in 20 µl of reaction volumes master-mix beads (Bioron®, USA) with a thermocycler (Bimetra®, Germany). Cycling conditions were as follows: initial denaturation at 95°C for 10 min followed by 49 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min followed by a final extension phase at 72°C for 10 min. Each PCR run included a positive control with purified DNA of *Aspergillus fumigatus*, and two negative controls with blank reagents.<sup>12</sup> Amplification products were separated by electrophoresis in a 1% agarose gel, subsequently stained with ethidium bromide, and analyzed with a gel electrophoresis. PCR products of 260 bp in length were interpreted as successful amplification process and indicated presence of fungal infection.

### Data collection and statistical methods

Predisposing factors, the results of direct microscopy, PCR and culture of corneal samples were obtained from chart review and recorded for each keratitis patient. All the data were entered and analyzed by using a Windows® based computer program, Statistical Package for the Social Sciences (SPSS) version 15.0. The corneal smear and PCR findings were compared to the culture results to analyze specificity, sensitivity, accuracy and predictive values of these techniques. An interrater reliability analysis using the Kappa statistic was performed to determine consistency between the tests. κ value <0.20 was interpreted as poor, 0.20-0.40 as

fair, 0.40-0.60 as moderate, 0.60-0.80 as good and 0.80-1.00 as perfect agreement. Tests were two-tailed and in comparing the sensitivity of the data,  $P < 0.05$  was considered significant.

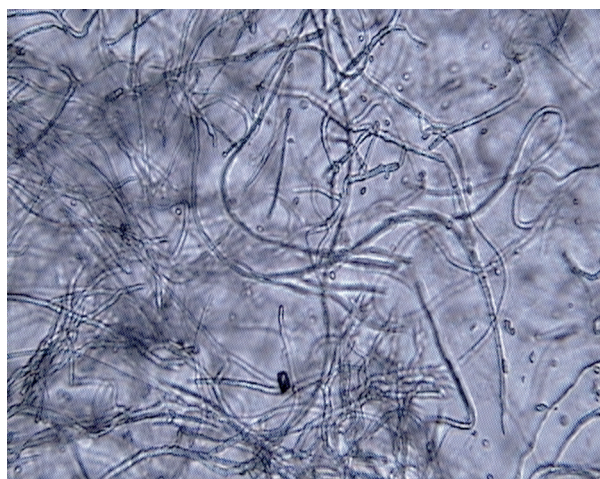
### RESULTS

During the six-month period, 350 patients met the inclusion criteria from which 60 patients were chosen by a systemic random sampling method. These 60 patients were included in the study. The fungal etiology was proven in 33 keratitis cases with a rate of 55% based on the culture results. When the predisposing factors analyzed, ocular trauma was the first predominant predisposing factor found in 21 (63.6%) out of 33 fungal keratitis cases followed by chronic liver diseases (Table 1).

**Table 1.** The predisposing factors for fungal keratitis cases (n=33)

Predisposing factors	Number (%)
History of trauma	21 (63.6)
Chronic liver disease	10 (30.3)
Diabetes mellitus	6 (18.2)
Contact lens use	4 (12.1)
Previous steroid use	2 (6.1)

The cultures of the corneal scraping samples revealed that the most frequent fungal pathogen isolated from the corneal ulcers were *Penicillium* spp. (Figure 1a, 1b) followed by *A. fumigatus* (Figure 1), *Candida* spp. and *Scopulariopsis* (Figure 2a, 2b). The results are presented in Table 2.

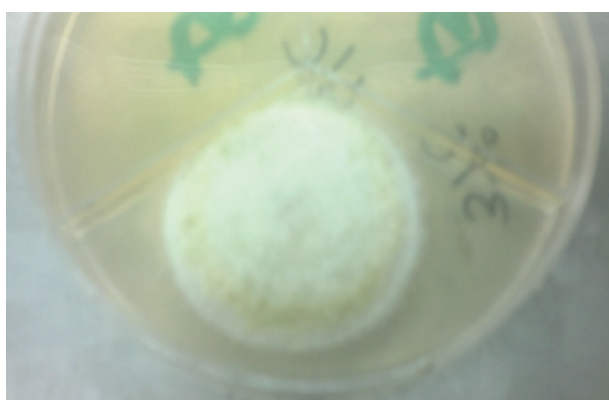


**Figure 1 a.** Microscopic appearance (X40) of KOH smear showing the hypha growth of *penicillium*

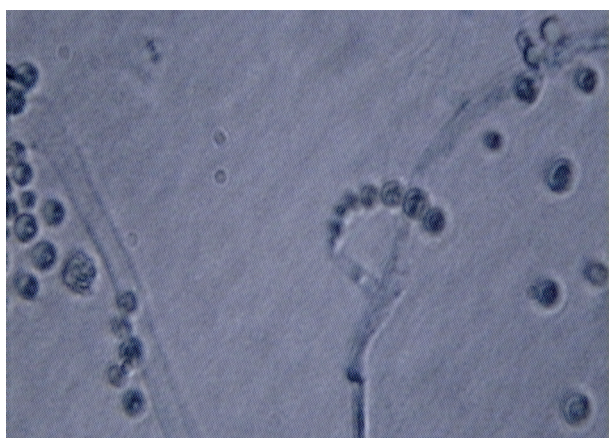




**Figure 1 b.** Corn meal agar showing the four days growth of penicillium colonies



**Figure 2 a.** Corn meal agar showing the four days growth of scopularopsis colonies



**Figure 2 b.** Microscopic appearance (X40) of transparency tape preparation showing growth of scopularopsis

**Table 2.** The causing microorganisms isolated from corneal samples of fungal keratitis cases by culture technique (n=33)

Microorganisms	Number (%)
Pure hyphal infection	
<i>Penicillium</i> spp.	8 (24.2)
<i>Aspergillus fumigatus</i>	7 (21.2)
<i>Scopulariopsis</i> spp.	4 (12.1)
<i>Fusarium</i> spp.	3 (9.1)
Zygomycetes	2 (6.1)
Mixed infection of two hyphae ( <i>Penicillium</i> sp. and <i>Absidia</i> sp.)	1 (3.0)
Pure yeast infection ( <i>Candida</i> spp.)	5 (15.2)
Mixed with hyphae and yeast ( <i>Penicillium</i> spp. and <i>Candida</i> sp.)	3 (9.1)

The culture technique was considered as the gold standard for diagnosing of fungal keratitis. After comparing the three different methods used for establishing diagnosis, we found that direct microscopic examination with 10% KOH has a sensitivity of 100%, specificity of 66.7%, a positive predictive value of 78.6% and a negative predictive value of 100%. PCR, on the other hand, has a sensitivity of 100%, specificity of 88.9%, a positive predictive value of 91.7% and a negative predictive value of 100%. There was a good and a positive agreement between the results of culture and direct microscopy ( $\kappa$ , 0.687;  $p=0.005$ ). Further, a perfect and a positive agreement was also found between the results of culture and PCR ( $\kappa$ , 0.898;  $p=0.0005$ ). The agreements between the diagnostic tests are presented in Table 3.

**Table 3.** The efficacy of diagnostic test methods for fungal keratitis diagnosis in terms of positivity of corneal samples cultures

Variables	Direct microscopy with KOH (10%)	Polymerase chain reaction (PCR)
Sensitivity (%)	100	100
Specificity (%)	66.7	88.9
Positive predictive value (%)	78.6	91.7
Negative predictive value (%)	100	100
Kappa ( $\kappa$ ) and (P-value)	$\kappa$ , 0.687 ( $p=0.005$ )	$\kappa$ , 0.898 ( $p=0.0005$ )

## DISCUSSION

Corneal infection is a leading cause of ocular morbidity and blindness worldwide. Early diagnosis and treatment are important in preventing vision threatening complications.<sup>13</sup> Early diagnosis of mycoses in patients is crucial for prompt antifungal therapy.<sup>14</sup> In this study, the frequency of fungal keratitis was 55% which was high as in previous study who found that the frequency of fungal keratitis in Zagazig University, Egypt was 75%.<sup>15</sup> A comparable frequency of fungal keratitis was also reported in upper part of Assam, India (60.6%) which is a subtropical area with similar climatic conditions to Egypt.<sup>2</sup> However, a minimum average annualized incidence of fungal keratitis in 2009 was found to be UK was 0.32%. Also found that the rate of infection with fungal keratitis is usually higher in the warmer half of the year (April to September).<sup>16</sup> This can be explained by the difference in climatic conditions. A study done in the National Research Centre, Cairo in Egypt to detect the association between fungal keratitis and the climatic changes concluded that the climatic conditions directly affect the frequency of fungal keratitis and that the incidence of this disease will continue to rise as long as the global warming is increasing and the green house gases will continue to rise.<sup>4</sup> Another apparent reason for the high frequency of fungal keratitis in our study might be due to the inclusion of referred cases from peripheral hospitals which were treated empirically with antibacterial drugs and failed to respond to treatment initially.

In this study, fungal culture of corneal samples revealed positive result in 33 eyes (55%), these cases were distributed as following: pure filamentous fungi in 24 (72.7%) cases, mixed mold species in only one (3%) case, candida in five cases (15.2%), and mixed mold and yeast in three cases (9.1%). The study found that a high frequency of cases had single species of isolates as it was found in a previous study.<sup>17</sup> The most prevalent molds isolated in this study were *Penicillium* spp., *A. fumigatus*, *Candida* spp. and *Scopulariopsis* spp. (Table 2). In contrast to other works which demonstrated that *Aspergillus flavus* was the most common fungus isolated followed by *Fusarium solani*.<sup>1,18</sup> Others revealed that the most predominant fungal species was *Fusarium* spp. (42.8%).<sup>2,19</sup> It was previously reported that the yeast isolation from the corneal ulcers was as low as 15% especially in hot areas.<sup>2</sup> However, in a study from the United Kingdom more than half of the isolates were reported as *Candida* spp. This finding confirms the importance of yeast as a cause of fungal keratitis in urbanized and colder areas.<sup>16</sup>

In the present study, history of ocular trauma is the most important predisposing factor (63.6%), as it was the main cause in many previous studies.<sup>2,16,17</sup> However, others found that trauma with plant debris and straws were noted in only 28.6% of patients with fungal keratitis.<sup>3,20</sup> In the current study, 30% of fungal keratitis patients were suffering from chronic liver disease. Diabetes mellitus was an associated factor in six cases, in which one case was affected with pure candida infection, one was with mixed candida and hyphal infection and the other four cases were infected with hyphae. These findings were also supported by the other reports which found that chronic systemic diseases were important predisposing factor for fungal keratitis due to suppressed immune system.<sup>17,21</sup> The results of the study suggested that nearly 12% of the cases were associated with refractive contact lens wear. This factor was more predominant in other studies.<sup>3,22</sup> The lesser incidence could be explained in view of the low socioeconomic level of the patients included in our study.

We found that the sensitivity of direct microscopic examination of KOH preparation in fungal keratitis was 100% (Table 3). This result was supported with other studies.<sup>15,17,23</sup> Although some researchers found that the sensitivity of direct corneal smear was lower.<sup>21</sup> The specificity was 66.7% which is lower than previous studies which revealed a specificity of 96.8%.<sup>2</sup> The probable cause of this may be due to presence of non-viable fungal elements at the time of culture due to prior administration of antifungal drugs, but the exact cause in our cases could not be ascertained. DNA amplification with universal primers is a promising diagnostic tool in cases of infectious keratitis where routine laboratory culture failed to identify the pathogen. PCR may be performed in cases where the results of corneal scraping stains are negative without waiting for the results of the culture.<sup>24</sup> Total time taken for PCR assay was 4-8 h whereas positive fungal cultures took 2-10 days. PCR has a sensitivity of 100% and a specificity of 88.9% (Table 3). These results agreed with results obtained by others.<sup>24,25</sup> However, another study revealed that PCR had a sensitivity of 90.9% and a specificity of 94.7% for the detection of fungal etiology, using primers based on the conserved region of 18S rRNA gene. This could be due to insufficient fungal elements present in corneal scraping or due to sequence variation of 18S rRNA gene of this fungus.<sup>26</sup> Our findings suggest that the PCR can be a useful adjunct to smear and culture in the rapid diagnosis of fungal keratitis, particularly in cases of failed detection with routine examination method. The study also found a good

and positive agreement between the results of culture and direct microscopy ( $\kappa$ , 0.687;  $p=0.005$ ) and a perfect and positive agreement between the results of culture and PCR ( $\kappa$ , 0.898;  $p=0.0005$ ) suggested these rapid diagnostic tests worked together with corneal sample cultures.

In conclusion, fungal corneal ulcer is common in Egypt due to the hot humid climate. Trauma and chronic systemic diseases are the most common predisposing factors. Due to the potential serious complications from fungal keratitis, it is important to know the exact etiology of corneal ulcer to institute appropriate therapy in time. Although, PCR is able to detect fungal DNA in a high proportion of culture negative cases, it is difficult to be used as a routine diagnostic test in our hospitals due to the economic reasons. Therefore, we strongly recommend the use of direct corneal smear as a rapid, economic and sensitive method for screening of fungal keratitis.

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