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Original article

Rapid diagnosis of fungal keratitis in patients with corneal ulcer using calcofluor white stain

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ABSTRACT

Background: The prevalence of fungal keratitis has risen in recent years significantly. To avoid additional complications, diagnosing and treating fungal keratitis is crucial. This study aims to measure the efficacy of a calcofluor white (CFW) stain for the quick diagnosis of fungal keratitis and to contrast the positive rates, sensitivity, and specificity with a 10% potassium hydroxide (KOH)-based smear and culture technique. **Methods:** From individuals with clinically suspected corneal ulcers, 30 corneal scrapings had been collected. Data on demographics had been analyzed. **Results:** Of the 30 patients, 40% were women and 60% were men. There was a 1.5:1 man-to-woman ratio. The age of patients ranged from 29 to 71 years (mean 46.67 ± 10.90). The age presentation of those between the ages of 41 and 50 years was the most frequent (36.7%). The majority of cases were farmers (43.3%). Trauma was the most common predisposing factor (46.6%). Twenty-four (80%) cases were culture positive. Eleven (36.7%) were fungal, 13 (43.3%) were bacterial and 6 (20%) showed no growth. *Fusarium* was the most common fungal isolate (36.4%), followed by *Aspergillus* (27.3%). While *Staphylococcus aureus* was the most common bacterial isolate (46.2%), followed by *Pseudomonas* (38.4%). The sensitivity of KOH wet mount and CFW stain was 72.7% and 90.9%, respectively. The specificity of both KOH wet mount and CFW stain was 100%. **Conclusion:** The early diagnosis of fungal keratitis can be made rapidly by direct microscopic examination of fungal elements using CFW stain. When diagnosing fungal keratitis, CFW has higher sensitivity to KOH.

Introduction

Fungal keratitis is one of the primary causes of serious ocular morbidity as well as blindness [1]. It is prevalent worldwide, but it is more common in the tropics and subtropics [2]. The incidence of fungal keratitis in Egypt is 20-55% (mean 40%) [3]. Increases in the lowest temperature and the high levels of atmospheric humidity in the area are causing this incidence to increase in proportion to these climatic changes [4]. Early detection and antifungal treatment are essential to prevent further complications, which include the formation of hypopyon, endophthalmitis, or vision loss [5]. *Fusarium* spp. and *Aspergillus* spp. are the

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most commonly recognized causative agents of fungal keratitis [6]. Several factors; such as the widespread use of antibiotics and steroids, contact lens usage, ocular trauma with agricultural materials, systemic immunosuppression, diabetes mellitus, and postoperative infection, are linked to an increase in the frequency of fungal keratitis [7].

The diagnosis of fungal keratitis remains problematic to make. Numerous clinical features are not exclusive to fungal ulcers, so antifungal treatment has to be withheld till a diagnosis has been established through laboratory tests. The most critical step in the early therapy of suspected fungal keratitis is obtaining corneal material for guided smears and media inoculation. Smears are employed to gain quick information on the disease. Even though culture aids in accurate identification and diagnosis, direct microscopic detection of fungal elements in scrapings of the cornea allows for a quick presumptive diagnosis [4].

This study aims to measure the efficacy of a calcofluor white (CFW) stain for the fast diagnosis of fungal keratitis and to contrast the positive rates, sensitivity, and specificity with a 10% potassium hydroxide (KOH)-based smear and culture technique.

Material and Methods

Study settings and population

A total of 30 patients with clinically suspected corneal ulcers attending an ophthalmology clinic in Ain Shams University Hospitals over the period from May 2021 to March 2022 had been involved in the research. Everyone who participated provided their informed consent. Ages, sex, occupations, history of trauma, the mode of trauma, history of prior treatment, usage of steroid eye drops or contact lenses, any related systemic illnesses like diabetes mellitus, and ocular abnormalities or disorders had all been recorded in the patient's demographic profile.

Study design

A cross-sectional descriptive study.

Ethical approval

The Faculty of Medicine at Ain Shams University's Ethics and Research Committee gave the study acceptance with approval No. MS 246/2021. Informed consent was obtained from each patient before enrollment. Identification codes had been utilized for data administration to preserve information confidentiality and privacy; these findings had been employed only for scientific purposes.

Inclusion criteria

The study included patients attending the ophthalmology clinic who had signs and symptoms suggestive of corneal ulcer. It also included patients who had a resistant corneal ulcer and were unresponsive to treatment.

Exclusion criteria

History of autoimmune disease and impending perforated corneal ulcer.

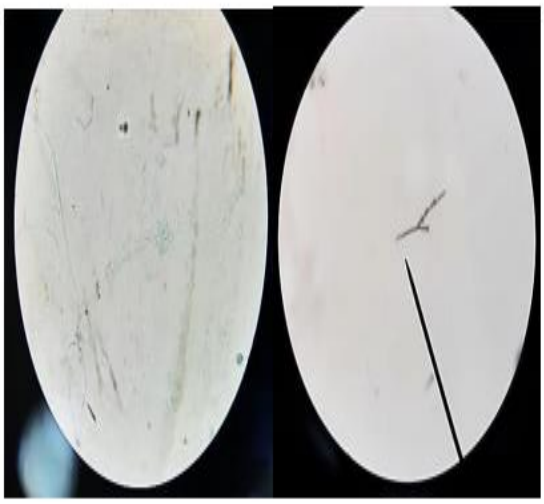
Specimen collection

Corneal scrapings were collected with a No.15 Bard Parker blade by an experienced Ophthalmologist under complete aseptic conditions following 4% lignocaine instillation. Scraping was done from the leading edge and the base of each ulcer and was collected in a sterile container and sent to the central Microbiology lab in Ain Shams University Hospitals. The samples were subjected to 10% KOH wet mount examination, CFW examination, and cultivation on blood agar and Sabouraud dextrose agar in a C-shaped manner.

KOH wet mount examination

The scraping material was put onto a spotless glass slide. One or two drops of sterile 10% KOH (Alpha Chemika Laboratories, Mumbai, India) were applied and covered using a clean coverslip to avoid the formation of air bubbles. The slide was left for 5-10 minutes and was examined under the microscope for the presence of hyphal elements or budding yeasts **(Figure 1).**

Figure 1. 10% KOH wet mount examination shows fungal hyphae.

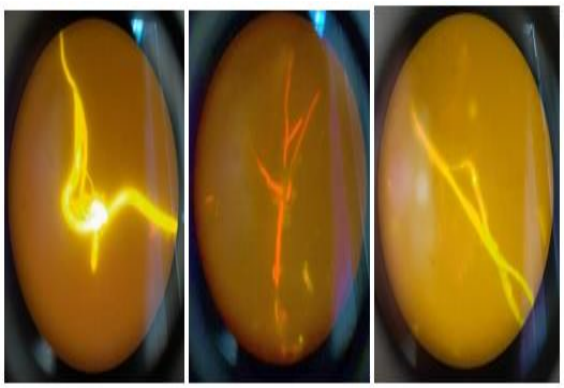


Calcofluor white stain examination

The specimen was added to a clean glass slide. Then a drop of CFW stain was added. This stain is composed of 0.5 g/l Evans blue dye and 1 g/l Calcofluor white stain M2R from Sigma-Aldrich in St. Louis, Mo., USA. A cover slip was placed on the specimen and then was left for 1 minute. The slide was covered with a paper towel and was gently pressed to remove any excess fluid and was examined under UV light at x100 to x400 magnifications. A filamentous structure was thought to be a fungal filament if it showed branching and septation, seen in the center of the smear, was identified at low power (× 100) and was confirmed at higher power (× 400 or × 1000).

Fungal elements appear as bright apple-green fluorescence with typical morphology **(Figure 2).**

Figure 2. Detection of fungal elements using calcofluor white stain.



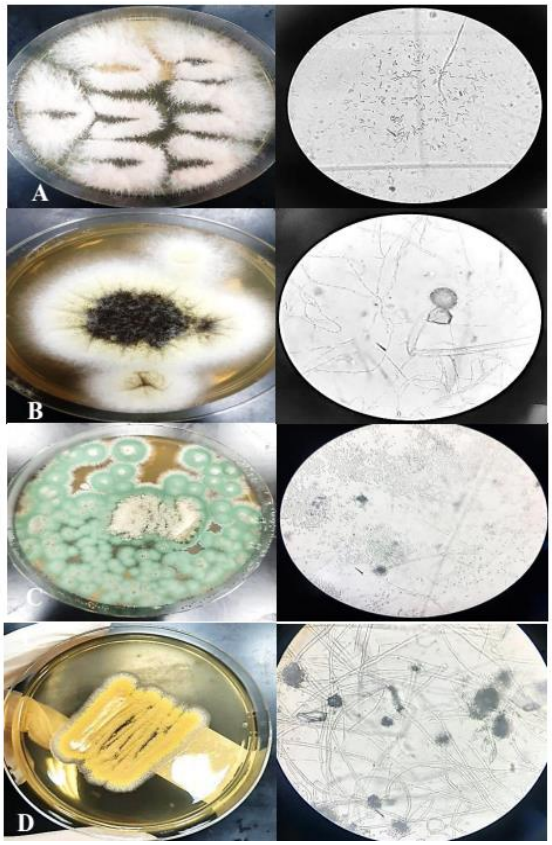
Culture

The scraping material was inoculated directly onto the blood agar plates (Oxoid, UK) and Sabouraud dextrose agar (SDA) plates (Oxoid, UK) in a C-shaped manner. The SDA plates were incubated for up to 2 weeks at 28°C and 37°C [8] and were examined daily during the first week and twice a week during the second week [9]. Failure of growth after two weeks was diagnosed as negative for fungal growth and the plates were discarded [10]. The blood agar plates were incubated at 37°C for 48 hours and were checked daily for fungal and bacterial growth before being discarded after 48 hours [10].

Following growth on plates, isolated fungi were identified based on the rate of growth, macroscopic morphological characteristics (colony color on the obverse and reverse side, colony textures, and submerged hyphae), and microscopic characteristics (such as conidiogenous cells and conidiophores, the shape and organization of conidia, macro-microconidia and blastoconidia and the absence or presence of chlamydospores) **(Figure 3)**.

Positive microbial culture was diagnosed if semi-confluent growth was seen at the inoculation site on a solid medium, it was compatible with clinical findings, and smear findings were compatible with that of the culture.

Figure 3. Different fungal strains on SDA and scotch tape mount: (**A)** Fusarium (**B)** *Aspergillus niger* (**C)** *Aspergillus fumigatus* (**D)** *Aspergillus flavus*.



Statistical analysis

A Statistical Package for Social Science had been employed to perform the statistical analysis **(SPSS version 23.0 for Windows; SPSS Inc., Chicago, IL, USA)**. In terms of sensitivity and specificity, the findings of direct microscopy analysis of smear specimens had been compared to those of fungal culture employing the Chi-square ($\chi 2$) test. *p*-values lower than 0.05 had been regarded as significant (S). The staining methods' sensitivity (true positive rate), specificity (true negative rate), PPV, NPV, and accuracy had been computed employing standard statistical formulas.

Results

Out of 30 samples collected from patients with clinically suspected corneal ulcers, 18 (60%) were men and 12 (40%) were women, with a man-to-woman ratio of 1.5:1 as shown in **figure (4)**. The patients' mean age was 46.67 ± 10.90 years, ranging from 29 to 71 years. The age presentation of 41 to 50 years was the most frequent age group (36.7%) followed by 31 to 40 years (26.7%) as seen in **figure (5)**. The occupation profile of the study group mainly consisted of farmers (43.3%) followed by housewives (23.3%), carpenters (16.7%), drivers (6.7%), cleaners (3.3%), construction workers (3.3%), and plumbers (3.3%) as illustrated in **table 1**. When predisposing factors were analyzed, it was shown that history of trauma was the most common predisposing factor (46.6%) followed by steroids (20%), Diabetes mellitus (10%), post-operative (6.7%), and contact lens use (6.7%) as seen in **the table (2)**. The major cause of corneal ulcers is trauma, and 50% of the fungal corneal ulcer cases gave a history of having sustained trauma due to vegetable matter as presented in **table (3)**. More than half of the patients (83.3%) had a history of previous treatment before their presentation at the outpatient clinic as shown in **table (4)**.

As seen in **figures (6 and 7)**, culture was positive in 24/30 (80%) cases in which 11/24 (36.7%) cases were pure fungal, and 13/24 (43.3%) cases were pure bacterial and the remaining 6 (20%) cases showed no growth. The predominant fungal pathogen isolated was *Fusarium* spp. 4/11 (36.4%) followed by *Aspergillus* spp 3/11 (27.3%) (Aspergillus niger 1/11, Aspergillus fumigatus 1/11 and Aspergillus flavus 1/11) and *Candida* spp. *2/11* (18.1%). Other fungi isolated were *Penicillium* spp. 1/11 (9.1%) and *Alternaria* spp. 1/11 (9.1%) as seen in **table (5).** The predominant bacterial pathogen isolated was *Staphylococcus aureus* 6/13 (46.2%) followed by *Pseudomonas* spp. 5/13 (38.4%) and *Coagulase-Negative Staphylococcus 2/13* (15.4%) as seen in **table (6).**

The culture technique had been regarded as the gold standard to diagnose fungal keratitis. We discovered that the positive rates of direct microscopic inspection of 30 corneal scraping samples for fungal components by 10%KOH and CFW stain were 26.7% and 33.3%, respectively. These results were compared to the two different quick detection techniques utilized for diagnosing fungal keratitis. When the direct microscopic inspection was compared to culture on SDA for sensitivity and specificity, CFW had greater sensitivity (90.9%) and specificity (100%), followed by 10% KOH sensitivity (72.7%) and specificity (100%). In addition, when direct microscopic inspection and culture on SDA were compared for accuracy, CFW exhibited better accuracy (96.7%) followed by 10% KOH (90.0%) as reliable tools in the diagnosis of fungal keratitis as illustrated in **table (7)**

Table 1. Distribution of occupation among the studied group.

Occupation	Total Number of cases	%	Fungal cases
Farmer	13	43.3%	6
Housewife	7	23.3%	2
Carpenter	5	16.7%	2
Cleaner	1	3.3%	-
Construction worker	1	3.3%	1
Driver	2	6.7%	-
Plumber	1	3.3%	-
Total	30	100%	11

Table 2. Distribution of risk factors among the studied group.

Risk Factors	Total Number of cases	%	Fungal cases
Trauma	14	46.6%	6
DM	3	10%	-
Steroids	6	20%	3
Post-operative	2	6.7%	-
Contact lens	2	6.7%	1
No risk factors	3	10%	1
Total	30	100%	11

Table 3. Distribution of mode of trauma among the studied group.

Mode of Trauma	Total Number of cases	%	Fungal cases
Vegetative matter	4	28.6%	3
Wooden object	2	14.3%	1
Iron particle	3	21.4%	1
Foreign body	2	14.3%	1
Animal tail	2	14.3%	-
Dust	1	7.1%	-
Total	14	100%	6

Table 4. Distribution of history of previous treatment among the studied group.

Treatment history	Number	%
No treatment	5	16.7%
Antibiotic only	7	23.3%
Antibiotic + Antifungal	12	40%
Antibiotic + Antiviral	6	20%
Total	30	100%

Table 5. Distribution of fungal isolates among corneal ulcer cases.

Fungal Isolate	Number	%
Fusarium spp.	4	36.4%
Aspergillus spp.	3	27.3%
Candida spp	2	18.1%
Penicillium spp.	1	9.1%
Alternaria spp.	1	9.1%
Total	11	100%

Table 6. Distribution of bacterial isolates among corneal ulcer cases.

Bacterial Isolate	Number	%
Staphylococcus aureus	6	46.2%
Staphylococcus Coagulase Negative	2	15.4%
Pseudomonas spp.	5	38.4%
Total	13	100%

Table 7. Diagnostic performance of 10% KOH and CFW compared to culture on SDA.

		Culture on SDA		Sensitivity	Specificity	PPV	NPV	Accuracy
		Negative	Positive	% (95% CI)	% (95% CI)	% (95% CI)	% (95% CI)	
10% KOH	**Negative**	19	3	72.7% (39.0 - 94.0)	100.0% (82.4 - 100.0)	100.0% (63.1 - 100.0)	86.4% (64.4 - 97.2)	**90.0%**
	Positive	0	8					
CFW	**Negative**	19	1	90.9% (58.7 - 99.8)	100.0% (82.4 - 100.0)	100.0% (69.2 - 100.0)	95.0% (75.1 - 99.9)	**96.7%**
	Positive	0	10					

Figure 4. Sex distribution among the studied group.

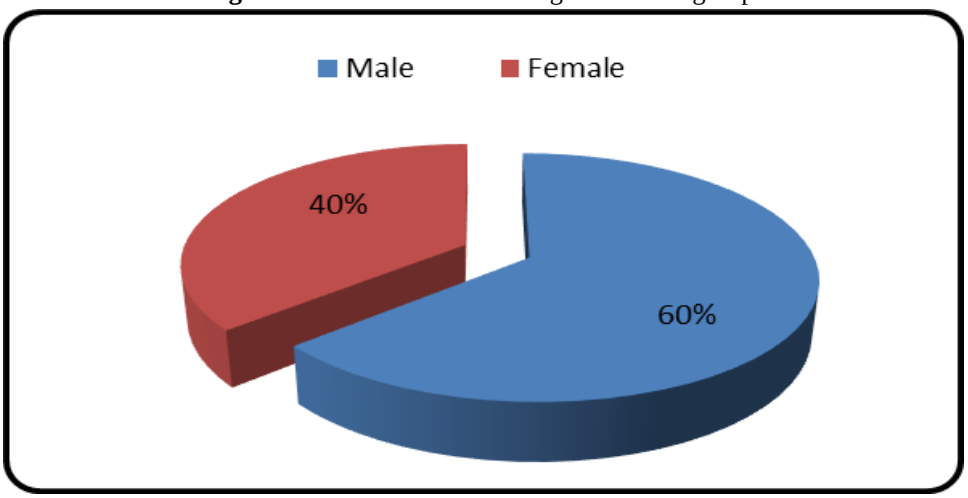


Figure 5. Age distribution among the studied group.

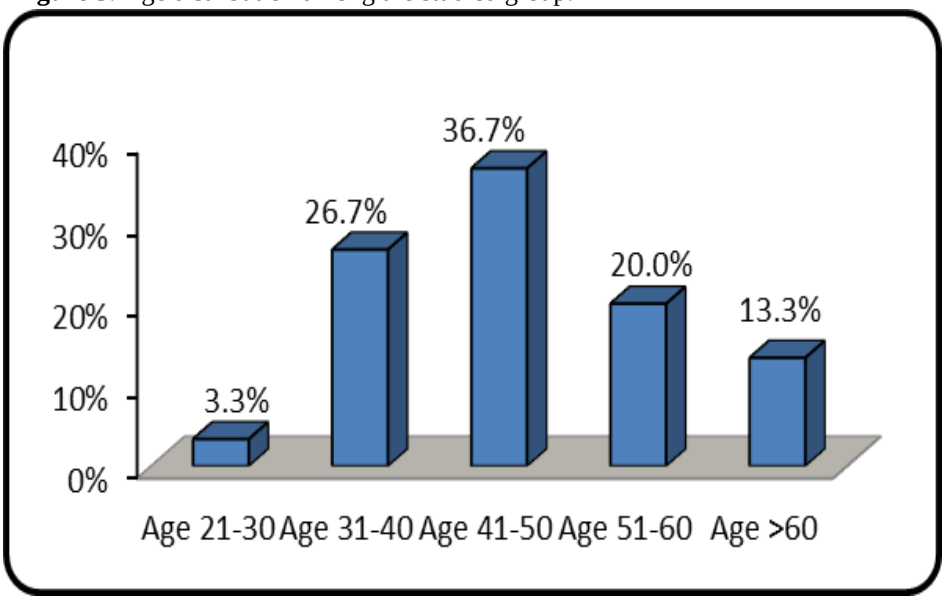


Figure 6. Culture positivity in the corneal scraping samples.

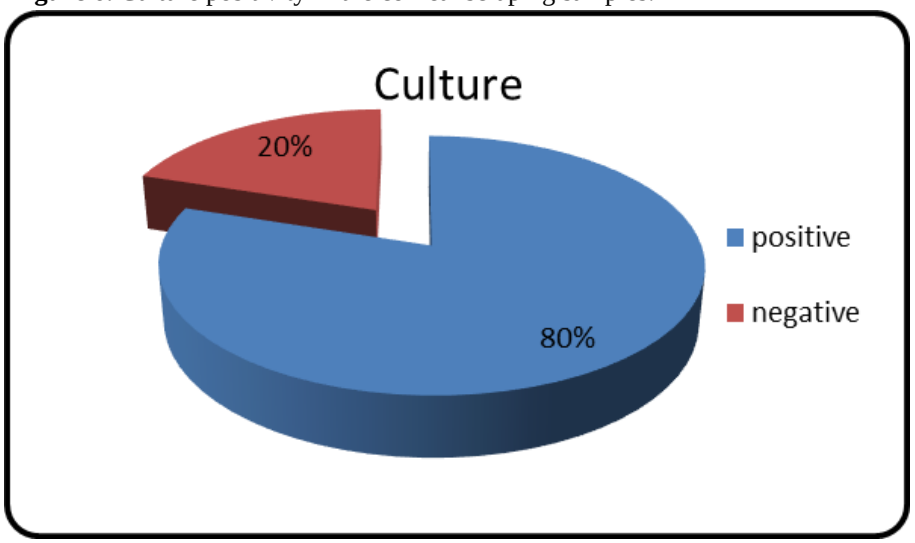
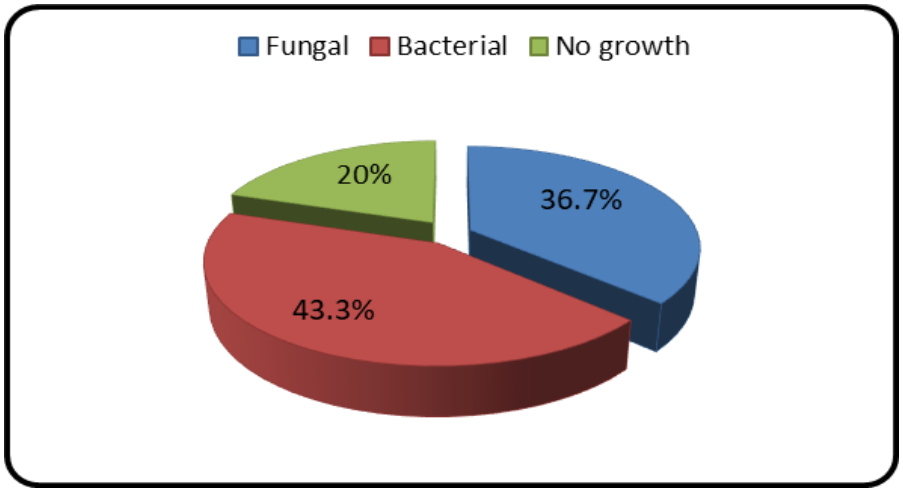


Figure 7. Distribution of culture isolates among corneal ulcer cases.



Discussion

Microbial keratitis is a frequent and possibly vision-threatening ocular infection that is caused by fungi, viruses, bacteria, or parasites. The eyes are normally protected by many natural defense mechanisms from invasion by these microorganisms. However, predisposing variables, including contact lens usage, corneal trauma, and ocular abnormalities might change the eye's resistance and allow fungi to invade the cornea [11]. A Fungal corneal ulcer is an important ophthalmologic problem, especially in developing countries. It is responsible for the significant prevalence of morbidity and blindness worldwide. Fungi, however, are one of the most elusive as well as difficult organisms to treat and diagnose among the organisms that cause keratitis [12].

Men's predominance had been seen in this research (men: 60% and women: 40%). This was in accordance with results obtained by **Khadka et al.** [13], **Furlanetto et al.** [14], **Khor et al.** [15], **Rahimi et al.** [16], and **Manikandan et al**. [6] who revealed a higher preponderance of males diagnosed with microbial keratitis. This may be explained by the fact that, in comparison to female patients, male patients are more susceptible to infectious keratitis owing to their occupations [17, 18].

The most affected age group in our study was 41-50 years (36.7%). This was in concordance with the study done by **Satpathy et al.** [19] (17.9%) and **Nathiya** [20] (32%). This may be explained as people of the age group of 40-50 years are the primary force in manual work, particularly in agricultural work, and are more engaged in outdoor activities [4]. However, **Khor et al.** [15] and **Bou et al.** [21] reported discrepant results as they found that the peak age group presentation among infectious keratitis cases was from 21 to 30 years.

Most of the suspected cases in the current study were farmers 13 (43.3%) which came in line with other studies done by **Reza et al.** [22], **Paty et al.** [23], **Ting et al.** [24], **Chidambaram et al**. [25], and **Park et al.** [26], (34.4%),(66.6%),(62%),(34.5%) and (42%) respectively.

The most frequent predisposing factor related to infectious keratitis in the current research was corneal trauma, with 14 (46.6%) cases, followed by steroid use, with 6 (20%), and DM, with 3 (10%). This came in agreement with a study conducted by **Harbiyeli et al.** [27], **Alekhya et al.** [28], and **Mahmoud et al.** [29]. On the other hand, other publications showed different results as a study conducted by **Gorski et al.** [30] who found that among 155 patients with microbial keratitis, contact lens wear was (39%) followed by corneal trauma (8%). Another study conducted in South Texas by **Puig et al.** [31] found that contact lens wearing (32.4%) is the most common risk factor related to infectious keratitis. These results could be explained by the fact that usage of contact lenses is more common in females and ocular trauma is more in males most probably because of their occupation [24].

Our study revealed that the most common mode of trauma was a vegetative matter, which was seen in (28.6%) of cases. This came in line with the findings of **Chidambaram et al.** [25] that reported that the source of trauma was vegetative matter (46%), among keratitis cases.

Total culture positivity was 80%. This was similar to a study by **Manikandan et al.** [6] (82.2%) and different from other studies done by **Acharya et al.** [32], **Termote et al.** [33], and **Tuft et al.** [34] who reported a lower rate of isolation, 62.9%, 37.5% and 51.6% of culture-positive cases, respectively. Positive corneal ulcer cultures range between 43% and 77%. The differences between studies might be due to variations in the techniques employed to determine positivity; sample collection techniques; prior empirical topical antibiotics; and the inability to employ selective media for isolation of the organisms [29].

Fusarium spp. (36.4%) was the most common fungal pathogen isolated, followed by *Aspergillus* spp. (27.3%) and *Candida* spp. (18.1%). This is comparable to the reported studies conducted by **Manikandan et al.** [6], **Khor et al.** [15], and **Jiragyal et al.** [35]. While **Jacob et al.** reported that *Aspergillus* spp. was the commonest cause in most of the cases (61.8%), followed by *Fusarium* spp. (14.7%). But **Ting et al.** [24] found that *Candida* spp. (62.3%) was the commonest isolate among fungal keratitis patients.

The most common bacterial pathogen isolated was *Staphylococcus aureus* (46.2%) followed by *Pseudomonas* spp. (38.4%). This is consistent with the findings of **Waghmare et al.** [36] and **Ferreira et al.** [37]**.** However, other investigators observed different distribution as in a study by **Khadka et al.** [13] who found that among bacterial isolates, the most prevalent organism was *Streptococcus pneumoniae* (22.1%) followed by *Staphylococcus aureus* (14.7%). **Puig et al.** [31], **Xu et al.** [38] and **Mun et al.** [39] also reported discordant results as they found that Coagulase-*Negative Staphylococcus* was the most common isolated agent, (25.7%), (58.3%) and (15.9%) respectively.

In the current research, we found that Calcofluor white stain had the highest sensitivity (90.9%), specificity (100%), PPV (100%), NPV (95%), and accuracy (96.7%) followed by KOH wet mount which had sensitivity (72.7%), specificity (100%), PPV (86.4%), NPV (86.4%), and accuracy (90%) when compared to culture on SDA. This came in line with **Moemen et al.** [4] who reported that CFW stain had a higher diagnostic performance than 10% KOH wet mount in the diagnosis of fungal keratitis.

Isolation is a conclusive technique for diagnosing fungal keratitis. Much of the research deemed SDA culture to be the gold standard. Direct microscopy using KOH wet mount and CFW stain, when compared with clinical presentation, may, according to reports, be able to detect more instances than culture alone. 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 and time-consuming. So, as a quick, simple, reliable, easy visibility at low power, with high sensitivity and specificity and inexpensive if fluorescent microscope is available in the laboratory, we heartily recommend the use of Calcofluor white stain.

Conclusion

One rapid and efficient way to make an early diagnosis of fungal keratitis is by performing a direct microscopic examination of fungal elements in corneal scrapings using a Calcofluor white stain. When diagnosing fungal keratitis, 10% potassium hydroxide mount is less sensitive than calcofluor white stain with fluorescence microscopy.

Conflict of interest: There aren't any conflicting interests.

Financial disclosure: None.

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