



A Comparative Study of Potassium Hydroxide Wet Mount, Calcofluor White Staining and Culture for the Diagnosis of Keratomycosis

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Abstract

Background: The incidence of keratomycosis has increased dramatically in recent years. Early diagnosis and treatment of keratomycosis are important in preventing further complications. Direct microscopic techniques are time-saving for diagnosing keratomycosis when compared to culture methods. This study was carried out to determine the sensitivities of potassium hydroxide (KOH) wet mount, Gram stain and Calcofluor white (CFW) stain plus KOH wet mount by taking culture as the gold standard.

Methods: Corneal scrapings were collected from 150 clinically suspected patients with keratomycosis. Demographic profile was collected and analyzed.

Results: Of these patients, 67.33% were male, 24% were in the age group of 51-60 years, 70% were rural residents, 44% were agricultural workers, and 60% presented with a history of corneal trauma. Laboratory investigations have revealed that 29.33% (44 cases) were culture positive. In other words, *Fusarium* spp. was isolated in 17 cases, *Aspergillus* spp. in 14 cases, phaeoid fungi in 3 cases and unidentified fungi in 10 cases. The positivity of CFW stain plus KOH wet mount, KOH wet mount, and Gram stain was 30%, 23.3%, and 20%, respectively. Sensitivities of CFW stain plus KOH wet mount, KOH wet mount, and Gram stain were 79.55%, 54.55%, and 47.62%, respectively.

Conclusion: Post-investigative analysis has revealed that CFW stain plus KOH wet mount was better than KOH wet mount alone in demonstrating fungal pathogens. Therefore, early diagnosis of keratomycosis by meticulous examination of corneal scrapings by direct microscopy specifically using CFW stain plus KOH wet mount and institution of antifungal therapy may limit ocular morbidity and disastrous sequelae among these patients.

Keywords: Calcofluor white stain, Potassium hydroxide wet mount, Keratomycosis, Direct microscopy, Gram stain

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Background

Microorganisms including bacteria, fungi, viruses, and parasites penetrate deep into the corneal layers, resulting in inflammation causing keratitis (1,2). Keratomycosis represents 30%-40% of culture-positive infectious keratitis (3). Moreover, fungi have replaced bacteria as the predominant cause of infectious keratitis in developing countries. Epidemiology is complicated and encompasses a wide variety of infectious eye diseases because the prevalence of corneal blindness varies from country to country and even from one population to another, depending on many factors, such as availability and general standards of eye care (4).

Laboratory diagnosis is essential for the accurate diagnosis of etiological agents. Prompt and effective treatment not only slows the progression of the disease but also results in early healing of the ulcer. False negative diagnosis not only delays the specific antifungal therapy

but also injudicious use of medication leads to the rapid growth of organisms (5).

This study was undertaken at a regional institute of ophthalmology:

(i) To identify the fungal etiology of corneal ulcer by direct microscopy using Calcofluor white (CFW) staining, potassium hydroxide (KOH) wet mount, Gram stain, and culture on Sabouraud dextrose agar (SDA).

(ii) To evaluate the sensitivity, specificity, and predictive value of CFW staining and KOH wet mount.

Methods

A total of 150 patients of all age groups and both genders attending the outpatient department, who were clinically suspected of having keratomycosis by the ophthalmologist, were included in the study. Informed consent was obtained from all the participants. The demographic profile of the patients including age, gender, occupation, community,

signs, and symptoms was evaluated.

Inclusion Criteria

Clinically suspected cases of keratomycosis, on the basis of at least two of the following criteria:

1. Patients with a history of trauma to the eye with vegetable matter or organic matter
2. Patients with clinical signs and symptoms of fungal corneal ulcer
3. Ulcer with irregular and feathery margins
4. Ulcer with satellite lesion
5. Presence of an endothelial plaque, fibrinoid aqueous reaction, and hypopyon formation
6. Dry looking ulcer
7. Pigmented ulcers

Exclusion Criteria

1. Patients already on antifungal therapy.
2. Clinically suggestive of bacterial or viral corneal ulcer
3. Other extra ocular infections

Specimen Collection

Under aseptic conditions, after instillation of 4% lignocaine, corneal scrapings were obtained using a Bard-Parker knife (No. 15) to debride material from the base and edges of the ulcerated part of the cornea (6-8).

Specimen Processing

Fungal elements were detected in the clinical specimen by direct microscopic examination of material from lesion. Corneal scrapings were placed on 2 slides for preparing 10% KOH wet mount (Himedia Laboratories L.B.S Marg, Mumbai, India) and Gram stain (Himedia Laboratories L.B.S Marg, Mumbai, India). After reporting KOH wet mount, one drop of CFW (CFW) comprising 1 g/L Calcofluor White M2R and 0.5 g/L Evans blue (Sigma-Aldrich, St. Louis, MO, USA) was then added to one edge of the cover slip and a filter paper was placed at the opposite edge to draw the stain over the smears between the slide and cover slip.

The scraping material obtained from leading edge and base of the ulcer was inoculated directly on to the surface of solid media such as SDA (Himedia Laboratories L.B.S Marg, Mumbai, India) and incubated in biological oxygen demand incubator (Bionics Scientific Technologies (P) Ltd. Sindhora Kalan, Delhi) at 25°C.

Compound microscope (Olympus CX23 – Olympus Medical Systems India Private Limited, Gurgaon, Delhi) was used for KOH wet mount and Gram stain smears. CFW plus KOH smear was visualized under a fluorescence microscope (LB-244, Labomed, Inc. Los Angeles, USA). In KOH wet mount (Figure 1A), fungi were reported as refractile filaments, in CFW plus KOH (Figure 1B) as apple green filaments and in Gram stain as Gram-positive granular fungal filaments or Gram-positive yeast cells

(Figure 1C).

Culture

Fungi on SDA were identified by a combination of:

- Growth rate
- Colonial morphological features: color of colony (on obverse and reverse side), colony topography, colony texture, aerial and submerged hyphae
- Microscopic features: identification of filamentous fungi was based on microscopic features like mycelium, conidium and relationship between hyphae and fruiting bodies. Slide cultures were used for the observation of conidiogenesis of filamentous fungi. Germ tube test and urease test were used for the identification of yeast.

Microbial culture was considered positive if:

1. There was semi-confluent growth at the site of inoculation on solid medium
2. It was consistent with clinical signs.
3. Smear results were consistent with culture.

Sensitivity (true positive rate), specificity (true negative rate), positive predictive value, negative predictive value, and accuracy of the staining methods were calculated using the standard statistical formulae.

Statistical analysis was performed using OpenEpi (version 3.01). The results obtained from direct microscopy of the smear samples were compared with those of fungal culture using the McNemar χ^2 test with respect to sensitivity and specificity. A P value of less than 0.05 was considered statistically significant.

Results and Discussion

In this study, male predominance was seen (males: 67.33% and females: 32.66%). This is in line with all the studies in which keratomycosis was investigated. The most affected age group was 51-60 years (24.0%). This is in accordance with the study of Srinivasan et al (9) (23.04%). According to studies by Deshpande and Koppikar (10) (79%), Kumari et al (11) (77%), Gopinathan et al (12) (64.4%), Bharathi et al (13) (66.85%), Chander et al (14)

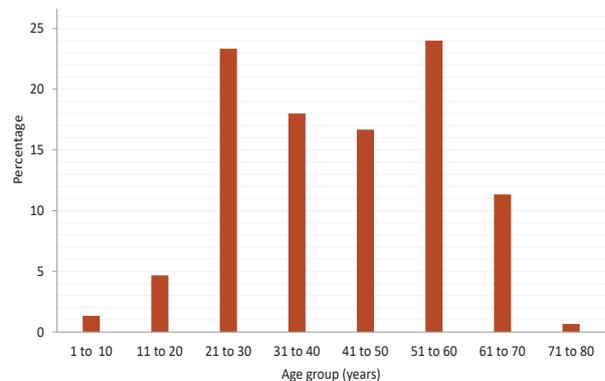


Figure 1. Frequency of Keratomycosis by Age Group.

Table 1 . Predisposing Factors Associated With Keratomycosis

Predisposing Factor	Number Of Cases	Percent	
Vegetable matter trauma	Thorn	3	2
	Stick	18	12
	Flower	1	0.6
	Others	24	16
Foreign body trauma	Stone	7	4.6
	Dust	13	8.6
	Cement	3	2
	Others	21	14
Diabetes mellitus	6	4	
Ocular surgery	6	4	
Steroid therapy	2	1.3	
Others	58	38.66	

(47.14%), and the present study (81.99%), most of the affected individuals are in the age group of 21-60 years (Figure 1).

Most of the suspected cases were from rural backgrounds (70%), which is in line with other studies (13-15). Out of 150 suspected cases, 66 (44%) were agriculture workers, 32 (21.33%) were laborers, 18 (12%) were households, 7 (4.6%) were students, 3 (2%) were garden workers, and 15 (10%) were people in other occupations. This is in accordance with the studies conducted by other researchers (9,16,17). The results of studies by Riaz et al (18) (Domestic group: 25.6%) and Jacob et al (19) (Housewife: 33.3%) are not in line with the present study.

In the present study, the most common predisposing factor associated with suspected keratomycosis was corneal trauma (60 %) (Table 1).

This is in accordance with studies conducted by Reddy et al (16) (52.8%) and Anusuya et al (20) (62.96%). Shokohi et al (17) reported most common predisposing factor as use of antimicrobials (22.7%), compared to corneal trauma (9.09%). It is well documented that corticosteroids may well potentiate these commensal fungi to pathogenicity (21). Agarwal et al (22) provided experimental proof of producing corneal ulcer after the injection of fungi and showed that corticosteroids enhance the virulence.

Clinical symptoms associated with keratomycosis are pain, photophobia, and decreased vision. The slit lamp examination reveals shaggy edged ulcer with satellite lesion, marked hypopyon, and endothelial plaque (24). In the present study, 16.6% of cases had stromal infiltrate, 4% had pigmented ulcer, 53.3% had conjunctival congestion, 46.6% had epithelial defect, and 46% showed hypopyon. Verma et al (25) reported that the corneal ulcers were associated with stromal infiltrate (43.47%), satellite lesions (13.04%), feathered margins (8.69%), hypopyon (60.86%), conjunctival congestion (56.52%), epithelial defect (43.47%), non-reactive pupils (30.43%),

and scleral congestion (26.08%).

Total fungal culture positivity in this study was 29.33% (Figure 2). This is in accordance with the studies by Saha et al (26) (25.6%) and Khorgade et al (27) (23.8%). However, Gopinathan et al (12) and Jacob et al (19) reported fungal culture positivity of 61.53%, 94.5%, and 62%, respectively. This variable incidence suggests the importance of ecological differences in temperature, humidity of the place, number of eyes investigated, and the methods employed.

In the present study, *Fusarium* spp. was isolated from 38.64% of the cases and *Aspergillus* spp. was isolated from 31.82% of the cases (Figure 3). This is in line with other similar studies (9,12,13,20). However, in the studies conducted by other researchers, *Aspergillus* spp. was the most common isolate (3,14,19,29-32). While Jampala et al (33) reported *Candida* spp. as the most common isolate. In the present study, yeast was not isolated; however, a study conducted by Prathiba et al (34) at the same regional institute reported a prevalence of 1% for *Candida*.

Isolation is considered a definitive method of diagnosis of keratomycosis. Culture on SDA was considered as the gold standard in many studies. However, reports suggest that direct microscopy with KOH wet mount when correlated with clinical presentation can reveal more cases than culture on SDA alone.

In the present study, the sensitivity using KOH wet mount was 54.55% (Table 2), in a study conducted by Jampala et al (33), the sensitivity of KOH wet mount was reported to be 33.33%. Positivity of CFW plus KOH wet mount in the present study is in line with a study conducted by Shokohi et al (17) (31.81%) and the

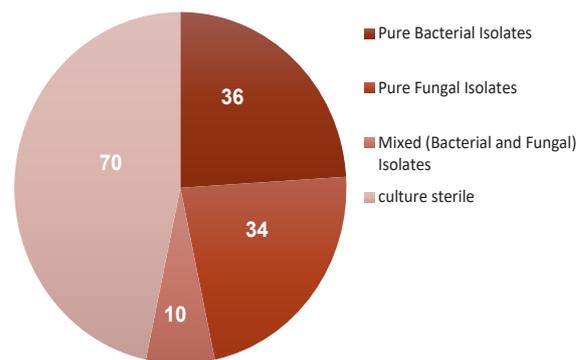


Figure 2. Incidence of Various Microbial Isolates .

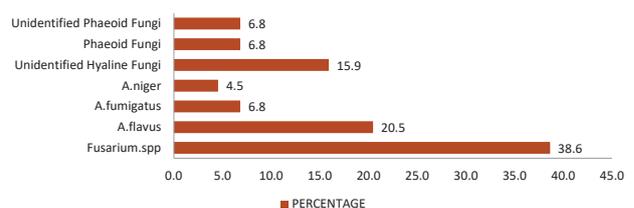


Figure 3. Etiological Agents of Keratomycosis.

Table 2. Comparison of the Results of Fungal Culture and Direct Microscopic Techniques for Sensitivity, specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV), and Accuracy

	Sensitivity	Specificity	PPV	NPV	Accuracy
KOH wet mount	54.55%	89.62%	68.57%	82.61%	79.33%
CFW plus KOH wet mount	79.55%	90.57%	77.78%	91.43%	87.33%
Gram stain	47.62%	90.38%	66.67%	81.03%	78.08%

P value = 0.0025, *P*<0.05 is considered statistically significant.

sensitivity of CFW plus KOH wet mount is in line with the study of Vemuganti et al (35) (84.6%).

The positivity of CFW plus KOH wet mount (30%) was the highest in the present study when compared to culture (29.33%), KOH wet mount (23.3%), and Gram stain (20%). This is in line with the results of studies conducted by other researchers (17,36-38).

Using KOH wet mount in combination with Gram stain smears resulted in an increase in positivity. In this study, 9 cases were KOH wet mount negative and gram stain positive and 10 cases were KOH positive and Gram negative. However, all the 19 cases were CFW plus KOH wet mount positive. This discrepancy in the results may be due to the quantity of the sample taken and the amount of fungal material present in sample. Other researchers did not analyze such a combination. Most of the laboratories in developing countries like India do not have expensive laboratory equipment to analyze samples using CFW plus KOH wet mount. In such setups, a simple KOH wet mount in combination with Gram stain can lead to a diagnosis which is as accurate as CFW plus KOH wet mount.

Conclusions

Keratomycosis continues to be an important cause of ocular morbidity, mostly in the individuals inhabiting rural areas and involving in outdoor and agricultural activities. Young male adults affected in these circumstances are often the breadwinners of their family and blindness in them leads to grave economic consequences. By performing direct microscopy using CFW plus KOH wet mount, treatment was initiated earlier (by 2-5 days) which helped in preventing complications in the patients. The sensitivity and specificity of CFW plus KOH wet mount were higher compared to those of KOH alone. The collection of corneal scrapings is an invasive procedure which increases risk of developing complications in the patients. Further, advancements like in vivo fluorescent imaging technology can be compared with direct microscopy as it is a non-invasive and rapid procedure.

Conflict of Interests

None.

Acknowledgments

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Ethical Approval

This prospective study was approved by the Ethics Committee of Osmania Medical College. It was conducted from December 2015 to May 2016 at Sarojini Devi Eye Hospital, Hyderabad, Telangana, India.

Authors' Contribution

PA: Concept and design of the study, acquisition, analysis and interpretation of data.

CAS: Final approval and monitoring of the study.

P: Critical revision of the study

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