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 cornal 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
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 χ² 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)
Clinical symptoms associated with keratomycosis are pain, photophobia, and decreased vision. The slit lamp examination reveals shaggy edged ulcer with satellite lesions, marked hypopyon, and endothelial plaque (25). In the present study, 16.6% of cases had stromal infiltrate, 4% had pigmented ulcer, 53.3% had conjunctival congestion, 46% had epithelial defect, and 46% showed hypopyon. Verma et al (26) 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 (27) (25.6%) and Khorgade et al (23) (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 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 (3,14,19,29-32). While Jampala et al (33) reported Candida spp. as the most common isolate.
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.

