Microbiological Diagnostic Methods and Spectrum of Fungal Keratitis in Eastern Nepal

Manish Pandey,[1] Pragya Luitel,[1] Amit Rajbanshi,[2] Amit Kumar Mehta,[3] Rajiv Ranjan Karn,[4] Mahesh Kumar Dev,[5] Binod Rayamajhee[6,7,8]

[1]Cornea Department, Biratnagar Eye Hospital, Rani, Biratnagar, Morang, Nepal
[2]Department of Ophthalmic Pathology and Laboratory Medicine, Biratnagar Eye Hospital, Rani, Biratnagar, Morang, Nepal
[3]Quality Department, Biratnagar Eye Hospital, Rani, Biratnagar, Morang, Nepal
[4]Research Department, Lahan Eye and Ear Care System, Biratnagar, Morang, Nepal
[5]Research Department, Biratnagar Eye Hospital, Rani, Biratnagar, Morang, Nepal
[6]Faculty of Medicine, Health and Human Sciences, Macquarie University, New South Wales (NSW) 2109, Australia
[7]School of Optometry and Vision Science, Faculty of Medicine, University of New South Wales, Sydney, NSW, Australia
[8]Department of Infection and Immunology, Kathmandu Research Institute for Biological Sciences, Saptakhel, Lalitpur, Nepal

ABSTRACT

Introduction: The most useful and the quickest diagnostic method for assessing fungal components in corneal scrapings in low-resource settings is microscopic assessment.

Objective: The aim of this study was to compare the diagnostic sensitivity of potassium hydroxide (KOH) and potassium hydroxide + calcofluor white (KOH+CFW) staining for detecting fungal keratitis (FK) using culture as the standard of reference, as well as to analyse their fungal culture results.

Methodology: This was a retrospective study where consecutive patients with microbial keratitis (MK) attending Biratnagar Eye Hospital from 2023 January to 2023 December were recruited. Corneal scrapes were sent for microscopic examination and culture using the standard protocol. Smear microscopy was performed using KOH wet mount, Gram stain, and KOH+CFW stain to determine the presence of microbial components in corneal scrapings. The sensitivity of KOH and KOH+CFW in diagnosing FK was compared.

Result: Among the 2,152 samples examined, 1,507 (70.02%) had pure fungal infections, 121 (5.62%) had pure bacterial infections, and 41 (1.9%) had mixed aetiology (bacteria + fungus). Smear microscopy revealed Acanthamoeba keratitis in eight (0.37%) samples. Direct microscopy of corneal scrapes revealed fungal elements in 1,420 (65.98%) and 1,523 (70.77%) from KOH and KOH+CFW preparations, respectively. Fungal culture was positive in 1,041 (48.37%) samples tested. The sensitivity of KOH+CFW (97.69%) was shown to be significantly higher than that of KOH alone (95.19%) ($p = 0.0013$) in diagnosing FK. Curvularia spp. was the most frequently isolated fungus, with 312/1,041 (29.97%) of positive fungal cultures.

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Corresponding Author
Dr. Manish Pandey
Consultant, Cornea Department, Biratnagar Eye Hospital, Biratnagar, Morang, Nepal.
E-mail: pandeymanish444@gmail.com

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Conclusion: KOH+CFW stain demonstrated to be more sensitive than KOH stain, as a point-of-care diagnostic tool for FK. Fluorescence microscopy with the KOH+CFW stain is recommended as a routine procedure in cases of MK in areas where FK is highly prevalent.

Key words: Calcofluor white; fungal keratitis; microbiological; Nepal; potassium hydroxide; sensitivity.

INTRODUCTION

Microbial keratitis (MK) is a major public health concern, especially in developing countries, and has been described as a "silent epidemic" due to the potential for severe visual impairment and lifelong impact on patients (Whitcher and Srinivasan, 1997). Nepal has a disproportionately high incidence of corneal ulcers, with rates seven times higher than those observed in South India, emphasising the region's unique epidemiological burden (Upadhyay et al., 2001; Gonzales et al., 1996).

Aspergillus and *Fusarium* species are frequently reported as the most common causes of mycotic keratitis worldwide (He et al., 2011; Kredics et al., 2015; Satpathy et al., 2019). However, emerging data from South Asia indicate that the prevalence of dematiaceous fungi, such as *Curvularia* spp., is increasing, which could be attributed to agricultural exposures and climatic factors (Hoffman, Yadav, Das Sanyam, et al., 2022). These regional variations in pathogen distribution complicate empirical treatment and emphasise the importance of microbiological confirmation.

While advanced diagnostic techniques such as polymerase chain reaction (PCR) and in vivo confocal microscopy optimise the accuracy and speed of diagnosing fungal keratitis (FK) (Ghenciu et al., 2024), traditional methods such as corneal scraping smears and cultures remain crucial in low-resource settings. Among staining techniques, the relative sensitivity of potassium hydroxide (KOH) and calcofluor white (CFW) for FK diagnosis has been debated (Hoffman, Yadav, Sanyam, et al., 2022; Zhang et al., 2010). Some studies suggest that CFW is more sensitive than KOH, but there is no definitive consensus.

This study aimed to compare the diagnostic sensitivity of KOH and KOH+CFW staining in detecting FK, using culture as the reference standard and characterise the mycological profile of FK in a tertiary eye hospital in eastern Nepal.

METHODOLOGY

Patients were recruited retrospectively from Biratnagar Eye Hospital (BEH), Biratnagar, Morang, Nepal, between 2023 January and 2023 December. The BEH is a tertiary eye hospital located in the warm, humid Terai region of eastern Nepal. The study comprised corneal scraping samples from non-viral MK that were sent for microbiological analysis. The ethical approval was obtained from the Institutional Review Committee of the BEH (Reference number: BEH-IRC-110/2024; Dated: 2024 July 25). A total of 2,152 corneal scraping samples of all age groups were collected during the period of 12 months.

Corneal scrapings from patients with microbial corneal ulcers were performed in the Cornea Department of BEH under local anaesthesia

(instillation of 4 % lignocaine) using a sterile surgical blade (15 number blade on a Bard Parker handle) (McGrath and Lee, 2014). The scrapings were taken from the leading edge and the base of each ulcer (Bharathi et al., 2006). These samples were sent for microbiological evaluation in the Department of Ophthalmic Pathology and Laboratory Medicine of BEH. Material from the corneal scraping was smeared on two glass slides. It was also inoculated directly into blood agar, chocolate agar, and Sabouraud Dextrose Agar (SDA).

Standard procedures were followed for the KOH wet mount, Gram stain, KOH+CFW stain, and cultures of the corneal scraping samples. The scrape material was transferred to a glass slide. In the first step, one or two drops of sterile 10% KOH were applied and covered using a clean coverslip. After 5-10 minutes, the slide was inspected under a microscope at 10X and 40X magnifications to detect hyphal elements or budding yeast (Zhang et al., 2010; Anwar et al., 2022)**.** In the second step, one drop of calcofluor white (1 g/l Calcofluor White and 0.5 g/l Evans blue; Sigma-Aldrich, St Louis, MO, USA) was then added. After allowing the slide to stand for one or two minutes, the slide was studied using fluorescence microscopy with blue light excitation (excitation at approximately 355 nm and emission wavelengths of 300–400 nm) (Zhang et al., 2010). Fungal filaments were recognised at low power (×100) and verified at higher power (×400 or ×1000) if they showed branching and septation in the centre of the smear (Anwar et al., 2022). Furthermore, Gram stain was performed on the second slide and examined under 100X oil immersion for bacteria (Poudyal et al., 2022)

Blood agar and chocolate agar plates were incubated aerobically at 37°C. They were checked for bacterial colonies twice at 24 hours and 48 hours and disposed of after 72 hours if no growth was seen. In a similar manner, a SDA plate was incubated at 25°C, and its fungal growth was monitored twice a week for the first week. If no growth was seen after two or more weeks, it was discarded as a non-fungal keratitis sample (Poudyal et al., 2022).

Microscopy and culture were used to determine the laboratory diagnosis of MK. The growth of the same strain on two or more solid culture media, semi-confluent growth at the site of inoculation, growth on one solid medium consistent with microscopy (appropriate staining and morphology with Gram stain), semi-confluent growth at the site of medium (if bacteria), or growth of the same organism on repeated scraping were all considered significant indicators of microbial culture (Thomas et al., 2005). Bacterial ulcers were diagnosed based on positive culture. Smear microscopy alone was not considered to be conclusive evidence (Hoffman, Yadav, Sanyam, et al., 2022; Thomas et al., 2005). The criteria for diagnosis of FK included the presence of fungal hyphae or yeast observed on microscopic examination or growth at the site of inoculation on solid culture media (Poudyal et al., 2022).

Data from microbiological records were entered into a Microsoft Office Excel spreadsheet in a systematic manner to ensure organisation. Demographic information (age, gender), staining results (KOH, KOH+CFW), fungal culture outcomes, and pathogen identification were all considered variables. Age was divided

into three categories: less than15, 15 to 59, and 60 years and above. The statistical analysis was carried out using IBM SPSS Statistics version 23 (IBM Corp., Armonk, N.Y., USA). Categorical variables (such as age groups and pathogen distribution) were summarised using frequencies and percentages. Continuous variables, such as age, were presented as means ± standard deviations.

The sensitivity of KOH and KOH+CFW stains was calculated as follows: sensitivity = [number of true positives (TP) / (number of true positives (TP) + number of false negatives (FN)] x 100, where TP = smear-positive cases confirmed by culture and FN = smear-negative cases with positive cultures. Z-test for proportions compared sensitivities of KOH vs. KOH+CFW. Results were deemed statistically significant at $p < 0.05$.

RESULT

Of the 2,152 samples of corneal scrapings performed among study participants, 1253 (58.2%) samples were from males and 899 (41.8%) were from females. Among the patients, 53 (2.5%) were younger than 15 years of age, 1,541 (71.6%) were 15 years to 59 years of age, and 558 (25.9%) were 60 years and above. Thus, the majority of patients belonged to the younger age group.

Curvularia species was the most commonly detected fungus, isolated in 312/1041 (29.97%) of positive fungal cultures (Table 1). A third of all fungal species that were cultured (352/1041, 33.31%) were dematiaceous fungus. *Fusarium* spp. (241/1041, 23.15%) and *Aspergillus* spp. (159/1041, 15.27%) were the second and third most frequently isolated genera, respectively. In 256/1041 samples (24.59%), the fungal

Table 1: Identification of fungi isolated from corneal samples of patients with microbial keratitis.

Fungi	n (%)
Curvularia spp.	312 (29.97)
Fusarium spp.	241 (23.15)
Aspergillus spp.	159 (15.27)
Acremonium spp.	25 (2.4)
Alternaria spp.	18 (1.72)
Cladosporium spp.	10 (0.96)
Exserohilum spp.	7 (0.67)
Bipolaris spp.	5(0.48)
Candida spp.	4 (0.38)
Penicillium spp.	3 (0.28)
Scedosporium spp.	1 (0.09)
Unidentified fungus	256 (24.59)
Total	1041 (100)

pathogen could not be identified. *Candida* spp. infection was found in four (0.38%) of the culture positive samples.

Fungal elements (hyphae, pseudohyphae, and yeast cells) were observed by direct microscopy of corneal scrapings in 1420 (65.98%) and 1523 (70.77%) from KOH preparation and KOH+CFW preparation, respectively (Table 2). Fungal culture was positive among 1,041 (48.37%) of the total sample. A total of 1,548 (71.93%) samples of fungal keratitis were detected, with the diagnosis being made if fungal elements were found on microscopic examination or growth on culture media. A total of 162 (7.5%) samples of bacterial keratitis (culture-positive samples) were identified. Mixed aetiology (bacteria + fungus) that is, co-infection was seen in 41 (1.9%) samples. Pure fungus was seen in 1,507 (70.02%) and pure bacteria in 121 (5.62%) samples. Also, using smear microscopy, eight samples of *Acanthamoeba* keratitis (observed as cysts or trophozoites) were identified.

The test result revealed that the sensitivity of KOH+CFW stain (97.69%) was significantly greater than that of KOH stain (95.19%) as an aid in the diagnosis of fungal keratitis ($p = 0.0013$).

Table 2: Comparison of results of fungal culture and microscopic examination after staining with KOH stain and KOH+CFW stain and the sensitivities of these stains relative to the culture results for the detection of FK in corneal scraping samples (n=2152) with MK.

	KOH		KOH + CFW	
Fungal culture	**positive**	**negative**	**positive**	**Negative**
Positive	991	50	1017	24
Negative	429	682	506	605
Total	1420	732	1523	629
Sensitivity		95.19		97.69

p=0.0013

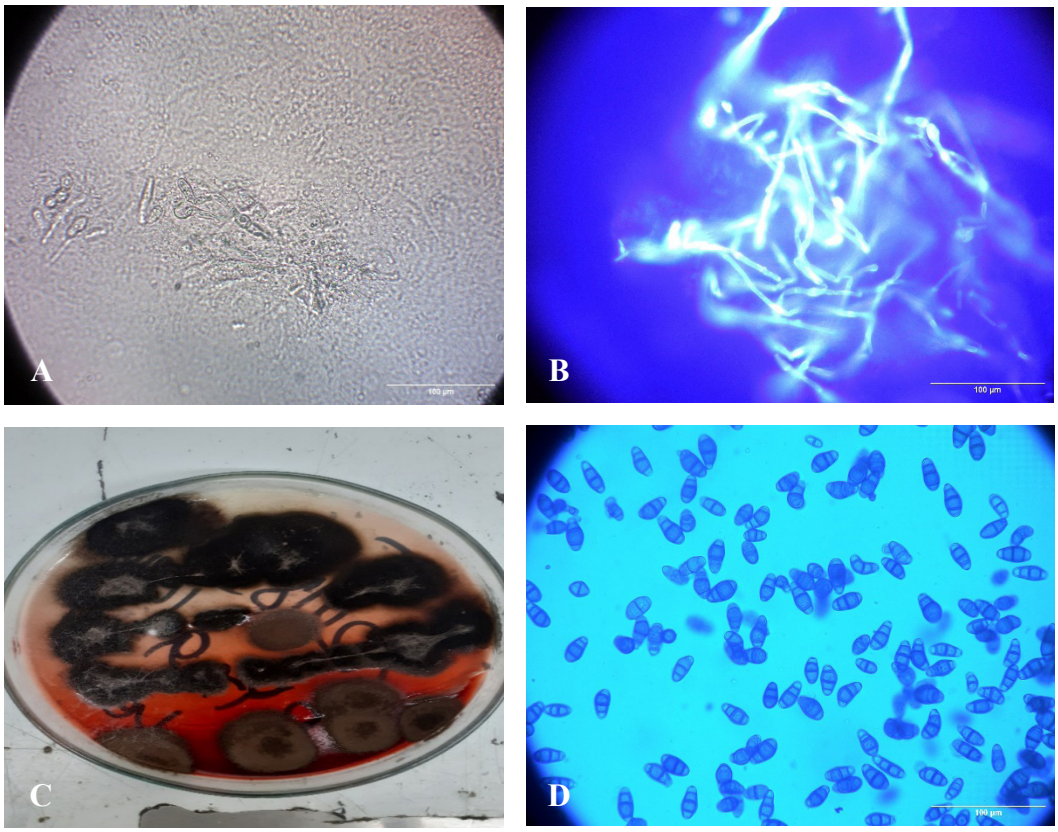

Figure 1: Microscopic and macroscopic images of filamentous fungi from study samples: a) A microscopic image of filamentous fungus in KOH mount revealing septate hyphae at 40X magnification; b) A fluorescent microscopic image of filamentous fungus stained with CFW, revealing brightly illuminated hyphal cell walls at 40X magnification; c) A macroscopic image of Curvularia spp. colony on SDA exhibiting colony's characteristic dark brown to black pigmentation; d) A microscopic image of Curvularia spp at 40x magnification demonstrating characteristic septate hyphae and conidiophores bearing curved multicellular conidia.

DISCUSSION

The findings of this study provided insights into the diagnostic efficacy of microscopic techniques and the mycological profile of FK in a large cohort of 2,152 patients. The data emphasised the importance of optimising diagnostic approaches and understanding the regional epidemiological trends to enhance clinical management of FK in low resource settings.

The high prevalence of FK, with 71.93% of samples containing fungal elements or growth, was consistent with previous studies (Sitoula et al., 2015; Hoffman, Yadav, Das Sanyam, et al., 2022; Suwal et al., 2016). This finding indicates that fungal infection is one of the major causes

of corneal morbidity, particularly in this region. The observed male dominance (58.2%) reflected that occupational or behavioural risk factors, as men are often subjected to environmental hazards associated with FK (Gopinathan et al., 2002). Moreover, the fact that the majority of patients (71.6%) were in the age group of 15 years to 59 years, highlights the significance of ocular trauma and exposure in this active population group (Sitoula et al., 2015).

This study placed greater emphasis on the sensitivity of the diagnostic tests (KOH and KOH+CFW) for diagnosing FK. This is due to the fact that the sensitivity of a diagnostic test was positively correlated with the disease's prevalence (Murad et al., 2023; Brenner and Gefeller, 1997). In high-prevalence settings, like in this study, where the prevalence of FK was 71.93%, the test's accuracy was heavily dependent on its sensitivity (Eisenberg, 1995). Also, the research in this region has shown the FK prevalence rates ranging from 70% to 82.9% (Sitoula et al., 2015; Hoffman, Yadav, Das Sanyam, et al., 2022). Thus, we compared the sensitivities of two microscopic stains (KOH and KOH+CFW) relative to the fungal culture results.

The diagnostic sensitivity of CFW-enhanced microscopy observed in this study was consistent with prior evidence supporting its superiority over conventional KOH staining (Zhang et al., 2010; Sharma et al., 2002). It has been demonstrated that CFW stain was able to identify fungal components even in low concentrations and in patients using antifungal medications (Marines, Osato and Font, 1987). Findings from the present study support this claim, as KOH+CFW had a significantly higher sensitivity (97.69%) than KOH alone (95.19%, p = 0.0013), reducing false negatives by 50% (50 vs 24). This improvement is crucial in clinical settings where prompt antifungal therapy, a major factor in determining visual outcomes in FK, depends on early diagnosis (Thomas et al., 2013). Utilising both the high sensitivity of CFW to identify fungi and the properties of KOH to digest contaminants, the combination staining procedure increases the likelihood of appropriate diagnosis of FK (Zhang et al., 2010). However, the gold standard for diagnosis remains fungal culture, which produced positive results in 48.37% of samples. The disparity between microscopy and culture positivity can be attributed to the fact that all samples with fungal filaments in smears may not show growth in culture (Sharma et al., 2002). In this study, combining smear microscopy and culture resulted in a diagnostic yield of 71.93%, demonstrating the complementary roles of rapid microscopy (for early intervention) and culture (for species identification).

In the current study, *Curvularia* spp. was the most frequently isolated fungus (29.97%), followed by *Fusarium* spp. (23.15%) and *Aspergillus* spp. (15.27%). These findings were in contrast to the reports from north and south India, where *Aspergillus* spp. and *Fusarium* spp. were predominant, indicating the regional variations in FK epidemiology (Ghosh et al., 2016; Chowdhary and Singh, 2005; Gopinathan et al., 2002). A Mexican study suggested that warm, humid climates (similar to our region) may favour Curvularia keratitis (Wilhelmus, 2005), a trend also observed in the Australian tropics (Kim et al., 2024). Along with other filamentous fungi like *Fusarium* spp. and *Aspergillus* spp., the study's preponderance of

dematiaceous fungi, especially *Curvularia* spp., suggests FK caused by agricultural exposure (Chowdhary and Singh, 2005). The rarity of *Candida* spp. (0.38%), which is typically associated with chronic ocular surface disease or contact lens wear (Tanure et al., 2000), further supports this hypothesis. The unidentified fungal isolates (24.59%) highlights the need for advanced molecular diagnostic techniques to enhance pathogen identification (Ghenciu et al., 2024).

The strengths of this study included a large sample size (2,152), which increased the statistical power and generalisability of the findings. This study employed multiple diagnostic methods: direct microscopy with KOH and KOH+CFW staining and fungal culture. This comprehensive approach has a high potential to improve the accuracy of FK detection. However, the major limitations were the retrospective nature of the study, the lack of clinical correlation, and the single-centre design that may limit generalisability. Future studies should correlate fungal speciation with antifungal susceptibility.

CONCLUSION

In conclusion, this study demonstrated that fluorescence microscopy using KOH+CFW stain was superior to KOH wet-mount in terms of sensitivity for diagnosing FK. Therefore, in regions where FK is highly prevalent, fluorescence microscopy with KOH+CFW stain should be the standard diagnostic procedure for all MK.

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