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Sensitivity and specificity of potassium hydroxide and calcofluor white stain to differentiate between fungal and *Pythium* filaments in corneal scrapings from patients of *Pythium* keratitis

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Purpose: To assess the sensitivity of potassium hydroxide and calcofluor white (KOH+CFW) mount in the diagnosis of *Pythium* keratitis and concordance among microbiologists. **Methods:** Three microbiologists evaluated the microscopic images of KOH + CFW mounts of confirmed cases of *Pythium* and fungal keratitis seen between January 2019 and February 2021. The filaments were compared using specific differentiating features. The sensitivity and specificity of KOH + CFW in diagnosing *Pythium* infection were evaluated along with concordance among the microbiologists. **Results:** Sixty consecutive cases with confirmed growth of fungus or *Pythium insidiosum* (n = 29) were evaluated. The sensitivity of KOH + CFW in the correct identification of *Pythium* filaments ranged from 79.3% to 96.5% among three microbiologists. There was good interobserver (k = 0.76–0.90) and intraobserver (k = 0.70–0.97) agreements among three microbiologists. The differentiating findings ($P < 0.0001$) suggestive of *Pythium* filaments were the absence of septae in 23 (79.3%) and collapsed walls in 22 (75.9%) cases. **Conclusion:** KOH + CFW has good sensitivity and specificity in the diagnosis of *Pythium* keratitis with good interobserver and intraobserver concordance.

Key words: Calcofluor white, fungal keratitis, potassium hydroxide, pythium keratitis, sensitivity

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Pythium insidiosum is a fungus-like pathogen classified as an oomycete.[1] It causes ocular as well as systemic pythiosis. The management of pythiosis has several challenges including its diagnosis and treatment. On microscopic examination, *Pythium* closely resembles fungal filaments. Due to a lack of ergosterol in cell membrane, it does not respond to antifungals and often responds to a combination of linezolid[2] and azithromycin applied topically.[3] Even with this understanding,[3,4] successful management needs timely diagnosis at an early stage. In late stages, the prognosis is very poor, even after surgical intervention, with higher rates of recurrences[5,6] and evisceration.[5,7]

Pythium keratitis often presents clinically as fungal keratitis that worsens on antifungal treatment. One of the challenges is the early identification of its salient microscopic features to differentiate from fungal filaments. At present, the gold standard in the confirmation of diagnosis is polymerase chain reaction (PCR) or culture along with the production of zoospores[8] in aquatic media. Potassium iodide-sulfuric acid has been used to make a diagnosis on smears under direct observation,[9] but the use of higher strength (65%) of sulfuric acid and the need for immediate examination make it cumbersome to use in general.[10] Various serological, molecular, proteomic, and microbiological assays have been used.[11-16] Confocal scan[17] has been used as a noninvasive method to diagnose the organisms such as *Nocardia* and fungal filaments. It cannot differentiate *Pythium* from *Aspergillus* or *Fusarium* filaments and should be substantiated with microbiological methods.

Although all the methods are very specific and sensitive, they cannot be used in general due to lack of resources, the cost involved, and a need for expertise. The potassium hydroxide and calcofluor white (KOH + CFW mount)[18,19] is found to have good sensitivity as well as specificity for early and late fungal keratitis.[20,21] As KOH is relatively simple and can be easily adopted by microbiologists and ophthalmologists in endemic areas, it will be easy to make use of this commonly used technique to increase the diagnosis rate with addition of CFW. The present study aims to evaluate the sensitivity of KOH + CFW mount for diagnosing *Pythium* and the salient differentiating features from fungi.

Methods

The images of KOH + CFW mounts were retrieved from the microbiological records of *Pythium* or fungal keratitis and were evaluated. KOH + CFW mount was prepared by using 10% KOH and 0.1% CFW and was seen under a fluorescence microscope (×400) and images taken. These images were acquired using the camera attached to the microscope, while

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the smears were examined by one of the three microbiologists. All the assigned microbiologists have >5 years of experience. The organisms belong to either *Pythium* or fungus as confirmed by growth in culture alone or additional zoospores production in cases of *Pythium*. The images studied were in clear focus and highlighted the special features of filaments, while unclear images or cases with mixed infection in growth were excluded. The images included for the analysis were from cases seen from January 2019 to February 2021.

All the images were presented in a random order to the three microbiologists. The microbiologists were asked to identify if the organism is *Pythium* or fungus and this exercise was repeated again with images presented in a different order. Features such as aseptate or sparsely septate, broad, collapsed wall, thin-walled, vesicular expansion, and ribbon folds were also noted down for each image.

The statistical analysis was performed using the software STATA v14.2 (StataCorp, College Station, TX, USA). Categorical data were described in proportions. Data on KOH + CFW staining were recorded as true positive, false positive, true negative, or false negative with respect to culture. Sensitivity, specificity, and their 95% confidence intervals (CIs) were estimated after cross-classification. Linear weighted kappa scores were estimated to assess the intraobserver and interobserver concordances. A value of 0.75 or more was considered a measure of excellent concordance. The proportions of filament features between two organisms were compared by Chi-square or Fisher Exact test and odds ratios were evaluated. P <0.05 was considered statistically significant.

Table 1: The number of species of fungus included for image examination and comparison from *Pythium* species

Name of the fungus species	Numbers (*n*)
Fusarium	9
Aspergillus	9
Alternaria	3
Acremonium	1
Nigrospora	1
Colletotrichum	1
Humicola	1
Scedosporium	1
Bipolaris	1
Curvularia	1
Unidentified fungus	3

Results

A total of 60 images of KOH + CFW mounts were evaluated, among which 31 were confirmed fungal growth while 29 were *Pythium*. The details of fungal species grown in culture are mentioned in Table 1. The microbiological signs [Fig. 1] of filaments that were used in the identification and their comparison are shown in Table 2. The features exclusively found in higher proportions (P < 0.0001) in *Pythium* were absence of septae (aseptate) in 23 (79.3%) and collapsed wall of filaments in 22 (75.9%). Broad, thin-walled, vesicular expansion and ribbon folds were also significantly higher in *Pythium*. Gram stain confirmed the presence of filaments in 24/29 (82.7%) and 26/31 (83.8%) smears of *Pythium* and Fungal, respectively.

The sensitivity of KOH + CFW mount in diagnosing *Pythium* filaments by three microbiologists was 96.55% (95% CI: 80.37%–99.82%), 89.66% (95% CI: 71.50%–97.29%), and 79.31% (95% CI: 59.74%–91.30%) respectively. The specificity was 100% (95% CI: 86.27%–100%), 100% (95% CI: 86.27%–100%), and 93.55% (95% CI: 77.16%–98.88%). The interobserver concordances among three microbiologists were 0.87 (95% CI: 0.74–0.99), 0.83 (95% CI: 0.69–0.97), and 0.76 (95% CI: 0.60–0.93), respectively. The intraobserver Kappa scores within each microbiologist was 0.97 (95% CI: 0.90–1.00), 0.76 (95% CI: 0.60–0.93), and 0.69 (95% CI: 0.50–0.88), respectively.

Discussion

This study highlighted the utility of KOH + CFW mount as a useful method to detect *Pythium* and differentiate it from fungal filaments. Specific features of filaments of *Pythium* are also described in detail, which will be helpful in recognition of the organism in corneal scrapings. Recently, there has been increased use of nested PCR, AFLP fingerprinting,[22] loop-mediated isothermal amplification (LAMP),[12] immunohistochemical assays for antibodies,[13] and MALDI-TOF[15] for the diagnosis of *Pythium* keratitis. These techniques are very specific as well as sensitive for diagnosis. While the sensitivity of LAMP[12] is approximately 88%, anti-elicitin antibody detection[13] from histopathological sections is found to be 100% specific. Nested PCR[14] has a high sensitivity (50%) and high specificity (94.7%) in comparison with culture.[14] We found that the sensitivity of KOH + CFW varied from 79.3% to 96.5% among three microbiologists. This is comparable to all the other newer methods. Although the difference in the sensitivities is not much, there is a significant difference in the cost involved. This suggests its usefulness as a first-line screening tool for *Pythium* keratitis in all the clinically suspected[4,5] cases. *Pythium* has coenocytic filaments with no cross walls that appear as aseptate filaments under the microscope. These along with collapsed

Table 2: Microbiological features of filaments of *Pythium* filaments and their comparison with fungal filaments

Feature	Pythium (*n*=29)	Fungus (*n*=31)	*P*	Odds ratio (95% CI)
Aseptate	23 (79.3%)	0 (0%)	<0.0001	Infinity
Sparsely septate	6 (20.7%)	4 (12.9%)	0.50	
Broad	13 (44.8%)	5 (16.1%)	0.03	4.23 (1.27-14.10)
Collapsed	22 (75.9%)	0 (0%)	<0.0001	Infinity
Thin-walled	19 (65.5%)	2 (6.5%)	<0.0001	27.55 (5.43-139.87)
Vesicular expansion	12 (41.4%)	2 (6.5%)	0.004	10.24 (2.04-51.32)
Ribbon folds	25 (86.2%)	1 (3.2%)	<0.0001	187.5 (19.67-1787.35)

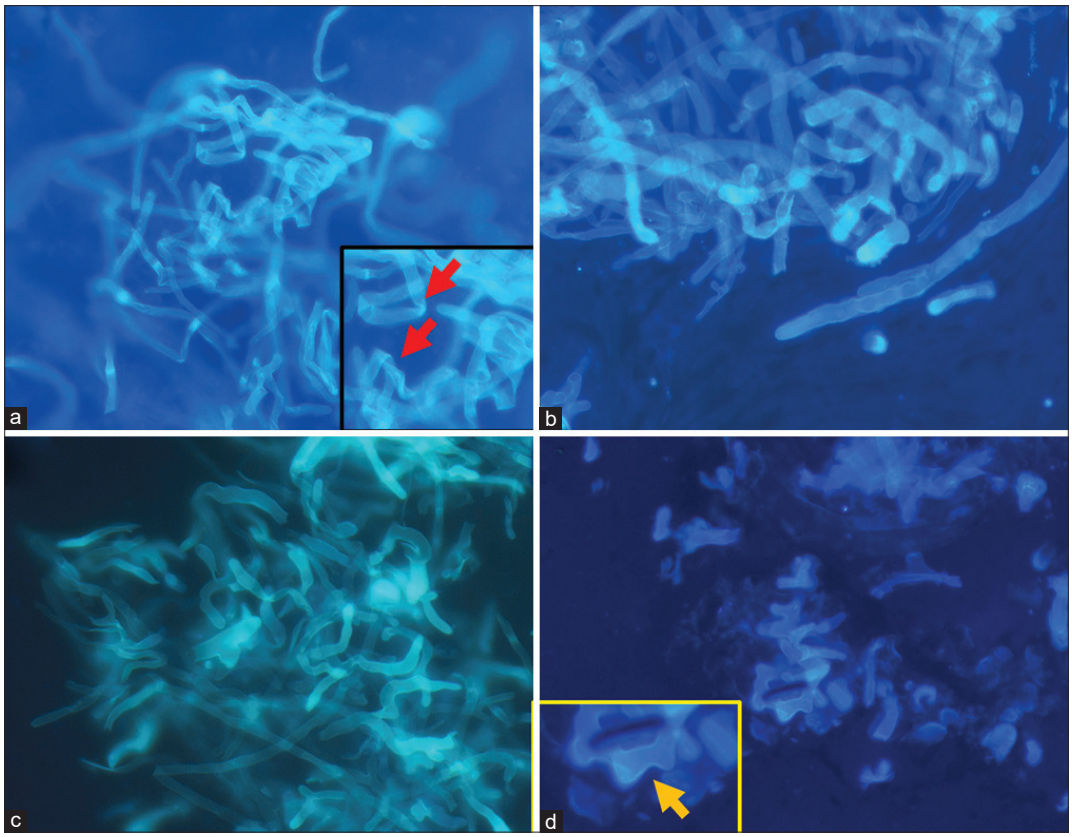

Figure 1: Highlights the differentiating microbiological features of *Pythium* filaments: (a) Aseptate, ribbon-like folds (inset, red arrow) with collapsed walls; (b and c) Broad filaments (d) Vesicular expansions (inset, yellow arrow) from the filaments

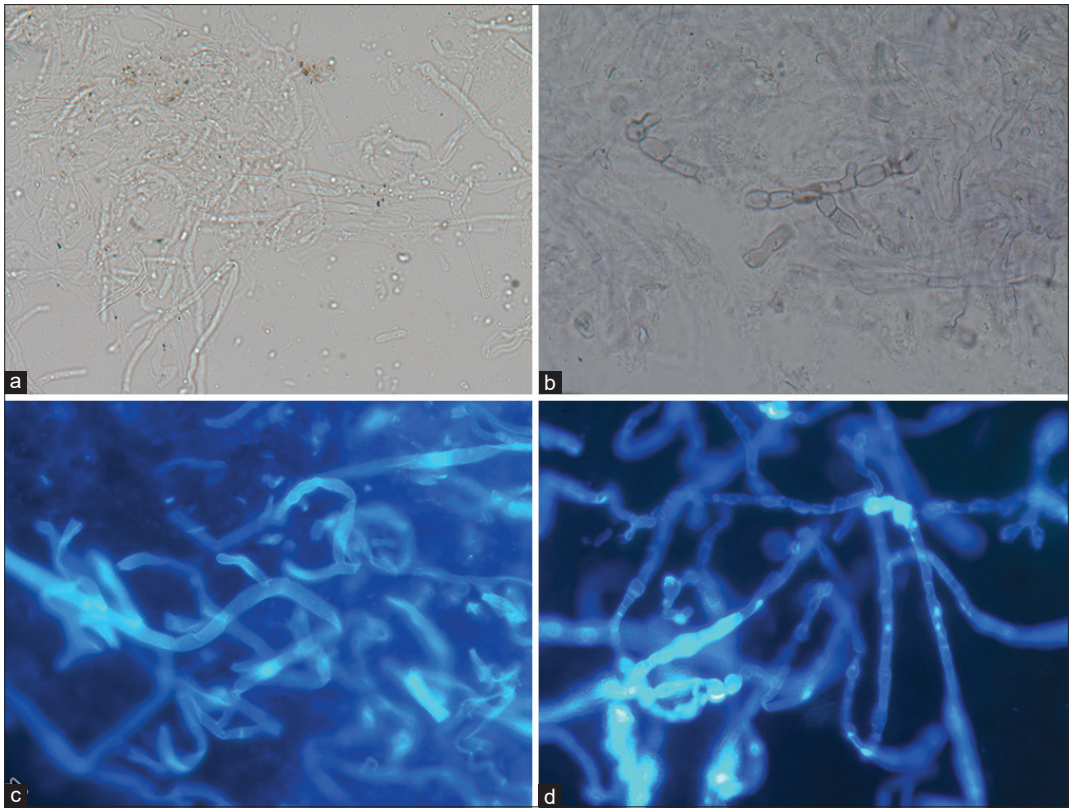

Figure 2: Shows comparison between Fungal (b and d), Pythium (c and d) filaments in KOH (a and b) and KOH+CFW (c and d) mounts

walls were the most important features in differentiation from fungal filaments which are parallel, thinner in most species (some may be broad), and have well-delineated margins.

The technique has its limitation being a subjective test and depends on the experience of the microbiologist or observer. As with any other test, KOH + CFW can be improved with repeated use. As illustrated in Figs. 1 and 2, the difference between filaments of *Pythium* and fungus can be made out in KOH + CFW by the unique features of *Pythium* filaments. The other limitation is the requirement of a fluorescence microscope, which is not available in all centers.

Conclusion

In summary, the results of the present study can help ocular microbiologists to screen for *Pythium* and differentiate from closely resembling fungal filaments and help in improving the prognosis of this deadly infection. The results of the study cannot be extrapolated for ophthalmologists but definitely can be a useful guide for ophthalmologists particularly those from endemic areas. Furthermore, while using this technique, we need to consider the possible discrepancies[23] between direct smear examination and its correlation with culture as a gold standard.

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Conflicts of interest

There are no conflicts of interest.

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