Chicago Sky Blue Stain, Calcofluor White Stain, and Potassium Hydroxide Mount for Diagnosis of Dermatomycosis

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ABSTRACT

Objective: To evaluate Chicago Sky Blue (CSB) stain, Calcofluor white (CW) stain, and Potassium Hydroxide (KOH) mount for rapid diagnosis of dermatomycosis, using fungal culture as the gold standard.

Study Design: Cross-sectional analytical study.

Place and Duration of the Study: This study was conducted in the Department of Microbiology, Armed Forces Institute of Pathology / National University of Medical Sciences, Rawalpindi, Pakistan, from July 2023 to February 2024.

Methodology: Clinical specimens were collected from patients suspected of having dermatomycosis, including skin scrapings, hair, and nails. Each sample was divided into two parts. The first part underwent three microscopic techniques, namely 10% KOH mount, CSB stain, and CW stain. The presence and absence of hyphae were noted by each of the techniques. Second part was inoculated onto Sabouraud Dextrose agar (SDA) with and without antibiotics, along with Dermatophyte test medium (DTM). Culture plates were incubated at 30°C for four weeks. True-positive, true-negative, and diagnostic accuracy of the microscopy methods were calculated against fungal culture.

Results: Out of 121 patients, the majority were females constituting 65.3% (n = 79) while males were 34.7% (n = 42). The average age of participants was 32.98 ± 16 years. Diagnostic accuracy was 82.6% for CSB, 87.6% for CW stain, and 76.8% for 10% KOH mount.

Conclusion: CW stain proved to be superior to 10% KOH mount and CSB stain for rapid dermatomycosis diagnosis, with enhanced sensitivity and diagnostic accuracy. Both CW and CSB stains are recommended for laboratory use to improve reporting accuracy as compared to the conventional 10% KOH mount technique.

Key Words: *Calcofluor white*, *Chicago sky blue*, *Dermatomycosis*, *Fungal culture*, *Fungal hyphae*.

How to cite this article: Khalid A, Gill MM, Khan MA, Naqvi SH, Roshan M, Imtiaz A. Chicago Sky Blue Stain, Calcofluor White Stain, and Potassium Hydroxide Mount for Diagnosis of Dermatomycosis. *J Coll Physicians Surg Pak* 2025; **35(01)**:44-48.

INTRODUCTION

Fungal infections are becoming an increasing concern in both healthcare and community settings globally. These infections are typically categorised by their location in the body: Superficial, subcutaneous, and systemic.[1] Superficial mycoses or dermatomycoses affect the outer keratinised layer of the skin, nails, and hair. They are caused by the dermatophytes, non-dermatophytic moulds or yeasts.[2] They cause morbidity by cosmetic disfigurement, chronicity and recurrences, thus representing a significant health concern.[3]

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Received: August 19, 2024; Revised: October 25, 2024; Accepted: December 20, 2024
DOI: https://doi.org/10.29271/jcpsp.2025.01.44

Apart from causing everlasting damage to the affected area, severe dermatomycosis in immunocompromised individuals can pose a life-threatening risk.[4]

Recently, the occurrence of superficial fungal infections has been steadily increasing, impacting roughly 20-25% of people worldwide.[5] A study conducted in Sindh, Pakistan, found that acne was the most common dermatological condition, occurring in 23.3% of cases, while fungal infections followed at 16.3%.[6]

Identifying dermatomycosis clinically can present challenges due to symptoms that may resemble those of other skin conditions.[5] Therefore, laboratory testing plays a crucial role in reaching a conclusive diagnosis.[5] Microscopy involves examining specimens of hair, skin, and nails under a microscope after treatment with 10% Potassium Hydroxide (KOH) to detect the presence or absence of fungal elements.[7] Additional stains, such as Calcofluor white (CW) and Chicago Sky Blue (CSB), can be employed alongside 10% KOH as a clearing agent.[8,9] Fungal culture, though gold standard, can delay diagnoses as it requires several weeks.[7,10]

The potential for delayed or inadequate treatment and high patient morbidity underscore the importance of swift and precise diagnosis by an economical method. The conventional microscopy using 10% KOH has been shown to give false negative results.[10] Two stains are mentioned in the literature namely CSB and CW stain which are claimed to have better sensitivities than 10% KOH mount.[11] This study aimed to evaluate the effectiveness of CW and CSB stains relative to the conventional method, with fungal culture serving as the gold standard. The rationale was to find a quicker and more reliable tool for detecting dermatomycosis.

METHODOLOGY

This cross-sectional, analytical validation study was conducted at Department of Microbiology of a Referral laboratory from July 2023 to February 2024 following ethical approval from institutional review board. The sample size was calculated by Beuder-er's formula.[12] Taking 95% confidence level, 5% margin of error, 51% population proportion of dermatomycoses, 93.3% sensitivity, and 100% specificity, the minimum sample size was calculated as 78 but 121 specimens were included due to the easy availability of samples and reagents.[12-14]

The sampling technique for this study was a non-probability consecutive technique. Patients of all age groups and both genders having lesions of skin, nails or hair and a clinical suspicion of dermatomycosis were included. However, samples from partially treated patients and duplicate samples from the same site of the same patient were excluded. After informed consent, the demographic data of the patient was recorded. Clinical specimens, including skin scrapings, hair, and nail samples, were collected by a dermatologist at a tertiary care facility. These specimens were wrapped in black paper and transported in sterile containers to the microbiology laboratory for further processing.

The received sample was divided into two portions: One was inoculated on culture media, and the other was placed in 1 ml of 10% KOH in a 7 ml bijou bottle for one to three hours depending on the nature of the sample.[7,15] After the designated amount of time had elapsed, the contents were poured into a petri dish.

For microscopic examination, small portions of the sample (approximately 0.5-1 mm) were taken from the dish and prepared on slides for different stains and mounts.

The slides were coded and the observer was blinded to the exact identification of the patient and the results of the other two microscopic modalities. For 10% KOH mount for skin scrapings, nails, and hair, one or two small flakes or pieces, depending on the sample, were placed on a slide with a coverslip and viewed under a bright field microscope for the presence of hyphae.

For CSB stain, after placing a small sample of flakes, nail pieces, or hair on the slide, one drop of 1% w/v CSB stain (MACKLIN Direct blue 1) was added, followed by a coverslip. The slide was incubated in a humidification chamber for 30 minutes, and then examined under a bright field microscope for the presence of fungal hyphae.

For Calcofluor White Stain, similar-sized samples were placed on slides, one drop of 0.1% w/v CW stain (Sigma-Aldrich Calcofluor white stain). After 10 minutes, the slides were viewed under a fluorescence microscope (Zeiss Primo Star Microscope) using blue light excitation which had a wavelength of approximately 355 nanometre (nm) (300–400 nm) and the presence or absence of fungal hyphae was noted (300-400 nm) and the presence or absence of fungal hyphae was noted.

Hair samples (four from each patient) were processed in the same manner as the skin and nail samples. One hair was used for culture, and the remaining three were examined using the three staining techniques described above. Each stain followed the same procedure, with the specific slide preparation and observation steps as outlined for skin and nail samples.

The sample portion reserved for culture was inoculated onto sabouraud dextrose agar (SDA) used as a non-selective media, SDA supplemented with 5% chloramphenicol selective media and dermatophyte test medium (DTM) used as a specialised media. Culture plates were sealed with tape to prevent drying and contamination. They were incubated at 30°C in an incubator designated only for fungal culture. The incubator was maintained routinely. The plates were incubated for four weeks and were examined after every 3 days for the first 2 weeks and weekly afterwards.[16] Any growth observed was identified up to genus level based on colony characteristics and microscopic morphology.

Data were analysed using the Statistical Package for the Social Sciences (SPSS) version 26. Mean and standard deviation (SD) were computed for quantitative variables such as age, while frequencies and percentages were calculated for qualitative variables, including gender, type of specimen, clinical presentation, and the percentage of different fungi isolated. The presence of fungal hyphae in 10% KOH mount, CSB, and CW stained slides was compared to the presence or absence of fungal growth on culture. Cohen Kappa agreement (0.41-1 showing moderate-to-good agreement), sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy were calculated for the three microscopy methods using fungal culture as reference method.

RESULTS

Out of the total 121 patients suspected of having dermatomycosis included in this study, the majority were females, constituting 65.3% (n = 79), while 34.7% (n = 42) were males. The average age of the participants was 32.98 ± 16 years. Skin scrapings were 54 in number (44.6%), 43 (35.5%) were nail clippings, and 24 (19.8%) were hair. The primary complaint reported by the patients providing skin scraping samples was itching, which affected 52% (n = 28) of individuals. This was followed by a combination of itching and skin discolouration, which was experienced by 32% (n = 17) of the patients, and

persistent flakiness was reported by 7% (n = 4). However, in 9% (n = 5) of the skin scraping samples, the clinical details were unavailable. Amongst the patients who provided nail samples, a significant 90.7% (n = 39) complained of nail discolouration, while clinical information could not be retrieved for 9.3% (n = 4) of these patients. For those who submitted hair samples, 87.5% (n = 21) reported experiencing itching on the scalp. Clinical details could not be obtained for three patients, accounting for 12.5% (n = 3) of the hair sample group.

Out of 121 samples, 57 samples showed fungal growth on culture (47.1%). KOH mount 10% was able to reveal fungal hyphae in 30 samples, CSB in 42 samples and CW in 50 samples (Table I).

Table I: Comparison of 10% potassium hydroxide (KOH) mount, Chicago Sky Blue (CSB), and Calcofluor White (CW) with fungal culture.

N = 121		Fungal Culture		Total
		Positive	Negative	
10% Potassium Hydroxide	Positive	30	1	31
	Negative	27	63	90
	Total	57	64	121
Chicago Sky Blue stain	Positive	42	6	48
	Negative	15	58	73
	Total	57	64	121
Calcofluor White stain	Positive	50	8	58
	Negative	7	56	63
	Total	57	64	121

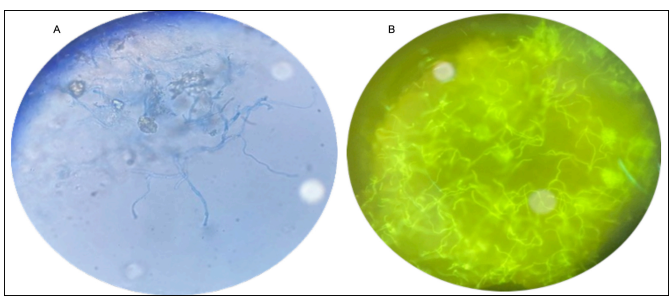

Figure 1: (A) Fungal hypha on Chicago Sky Blue stain under bright field microscope (B) Fungal hypha using Calcofluor white under florescent microscope.

Additionally, the comparison between the two staining techniques for detecting the fungal elements can be seen in the side-by-side images in Figure1 (A,B).

The sensitivity, specificity, PPV, NPV, and accuracy of 10% KOH mount for the diagnosis of dermatomycosis by detection of fungal hyphae was 52.6%, 98.4%, 96.7%, 70%, and 76.8%, respectively. CSB stain was found to have a sensitivity, specificity, PPV, NPV, and accuracy of 73.6%, 90.6%, 87.6%, 79.4%, and 82.6%, respectively. CW stain, on the other hand, had a sensitivity, specificity, PPV, NPV, and accuracy of 87.7%, 87.5%, 86.2%, 88.8%, and 87.6%, respectively.

Compared to fungal culture, the Cohen kappa agreement value of 10% KOH mount was 0.524, CSB stain 0.649 while of CW stain was 0.751 and the p-value for all three methods was <0.001.

Amongst the three methods tested for diagnosing dermatomycosis, the CW stain diagnosed the most proportion of true positives with the highest sensitivity (87.7%) and accuracy (87.6%). The CW stain also showed the strongest agreement with fungal culture, as indicated by the highest Cohen's kappa value (0.751).

In comparison, the CSB stain, while slightly less accurate than CW, still performed better than the 10% KOH mount, with higher sensitivity (73.6%) and a reasonable kappa value (0.649). The 10% KOH mount, while highly specific (98.4%), had the lowest sensitivity (52.6%) and overall accuracy (76.8%).

Overall, CW stain is the most effective method for detecting fungal hyphae in dermatomycosis, followed by CSB stain, with the 10% KOH mount being the least sensitive but highly specific.

DISCUSSION

The increasing incidence of fungal infections along with difficult treatment and their contiguity makes it imperative for the healthcare providers to look for options for early and correct diagnosis. Since, culture takes a minimum of about 4 weeks to visually identify fungal species and the molecular techniques are expensive, therefore, microscopy appears to be an option for quick diagnosis.[7,10] In the literature CW has been proven to be a superior staining method as compared to CSB and 10% KOH mount.[11] In this study, the diagnostic accuracy of CW stain, CSB stain, and 10% KOH mount was compared keeping fungal culture as the gold standard.

Comparison of the findings in this study with others in the literature reveals a variety of observations. For instance, a study conducted by Baddireddy *et al*. reported that the sensitivity, specificity, PPV, NPV, and overall diagnostic accuracy of 10% KOH mount was 55%, 79%, 32%, 60%, 23%, and 55%, respectively. The sensitivity, specificity, PPV, NPV, and accuracy of the CSB stain was 98%, 34%, 68%, 93%, and 72%, respectively.[17] In contrast, the present study demonstrated better diagnostic accuracy for both 10% KOH mount (76.8%) and CSB (82.6%) than the study by Baddireddy *et al*. One possible reason for the lower accuracy in their study could be the lack of technical expertise on the part of the observer.

A study by Liu *et al.* reported the sensitivity, specificity, PPV, and NPV of 10% KOH mount to be 62.2%, 89.9%, 83.6%, and 74.2%, respectively. The sensitivity, specificity, PPV, and NPV of CSB stain were reported as 75.4% 65.4%, 87.7%, 81.5%, and 75.4%, respectively.[18] The patterns observed in Liu *et al*.'s study are similar to those in the present research which are that the 10% KOH has the higher specificity (98.4%) but lower sensitivity (52.6%) compared to the CSB stain which has a specificity of 90.6% and sensitivity of 73.6%.

Mourad *et al*. compared both CSB stain and CW keeping culture as the gold standard.[11] Their findings show the sensitivity, specificity, PPV, NPV, and accuracy of CSB to be 90%, 86%, 88%, 94%, and 90%, respectively. The p-value was calculated to be 0.123. The sensitivity, specificity, PPV, NPV, and accuracy of CW was 98%, 83%, 85%, 90%, and 92%, respectively, and p-value was 0.039. While the exact values of this study as compared to the present study are not the same but the pattern indicates CW

to have better sensitivity, lesser specificity but overall better diagnostic accuracy as compared to CSB. The same pattern is observed in the present study under discussion, making CW a more sensitive tool in these three methods, while CSB as a more specific tool to diagnose dermatomycosis. The p-value of all of the methods is less than 0.01 indicating this study to have a higher statistical significance. The low specificity can partly be attributed to the failure of culture growth. In the present study, the fungal growth on culture was 47.1%. KOH mount (10%) was able to reveal fungal hyphae in 30 (24.7%) samples and CSB in 42 (34.7%) samples. However, the culture positivity rate in a study conducted by Baddireddy *et al*. was 59% out of a total of 100 cases with 85 (85%) being positive on CSB stain and 70 (70%) on 10% KOH mount.[17] The discrepancy between microscopic findings and culture results can be attributed to several potential factors. One possible reason for negative culture and positive microscopy findings could be the lack of fungal elements in the specific portion of the specimen that was cultured which was considered a limitation of this study. Another contributing factor might be insufficient clinical material being provided for the culture. Additionally, it is also plausible that the patient may have used antifungal medications prior to sample submission, which could inhibit fungal growth and contribute to the lack of culture positivity.[19,20]

The gender distribution in this study, constituted 65.3% (n = 79) female patients while males accounted for 34.7% (n = 42), with an average age of 32.98 ± 16 years. The main age group of the sample was less than 50 years. These findings are in coherence with the findings in a study conducted by Nagar *et al*.[21] In this study from India, 51.11% were females and 48.88% were males. Around 73.8% of the patients were under 50 years of age, with the majority falling within the 21-30-year age group.[21] Nagar *et al*. attributed the high incidence of fungal infections in this age group to increased physical activity and high chance of exposure. However, the authors of the present study believe that the more plausible reason for the younger age group and female predominance can be the aesthetic stigma that this disease presents in the youth that leads females to seek treatment.

In a nutshell, though microscopic methods are rapid and reliable tests but they have the limitation that they do not give clue to species identification which is essential for prescribing antifungal treatment. However, in resource-limited settings where funds are a problem, these methods are a cornerstone of the diagnosis of dermatomycoses. The present study revealed better sensitivity and accuracy of both CSB and CW as compared to 10% KOH preparation. Though CW was found even better than CSB but CSB has the advantage that it provides a presumptive diagnosis without the need for expensive instruments such as a fluorescent microscope as required for CW staining. However, the use of this stain will indeed increase the yield of positive samples in superficial fungal infections, which frequently gets missed because of the low rate of growth of dermatophytes in culture and the lower sensitivity of conventional 10% KOH method.

CONCLUSION

The CW staining technique was found superior, in terms of sensitivity and accuracy, to both CSB staining and 10% KOH preparation for rapid diagnosis of dermatomycoses. CW and CSB stains are recommended for laboratory use to enhance the reporting accuracy as compared to the traditional 10% KOH mount technique.

ETHICAL APPROVAL:

Ethical approval was taken from the Institutional Review Board of the Armed Forces Institute of Pathology, Rawalpindi, Pakistan, (Reference No: FC-MIC21-2/READ-IRB|22|2890, Dated: 4-07-2022).

PATIENTS' CONSENT:

Informed consent was taken from all patients.

COMPETING INTEREST:

The authors declared no conflict of interest.

AUTHORS' CONTRIBUTION:

AK: Conception, interpretation, drafting, and analysis.
MMG: Analysis and revision.
MAK: Acquisition of data, and drafting.
SHN, MR, AI: Interpretation and revision.
All authors approved the final version of the manuscript to be published and agreed to be accountable for all aspects of the work.

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