Original Article

Accuracy of Interpretation of Fungi by Direct Microscopy using Chlorazol Black E versus Gold Standard Potassium Hydroxide

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Background: Superficial fungal infections are skin diseases that affect people worldwide. To confirm the diagnosis of these diseases, mycological investigation by direct microscopic examination and culture are required. Chlorazol black E (CBE) is an acid-based dye of the trisazo group of dyes that has a high affinity for chitin. Data specific to the efficacy of CBE for detection of fungi by direct microscopy is limited.

Objective: To investigate the accuracy of interpretation of fungi by direct microscopy using CBE versus potassium hydroxide (KOH).

Materials and Methods: The present study was a retrospective study, conducted at the Department of Dermatology, Faculty of Medicine, Siriraj Hospital, Mahidol University in 2018. Slide interpretations by 33 microscopists were reviewed. The interpretation scores of all participants in each specimen were recorded and compared between CBE and KOH.

Results: Thirty-three participants were included. The mean interpretation score of all participants was 17.2±4.9. The mean interpretation scores of overall specimens using CBE was significantly more than using KOH ($p<0.001$). For *Candida* spp. and dermatophytes, the accuracy of interpretation using CBE was statistically higher than KOH ($p=0.020$, $p<0.001$, respectively). Relative to negative findings, CBE yielded significantly more false-positive results than KOH ($p=0.003$).

Conclusion: CBE for direct microscopic examination of fungi specimens is helpful for increasing the accuracy of interpretation. However, false-positive results are more prevalent when using CBE.

Keywords: Accuracy, Interpretation, Fungus, Direct microscopy, Chlorazol black E, Potassium hydroxide

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Superficial fungal infections, including dermatophytosis, pityriasis versicolor, and candidiasis, are skin diseases that affect people worldwide[1-3]. To confirm the diagnosis of these diseases, mycological investigation by direct microscopic examination and culture are required, especially in cases with atypical presentation[1,4-8]. Other studies reported that direct microscopic examination alone is simpler, faster, and less expensive than fungal culture, and provided an immediate and precise diagnosis of cutaneous fungal infection that reduced the rate of misdiagnosis and facilitated the delivery of timely and accurate treatment[2,9-11].

The most common and widely accepted solution to use for specimen preparation for direct microscopic examination is potassium hydroxide (KOH)[3]. KOH is an alkaline agent that breaks down debris without interfering with chitin, the predominant structure of fungal cell wall. However, experience and skill are necessary when using KOH, because artifacts are often observed during KOH examination that can lead to false-negative and false-positive results[2,7,11].

Chlorazol black E (CBE), which is another solution that can be used for direct microscopy, is an acid-based dye of the trisazo group of dyes that has a high affinity for chitin[1,3]. CBE is a black crystalline powder that can dissolve in water and is stable under normal temperatures and pressures. The inhalation and contact with CBE may cause respiratory tract, skin, and eye irritation. It should be stored at room temperature in a closed container and dry area[12]. In addition, CBE has a greater solubility in alkaline than

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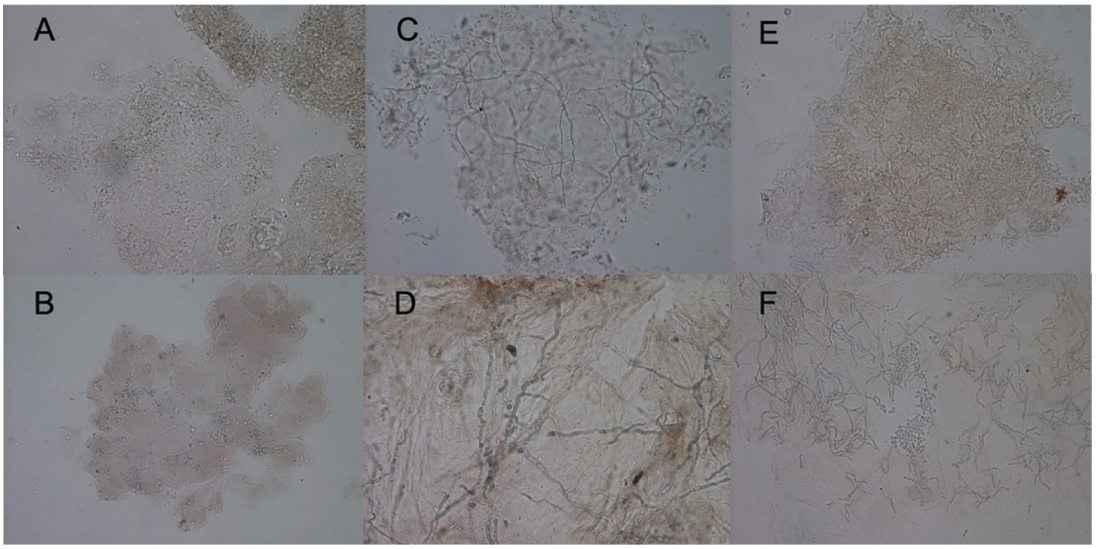

Figure 1. Direct microscopic appearance of fungi. A and B indicated pseudohyphae with budding yeast cells appearance of *Candida* mounted with potassium hydroxide and chlorazol black E, respectively. C and D indicated septate hyphae appearance of dermatophytes mounted with potassium hydroxide and chlorazol black E, respectively. E and F indicated spaghetti and meatball appearance of *Malassezia* mounted with potassium hydroxide and chlorazol black E, respectively.

in normal aqueous solution[13]. CBE stains the cell walls of filamentous fungi and of yeasts a blue-black color[1]. Time to stain fungi is different according to species of fungi and types of examined hyphae, which varies from minutes to hours[14]. Generally, for dermatophytes, CBE is ready for viewing under the microscope within two minutes after mixing CBE with the specimens[1]. Therefore, CBE can be considered as a simple and rapid technique for microscopic examination. Moreover, the CBE-stained slides can remain stained for nearly 12 months if the slides were protected from light exposure[3]. Data specific to the efficacy of CBE for detection of fungi by direct microscopy is limited. Therefore, the authors aimed to investigate the accuracy of interpretation of fungi by direct microscopy using CBE versus gold standard KOH at Siriraj Hospital, the largest national tertiary referral center of Thailand.

Materials and Methods

The present study was a retrospective study, conducted at the Department of Dermatology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand during 2018. Routine testing of microscopists in the department is performed every year to assess direct microscopy examination skills. The answer documents of participants, who underwent testing during 2018 were reviewed. Demographic data such as age, gender, and microscopy experience were collected. The study protocol was approved by the Siriraj Institutional Review Board (COA No. Si 803/2018).

For the examination, scale scrapings from glabrous skin of 15 patients were taken and prepared using 20% KOH or 20% CBE by two experienced microscopists. These two microscopists did not participate in the examination. No specimen obtained from nail lesion was included in this test. CBE (Delasco Dermatologic Lab & Supply, Inc., Council Bluffs, IA, USA) was composed of 0.1% CBE, 5% KOH, 10% dimethylsulfoxide, and purified water. Each type of sample was placed on each of two clean glass slides. Then, one drop of 20% KOH was deposited onto one slide, and one drop of CBE was deposited onto the other slide. All slides were covered with a coverslip and microscopically examined under 100x magnification for fungal detection and 400x magnification for fungal element observation. The clinical and laboratory diagnosis of patients that had positive result of KOH examination were confirmed as one of the following superficial fungal skin infections, dermatophytosis (branching septate hyphae), candidiasis (pseudohyphae with budding yeast), or pityriasis versicolor (spaghetti and meatball) (Figure 1). For negative KOH findings, the slides were prepared from patient skin lesion of psoriasis or eczema. Obviously, given that this was a test setting, all subjects were blinded to the type of slide. Participants were given three minutes to evaluate each slide using either KOH or CBE solution. The score

Table 1. Mean interpretation scores of findings and results of specimens compared between potassium hydroxide and chlorazol black E solution

Specimen	Mean±SD	p-value
Candida spp.		0.020
KOH	1.61±1.35	
Chlorazol black E	2.15±1.35	
Dermatophytes		<0.001
KOH	2.94±0.93	
Chlorazol black E	3.97±0.98	
Malassezia spp.		0.501
KOH	1.45±0.71	
Chlorazol black E	1.33±0.99	
Negative findings		0.003
KOH	2.18±0.95	
Chlorazol black E	1.67±0.92	

KOH=potassium hydroxide

A p<0.05 indicates statistical significance

from all answer sheets were analyzed.

The interpretation score was the ability to identify the causative organisms under microscopic examination. One point was given for a correct answer, and no points were given for an incorrect answer in each specimen evaluation so total score was 30. Correct interpretations included both true-positive and true-negative results. The interpretations consisted of six negative specimens and positive results of ten dermatophyte, eight *Candida*, and six *Malassezia* specimens. All specimens were confirmed by fungal culture. Fifteen specimens were prepared by KOH and another fifteen specimens were prepared from CBE. Interpreters were required to accurately describe microscopic findings in positive specimens. Then, the mean interpretation scores of all participants were calculated by adding the correct scores of each participant together and then dividing by the number of participants.

Data were analyzed using PASW Statistics for Windows, version 18.0 (SPSS Inc., Chicago, IL, USA). Descriptive statistics are reported as frequency and percentage or mean ± standard deviation (SD). Paired t-test was applied to compare correct results between the KOH and CBE methods. A p-value of less than 0.05 indicated statistically significant.

Results

Thirty-three test answer documents from 33 microscopists with the mean age of 29.3±2.6 years were included in the present study. Twenty-seven participants (81.8%) were female. The mean years of experience of interpretation of fungi by direct microscopy was 1.3±2.4 years.

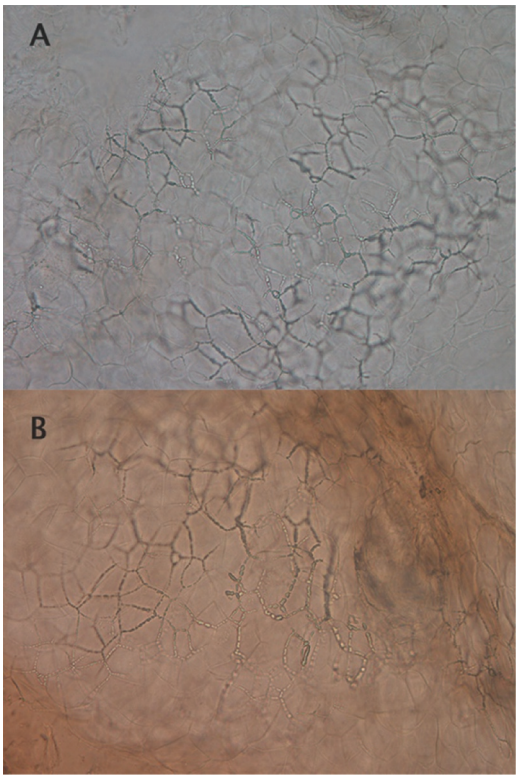

Figure 2. Direct microscopic appearance of mosaic fungi or cholesterol crystals from skin. A indicated direct microscopic with potassium hydroxide while B indicated direct microscopic with chlorazol black E.

The mean interpretation score of all participants was 17.2±4.9. The mean interpretation scores of overall specimens using CBE (9.1±2.4) was significantly more than that of specimens using KOH (8.2±2.6, p<0.001). The mean interpretation scores of findings among different specimens compared between KOH and CBE solution are shown in Table 1. For evaluation of *Candida* spp., the accuracy of interpretation using CBE was statistically higher than that of KOH (p=0.020). Similarly, for the evaluation of dermatophytes, the accuracy using CBE was statistically higher than that of KOH (p<0.001). However, for evaluation of *Malassezia* spp., the accuracy of interpretation using KOH was higher than CBE, although the results did not have a statistically significant difference. Regarding negative findings, CBE had a significantly lower mean score than KOH (p=0.003), thus, a false-positive finding was more frequently reported in slides prepared with CBE

(43.4%). Most false positive findings were mosaic fungi (cholesterol crystals) (Figure 2).

Discussion

In the present study, the mean interpretation scores of overall specimens using CBE was significantly more than that of specimens using KOH. Consistent with the recommendations of previous studies, the authors recommend the use of CBE for the identification of fungi[3,10,15]. This is probably due to the CBE-stained slides that can be read easily and rapidly. Various preparation solutions in direct microscopy yielded a range of different sensitivities and specificities[2,6,16]. Accuracy of direct microscopic examination to detect fungal element is essential for diagnosis of cutaneous fungal skin infection, and to facilitate the provision of timely and appropriate treatment[2].

The present study revealed that subjects gave more correct answers for *Candida* spp. and dermatophyte samples prepared with CBE than in those prepared with KOH. Since chitin was well stained with CBE showed a green appearance with dark shade background in microscopic view[3], large hyphae of dermatophytes or pseudohyphae with budding yeast appearance of *Candida* spp. could be easily observed with CBE.

The present study results showed CBE was not different from KOH in identifying the spaghetti and meatball appearance of *Malassezia* spp. This could be explained by the size and shape of this sign, which is quite unique and is easy to identify by any preparations. In addition, interpreters should be aware of the potential for false-positive results when using CBE because the interpretation of specimens with negative findings that were prepared using CBE had a lower mean correct score than the negative findings that were prepared using KOH. Previous studies revealed inadequate sample collection, inexperienced interpreter, or low color contrast might result in a false-negative rate of up to 15% in KOH examination[2,9,11]. Another study reported that CBE could detect even small numbers of fungal hyphae without staining or contaminants such as cotton or elastic fiber[17]. Therefore, the false-positive results from CBE may be due to artifacts that could be detected more easier with CBE. Therefore, morphologies of fungi such as branching septate hyphae should be integrated in the interpretation. Thus, in cases with highly suspicious clinical manifestations of superficial fungal infection and a negative result from direct examination, the test should be repeated[6].

The present study had some limitations. First, this was a retrospective study. Then, because the size of the study population was relatively small, the study may not have enough sufficient statistical power to identify all significant differences and associations.

In conclusion, CBE is helpful for use in specimens prepared for direct microscopic examination of fungi specimens, especially *Candida* spp. and Dermatophytes. However, observers should remain suspicious for false-positive results.

What is already known on this topic?

The most common and widely accepted solution used for direct microscopic examination is KOH. CBE is an acid-based dye of the trisazo group of dyes that can also be used for direct microscopy.

What this study adds?

This study described CBE for direct microscopic examination of fungi specimens as a helpful solution to increase the accuracy of interpretation. However, the interpretation based on CBE method should be cautious of false-positive results.

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Conflicts of interest

The authors declare no conflict of interest.

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