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Original Research Article

Comparison of different methods to determine the viability of dermatophytes in skin scrapings

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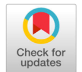

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ABSTRACT

Background: One of the most prevalent infectious dermatoses seen in dermatology outpatient clinics is superficial fungal infections of the skin, hair, and nails. Filamentous fungi, dermatophytes, which invade and colonise the keratinised layers of the skin, are the main cause of skin fungal infection. Sometimes there is a discrepancy between the results of microscopy and culture for dermatophytes. If the microscopy of scrapings is positive due to presence of non-viable fungus, no growth will take place in culture. Culture is the method used to determine the presence of viable dermatophytes. However, the problem with culture is the long time, up to 4 weeks, that is required for the growth of fungus to take place.
Aim: Aim of the present study is to develop a simple, rapid method(s) for identification of live dermatophytes in skin scrapings.
Materials and Methods: The novel methods used for identification of live dermatophytes in skin scrapings were direct microscopic examination using periodic acid-Schiff (PAS) stain, trypan blue stain, neutral red stain, and methylene blue stain; and their results were compared with culture on Sabouraud Dextrose Agar (SDA) and Dermatophyte Test Medium (DTM).
Results: Out of a total 91 samples, fungi were isolated in 81 (89%) in SDA and 66 (72.5%) in DTM. Sixty-eight (74.7%) showed evidence of viable fungi on microscopic examination after staining with PAS and 77 (84.6%) with trypan blue. In 84 patients, the culture was positive either by SDA or DTM or both culture media, out of which 64 (76.2%) were detected viable on staining with PAS and 74 (88.1%) on staining with trypan blue. Three skin samples that failed to grow on both SDA and DTM demonstrated viable fungi on microscopic examination after staining with PAS. Four skin samples that failed to grow on both SDA and DTM demonstrated viable fungi on microscopic examination after staining with trypan blue. Taking culture positivity in SDA or DTM or both media, as the gold standard, the sensitivity of trypan blue stain was 88.1% and specificity was 57.1%. The sensitivity of PAS stain was 76.2% and specificity was 42.9%.
Conclusion: Although culture remains the gold standard to identify viable dermatophytes; trypan blue and PAS stains, as described, may be used as simple and rapid alternatives to culture.

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1. Introduction

One of the most prevalent infectious dermatoses seen in dermatology outpatient clinics in India is superficial fungal infections of the skin, hair, and nails.[1,2] Over the last 3–4 years, the frequency of such cases has increased alarmingly.[3] In addition, there is an alteration in the disease presentation, disease severity, treatment response and relapse rate.[4] According to studies, these shifting patterns of the disease and response may be due to the emergence of Trichophyton mentagrophytes as the main causative organism and high terbinafine

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resistance.[5–7] Filamentous fungi, dermatophytes, which invade and colonise the keratinised layers of the skin, are the main cause of skin fungal infection. Sometimes there is a discrepancy between the results of microscopy and culture for dermatophytes. If the microscopy of scrapings is positive due to presence of non-viable fungus, no growth will take place in culture. Culture is the method used to determine the presence of viable dermatophytes. However, the problem with culture is the long time, up to 4 weeks, that is required for the growth of fungus to take place. Aim of the present study is to develop a simple, rapid method(s) for identification of live dermatophytes in skin scrapings.

2. Materials and Methods

The study was conducted in the Department of Dermatology in the outpatient department of a medical college in North India. Total 91 patients were enrolled in the study. Patients with tinea corporis, tinea cruris and tinea faciei or a combination of these conditions attending dermatology outpatient services were included. Selection criteria were:

1. Clinical diagnosis of tinea cruris, tinea corporis, and tinea faciei.
2. Microscopic confirmation (positive potassium hydroxide [KOH] microscopy) of diagnosis.
3. Affected body surface area 2% or more.
4. Scaling grade 2 or more on a scale of 0-4.

A witnessed, written and informed consent was given by the patients, or by a parent in case of minor patients. The study was performed in two parts (Part 1a, 1b and Part 2). Skin scrapings were taken from 20 patients fulfilling the selection criteria and the following procedures were performed (Part 1a): Culture in Sabouraud's Dextrose Agar (SDA), culture in Dermatophyte Test Medium (DTM), and direct staining by methylene blue. Skin scrapings were taken by scraping the edge of the affected area with a sterile blade after cleaning the site with 70% alcohol on sterilized black chart paper and were kept in envelopes. For direct microscopy the sample collected was placed on a new glass slide and a drop of 10% KOH was put on it. A glass cover slip was kept on the sample and pressed, taking care to prevent the formation of air bubbles. The sample was kept in KOH for a variable duration ranging from 5 minutes to 30 minutes, depending upon the thickness of the scales and examined every 5 minutes. Each slide was thoroughly examined for the presence of filamentous, septate, branched hyphae with or without arthrospores crossing the margins of the squamous epithelial cells of the skin.

2.1. Direct staining by methylene blue

Scrapings were placed directly on a clean slide and covered with 1 to 2 drops of methylene blue solution, prepared by dilution of 1 part of saturated 95% alcoholic solution of the dye with 9 parts of distilled water (resultant concentration of methylene blue 0.1%). The dye was allowed to act for 30 seconds and then was drawn off by small torn pieces of bibulous paper. Distilled water was put on the slide with a dropper, and was permitted to remain until dye ceased to flow from the specimen, and was drawn off as above, to dryness. One drop of 1 % acetic acid was put for discoloration and left for 15 seconds, and this procedure was followed by application of water. After drying, the slide was visualised under light microscope under low and high power objectives for the presence of viable fungal hyphae by dye-exclusion.

2.2. Culture and isolation of fungi

Skin scrapings taken at the time of enrolment were inoculated on media containing Actidione and Dermatophyte Test Medium (DTM) in separate plates. Actidione media is a combination of Sabouraoud's Dextrose agar (SDA) with cycloheximide (0.5 g/l). Cycloheximide was added to inhibit majority of non-dermatophytes. A pH indicator (phenol red) which converts from straw-yellow to vivid red under the alkaline conditions associated with the growth of dermatophytes, provided a differentiating quality to the DTM medium. The specimens were inoculated on the surface of the medium in culture plate with aseptic forceps or loop breaking the surface of medium without becoming completely embedded. For both cultures, plates were incubated at 30°C in Bio-Oxygen Demand (BOD) incubator for a maximum of 4 weeks. If no growth was found at 4 weeks, it was considered as negative for the growth of fungi. Identification of the dermatophyte was done on the basis of colony morphology and microscopic morphology.

2.3. Colonial morphologic features

Colony color (e.g. white, pearl, ivory and black), consistency (e.g. cottony, fluffy, suede like) and topography (e.g. flat, folded, plicate, rugose) were noted.

2.4. Microscopic morphologic features

Cultures were examined microscopically by removing a portion of growth with sterile wire slightly bent at the end. The materials were placed on a clean glass slide in a drop of lacto phenol cotton blue (LPCB) [lactic acid-100gm; phenol-100gm; glycerol-200ml, saturated aqueous solution of picric acid] and the matted mycelia mass was teased with dissecting needles. Cover slip was put, and the preparation was slightly heated over the flame to drive the air bubbles away and enhance the penetration of the dye into fungal filaments. The slides were examined microscopically under low and high power objectives with reduced lights. The lactic acid conserves fungi structures by provoking osmotic gradient changes with relation to the interior of the fungi.

Phenol deactivates lytic cellular enzymes; thus the cell does not lyse. Cotton blue gives the colour for visualization.

In Part 1b of the study, the skin scrapings taken were put on a glass slide, on which double-sided sticking tape had been fixed. Following staining methods were tried on these samples: PAS staining, neutral red staining, trypan blue staining and methylene blue staining.

2.5. *PAS staining*

The scrapings taken were dissolved in 10% KOH for 30 minutes in a sample container to digest keratin from the sample for easy visualization of hyphae. For staining, the sample was then put on the double-sided sticking tape which was fixed on a glass slide, then it was kept overnight at 25^0C in an incubator with fan for drying. 1-2 drops of 1% periodic acid were put on sample and allowed to act for 10 minutes, after which the slide was washed in running tap water for 1 minute and then immersed in Schiff reagent for 40 minutes. Subsequently, the sample was washed in tap water for 1 minute, counterstained with 1-2 drops of Mayer's haematoxylin for 1 minute and again washed in tap water for 1 minute. After drying, slide was visualised under light microscope under low and high power objectives for presence of viable fungi hyphae with the help of dye-exclusion.

2.6. *Trypan blue staining*

The scrapings taken were dissolved in 10% KOH for 30 minutes in a sample container to digest keratin from the sample for easy visualization of hyphae. For staining, the sample was then put on the double-sided sticking tape which was fixed on a glass slide and the slide was kept overnight at 25^0C in an incubator with a fan for drying. overnight for drying. 1-2 drops of trypan blue solution, prepared by dilution of 1 part of 0.4% trypan blue dye with 3 parts of distilled water, were put on the sample and allowed to act for 15 minutes, it was followed by washing in running tap water. After drying, the slide was visualised under light microscope under low and high power objectives for presence of viable fungal hyphae with the help of dye-exclusion.

2.7. *Methylene blue staining*

The scrapings taken were dissolved in 10% KOH for 30 minutes in a sample container to digest keratin from the sample for easy visualization of hyphae. For staining, the sample was then put on the double-sided sticking tape which was fixed on a glass slide and the slide was kept overnight at 25^0C in an incubator with fan for drying. 1-2 drops of 1% methylene blue dye were put on the sample and allowed to act for 30 seconds, it was followed by washing in running tap water. After drying, the slide was visualised under light microscope under low and high power objectives for presence of viable fungi hyphae.

2.8. *Neutral red staining*

The scrapings taken were dissolved in 10% KOH for 30 minutes in a sample container to digest keratin from the sample for easy visualization of hyphae. For staining, the sample was then put on the double-sided sticking tape which was fixed on a glass slide and the slide was kept overnight at 25^0C in an incubator with a fan for drying. Different concentrations of neutral red stain such as 1%, 2% and 5% were tried on the sample for staining for different durations such as 10, 15 and 20 minutes. After drying the slide was visualised under light microscope under low and high power objectives for presence of viable fungi hyphae.

An interim analysis was done after completion of Part 1 of the study. The top two methods with high positivity rate, out of the direct staining methods of Part 1b, were selected for Part 2 of the study. In Part 2 of the study these methods were compared with all methods of Part 1a of the study in 71 more patients who fulfilled the selection criteria.

3. Results

Skin scrapings from a total of 91 patients (29.4±13.5 years; 59 males, 32 females) with microscopically confirmed tinea were included in the study. Fifty patients had tinea corporis et cruris, 27 had tinea corporis et cruris et faciei, and 14 had tinea corporis. In the part 1a of the study, 20 microscopically confirmed skin samples [Figure 1] were subjected to staining with 0.1% methylene blue and culture in SDA and DTM. Fungi were isolated in 19 (95%) samples in SDA [Figure 2a,b], and in 14 (70%) samples in DTM [Figure 3a,b]. Ten (50%) out of 20 samples showed evidence of viable fungi on staining with 0.1% methylene blue [Figure 4a,b]. Methylene blue dye stained the dead fungal hyphae deep blue, leaving the viable hyphae colourless. Out of the 19 SDA and 14 DTM culture positive samples, 9 (47.3%) and 6 (42.8%) showed viable fungi on staining with methylene blue, respectively. In the part 1b of the study, 20 samples of Part 1a were further subjected to staining with trypan blue, periodic acid-Schiff, methylene blue and neutral red, on a double-sided sticking tape fixed on glass slide. Viable fungi were detected in 15 (75%) samples with trypan blue [Figure 5a,b,c,d] and 13 (65%) samples with periodic acid-Schiff [Figure 6a,b,c,d]. Trypan blue dye stained the dead fungal hyphae light blue, leaving the viable hyphae colourless. Periodic acid-Schiff stained the live fungal hyphae deep pink in colour. Out of 19 SDA culture positive samples, 15 (79%) and 13 (44.8%) were identified as viable fungi on staining with trypan blue and PAS, respectively. Out of the 14 DTM culture positive samples, 13 (92.8%) samples stained with trypan blue and 8 (57.1%) stained with PAS were identified as viable. On staining with methylene blue [Figure 7a,b] and neutral red

[Figure 8a,b] dyes, we were unable to differentiate between viable and dead fungi on microscopic examination. In the part 2 of the study, 71 skin scraping samples from additional patients were subjected to (i) staining with trypan blue and (ii) periodic acid-Schiff stains on a double-sided sticking tape fixed on glass slide and, (iii) culture in SDA and (iv) DTM. Fungi were isolated in 62 (87.3%) samples in SDA and in 52 (73.2%) in DTM. Viable fungi were detected in 55 (77.4%) samples on staining with PAS and in 62 (87.3%) samples with trypan blue. In 64 patients, the culture was positive either by SDA or DTM, out of which 51 (79.7%) were detected viable on staining with PAS and 59 (92.2 %) on staining with trypan blue. Out of total 91 samples, fungi were isolated in 81 (89%) in SDA and 66 (72.5%) in DTM. Sixty-eight (74.7%) showed evidence of viable fungi on microscopic examination after staining with PAS and 77 (84.6%) with trypan blue. In 84 patients, the culture was positive either by SDA or DTM or both culture media, out of which 64 (76.2%) were detected viable on staining with PAS and 74 (88.1%) on staining with trypan blue. Three skin samples that failed to grow on both SDA and DTM, demonstrated viable fungi on microscopic examination after staining with PAS. Four skin samples that failed to grow on both SDA and DTM, demonstrated viable fungi on microscopic examination after staining with trypan blue. Out of the 84 culture positive samples, Trichophyton mentagrophytes was identified in 83 samples and Trichophyton tonsurans in 1 sample. Culture positivity, in SDA or DTM or both media, was taken as the gold standard for the calculation of sensitivity and specificity of trypan blue and periodic acid-Schiff staining methods (Table 1).

Table 1: Sensitivity and specificity of trypan blue and periodic acid-Schiffstaining methods

	Trypan blue	Periodic acid-Schiff
Sensitivity	88.1%	76.2%
Specificity	57.1%	42.9%

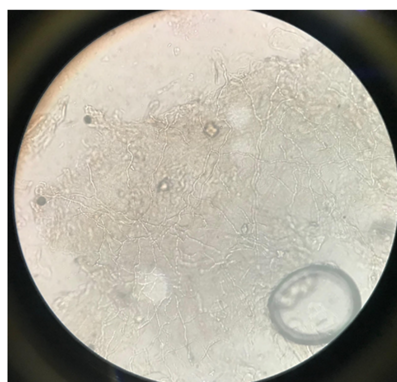

Fig. 1: Potassiumhydroxide (KOH) mount

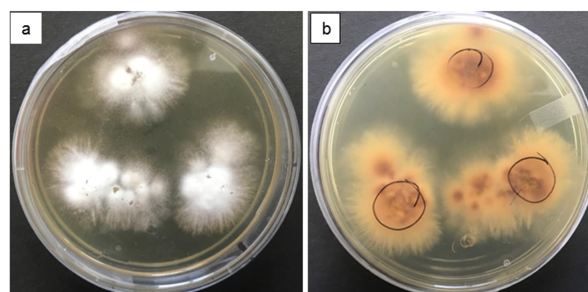

Fig. 2: a: Culture in sabouraud dextrose agar; **b:** Culture in sabouraud dextrose agar

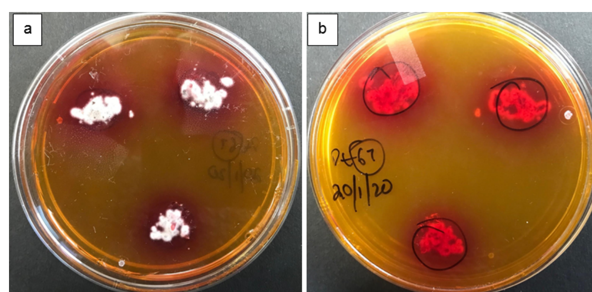

Fig. 3: a: Culture in dermatophyte test medium**; b:** Culture in dermatophyte test medium

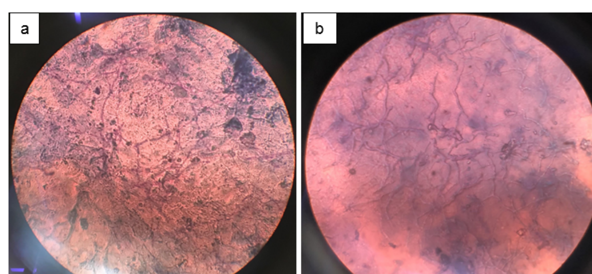

Fig. 4: a: Staining with 0.1% methylene blue;dead hyphae stained deep blue, leaving viable hyphae colourless; **b:** Staining with 0.1% methylene blue; dead hyphae stained deep blue, leaving viable hyphae colourless

4. Discussion

Singh et al demonstrated that out of total 260 skin, hair and nail samples examined, 157 (60.4%) showed evidence of fungal elements on direct microscopy using 10% KOH and 116 (44.6%) samples were culture positive. 144 (55.3%) did not show evidence of the fungi either on direct microscopy or culture. Fungi were isolated from SDA in 112 (96.6%) and DTM in 114 (98.3%) and the difference between SDA and DTM was statistically not significant.[8] Trypan blue and methylene blue staining have been studied for differentiating between live and dead yeast cells in colonies by Kucsera et al. The dead cells would have

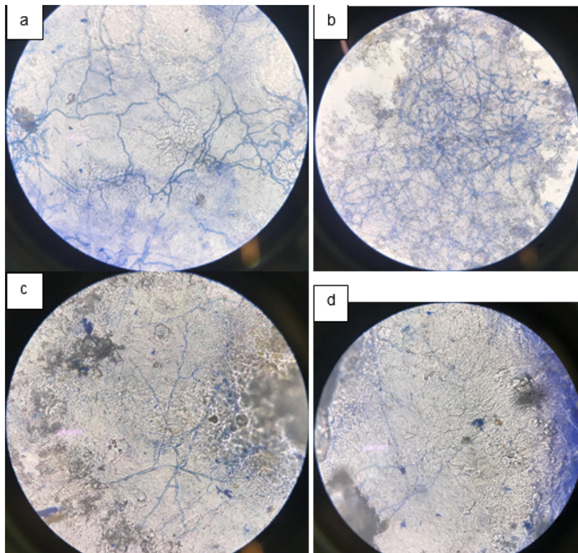

Fig. 5: a: Staining with trypan blue; dead hyphae stained light blue, leaving viable hyphae colourless; **b:** Staining with trypan blue; dead hyphae stained light blue, leaving viable hyphae colourless; **c:** Staining with trypan blue; dead hyphae stained light blue, leaving viable hyphae colourless; **d:** Staining with trypan blue; dead hyphae stained light blue, leaving viable hyphae colourless.

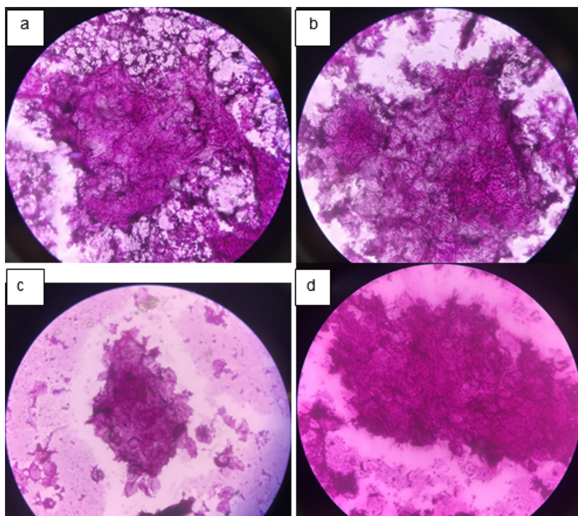

Fig. 6: a: Staining with periodic acid-Schiff; viable fungal hyphae stained deep pink;**b:** Staining with periodic acid-Schiff; viable fungal hyphae stained deep pink; **c:** Staining with periodic acid-Schiff; viable fungal hyphae stained deep pink; **d:** Staining with periodic acid-Schiff; viable fungal hyphae stained deep pink.

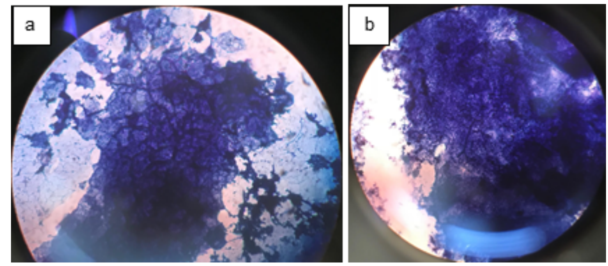

Fig. 7: a: Staining with 1% methylene blue; differentiation of viable and non-viable fungi was not possible; **b:** Staining with 1% methylene blue, differentiation of viable and non-viable fungi was not possible.

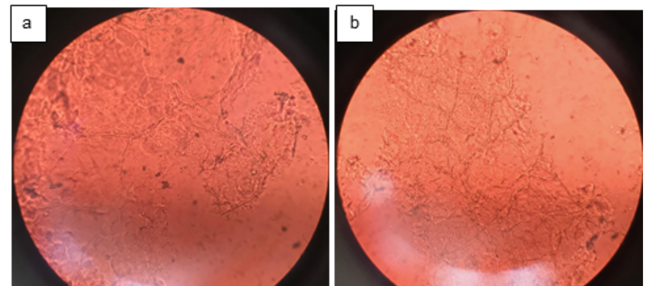

Fig. 8: a: Staining with neutral red, differentiation of viable and non-viable fungi was not possible; **b:** Staining with neutral red, differentiation of viable and non-viable fungi was not possible

non-functional membranes, through which the stains would penetrate quickly, whereas the living cells would exclude the dye. Thus, dead cells would stain, living cells would remain colourless.[9–12] The PAS stain is a simple stain that demonstrates polysaccharides. Cellulose and chitin, two substances rich in polysaccharides, are found in the cell walls of fungi. In this method, the periodic acid oxidizes the carbon to carbon bond forming aldehydes, which react to the fuchsin- sulphurous acid which forms the magenta colour.[13,14] Neutral red(NR) is a water soluble vital stain that can differentiate between viable and non-viable fungal elements. Neutral red is capable of passing through the intact plasma membrane and is stored in the lysosomes of viable cells. Therefore, the uptake of dye ceases when cell membranes and lysosomes are damaged. Neutral red staining after trypsinization showed fungal elements in 58 (48.3%) skin scrapings as it stained only viable fungal cells. Out of the total 58, KOH and NR positive only 29 (50%) grew on SDA. 5 (4.2%) specimens which were only NR positive showed growth on SDA. Deepashri et al. concluded that the isolation rate of fungus in patients with dermatophytic infection show increased isolation after trypsinization and simultaneously it was found that neutral red staining is a very useful technique to distinguish between viable and non-viable fungal elements.[15] In our study, the top two methods with high positivity rate, out

of the direct staining methods of Part 1b, were trypan blue and PAS stains. These two methods were selected for Part 2 of the study. In Part 2 of the study, these methods were compared with all methods of Part 1a of the study in 71 more patients who fulfilled the selection criteria. In this way, in total 91 patients, the results of the following methods were compared: culture in SDA, culture in DTM, PAS stain, trypan blue stain. Out of total 91 samples, fungi were isolated in 81 (89%) in SDA and 66 (72.5%) in DTM. Sixty-eight (74.7%) samples showed evidence of viable fungi on microscopic examination after staining with PAS and 77 (84.6%) with trypan blue. In 84 patients, the culture was positive either in SDA or DTM or in both culture media, out of which 64 (76.2%) were detected viable on staining with PAS and 75 (89.3%) on staining with trypan blue. Three skin samples that failed to grow on both SDA and DTM, demonstrated viable fungi on microscopic examination after staining with PAS. Four skin samples that failed to grow on both SDA and DTM, demonstrated viable fungi on microscopic examination after staining with trypan blue.

5. Conclusion

Microscopy of scrapings in patients with tinea are positive even when the fungus may be non-viable. Culture identifies viable dermatophytes, but has the drawback of requiring a long time, up to 4 weeks, for the fungal growth to occur. In the present study, different novel methods for identification of fungus were compared, with the aim of selecting method(s) which may identify the viable dermatophytes in skin scrapings. Staining with trypan blue and PAS were found to be effective. With these methods, viable and dead fungi were easily distinguishable by their colour on staining. Although culture remains the gold standard to identify viable dermatophytes, the above methods are less time consuming and easy to perform. These simple and rapid staining methods may be used as alternatives to culture.

6. Conflict of Interest

None.

7. Source of Funding

None.

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