



Neutral red staining and trypsin treatment to study viability and culture yield of Dermatophytes

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Abstract

Dermatophytosis is common fungal infection of human being. To diagnose dermatophytic infections microscopic examination should be followed by culture which is essential step. Many times fungus may fail to grow on culture even after direct microscopy is positive due to non-viability of fungus. In such cases, neutral red staining helps us to know about non-viability of fungus and it has been revealed that trypsinization enhances the rate of isolation of fungus on culture. Therefore, the study was undertaken with an aim to look for the viability and yield of dermatophytes on neutral red staining and trypsinization respectively.

Key words

Trypsinization, Dermatophyte, Neutral red.

Introduction

Dermatophytosis is one of the common fungal infection in patients which may result in the form of infections as onychomycosis, tinea corporis, tinea cruris etc. Direct microscopic examination of skin, hair and nail specimens is essential for their diagnosis [1, 2]. Clearing the

specimen with 10-40% potassium hydroxide, followed by microscopic examination, is the most widely used method [1, 3]. Culture is essential for confirmation of microscopically positive fungus for definitive identification of the etiological agent can be ascertained only by culture [4]. In many instances, the choice of

therapy may depend upon the species causing infection.

In Dermatophytosis, microscopic examination with KOH positive and culture negative is usual problem which infers the non viability of fungus. It is difficult to treat patients with negative microscopic examination. The researchers have used many methods for visualization of fungi using KOH, NaOH, Di-Methyl Sulphoxide (DMSO). One such method is trypsinization along with neutral red which increases sensitivity of culture. It is found that treatment with trypsin helps in digestion of tissue in a better way than KOH which indirectly helps in sensitivity of wet mount of fungal element. Neutral red helps in better visualization of fungal element as it stains only viable cell which was confirmed by autoradiographic study using 3H-thymidine [5].

In our laboratory, we routinely use KOH mount preparation for examination of fungus. But it does not infer about the viability of fungus. Hence this study was planned to look for viability of fungus using Neutral Red along with trypsin treatment.

Objectives

- To detect fungal elements by microscopic examination using KOH (10%) and Neutral red.
- To isolate fungus on SDA with or without treatment with trypsin.
- To compare the effect of trypsin on isolation of fungus.

Material and methods

The study was conducted in the department of Microbiology of tertiary care hospital for period of two months from 1st July to 31st August 2013.

Institutional Ethical Committee clearance was taken.

A total of 120 skin scrapings were collected from patients attending the department of Microbiology for routine investigation. Skin scrapings from these patients were collected from margin of the lesion on sterile white butter paper.

Each scraping sample was divided into 2 parts and processed as follows.

- First part was again divided into two, 1st portion mixed with 10% KOH for 30 minutes and then looked under microscope (40X) for fungal element and 2nd portion was cultured on SDA agar with and without chloramphenicol and tube was incubated at 25°C and 37°C for 24 hours.
- Second part of the scraping was treated with 2% trypsin and then looked for fungal element under microscope using 1% Neutral red and 10% KOH and cultured on SDA with and without chloramphenicol [6].

Second part of a skin scraping was treated with 1 ml of 2% trypsin solution and kept at 37°C for 2 hours. After 2 hours, scales were washed with phosphate-buffered saline (PBS) by centrifuging 3 times (1,500 g x 10 minutes). The deposit was suspended in 300 µl phosphate buffered saline (PBS). From this 300 µl of PBS, 200 µl was cultured on Sabouraud's dextrose agar (SDA) with and without chloramphenicol and incubated at 25°C and 37°C. Remaining 100 µl resuspended deposit was mixed with an equal amount of 1% neutral red reagent and kept at room temperature for 1 hour. This was then microscopically examined as a wet mount under microscope for fungal element. Also mixed with few drops of KOH before adding 1% neutral red

and looked under microscope for fungal element [6].

All the cultured SDA tubes were incubated at 25°C and 37°C for up to 1 month before declaring a negative result. The growth obtained was identified by gross appearance and microscopic examination.

Results

A total of 120 skin scrapping were observed under microscope for visualizing fungal element after mixing with 10% KOH as well as with and without trypsin treatment. After trypsinization neutral red was added. Out of 120 skin scraping, 75 (62.5%) samples before trypsinization and 98 (81.67%) samples after trypsinization showed fungal element by using 10% KOH. This increase in percentage of positivity on microscopy after trypsinization is because of trypsin causes extraction of fungal element embedded in tissue. Neutral red staining after trypsinization showed fungal element in 58 (48.33%) skin scrapings as it stained only viable fungal cells. Positivity of culture with both before and after trypsinization was 20 (16.66%) and 36 (30%) respectively. Positivity of culture after trypsinization was increased as compared to without trypsinization of samples. There was increase in positivity with trypsinization because fungal element which was embedded in specimen/ tissue got freely dispersed and could grow luxuriantly on SDA as compared to non trypsinised specimen. Majority of dermatophytes grown on culture was *Trychophyton mentagrophytes* followed by *Trychophyton rubrum* both before and after trypsinization.

Fungus positivity was increased after trypsinization as per **Table - 1**. Out of the 120 specimens, with KOH examination 98 (81.67%) and with Neutral red 63 (52.5%) showed fungal

elements. When we compared individually there was increased in microscopy by 19.17% in KOH and in Neutral red by 15% after trypsinization.

Table - 1: Comparison of microscopy and culture positivity with trypsinization.

N = 120	Smear positivity	
	KOH	Neutral red (NR)
Fungus positivity before trypsinization	75 (62.5%)	40 (33.33%)
Fungus positive after Trypsinization	98 (81.67%)	63 (52.5%)

Out of the total 58, KOH and NR positive only 29 (50%) grew on SDA as per **Table - 2**. This 50% positivity was because of less fungal element in specimen which was not able to grow on SDA and in patients who were taking antifungal therapy. 5 (4.17%) specimen which were only NR positive showed growth on SDA. 2 (1.67%) specimen which were KOH positive but NR negative were could also grew on SDA after culture because of less fungal element present in specimen was missed during processing and not detected in Neutral Red microscopic examination.

Table - 2: Comparison of culture with microscopy results of KOH and NR after trypsinization.

Procedure done (N= 120)	Total	Culture Positive	Culture Negative
KOH and NR positive	58	29 (50%)	29 (50%)
KOH positive and NR negative	35	02 (5.71%)	33 (94.29%)
KOH negative and NR positive	5	05 (100%)	Nil
KOH and NR negative	22	Nil	22 (100%)

Discussion

Definite diagnosis of Dermatophytosis can create a problem when there is discrepancy between microscopic findings and culture. Samples positive by direct microscopy may fail to grow on culture if the fungal elements are nonviable or some artifacts may be mistaken for fungal hyphae. Several modifications have been made in microscopic examination of fungus by 10% KOH preparation for more rapid detection of fungal elements in the specimen [1, 7]. Modification of the basic method by using 36% dimethyl sulphoxide (DMSO) to 20% KOH has also been used for clearing a specimen [8]. A study conducted by Naka, et al. [5], neutral red staining was used to evaluate the viability of fungal elements [5]. Neutral red 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 [5].

We found that, out of the 120 samples processed directly, 40 (33.33%) were positive by direct microscopic examination using Neutral red, but only 20 (16.67%) were grown on culture before trypsinization. After trypsinization culture positivity was increased up to 36 (30%) from the microscopy positives i.e. 63 (52.5%) by using neutral red. Amongst 63 neutral red positive, 27 (42.86%) specimens grown with contamination on SDA so they were excluded.

Similar type of results was shown by Xess, et al. in 2004, they found 66.67% growth on SDA after trypsinization. They also found that 3 of their isolates not grown on SDA even though they are positive in Neutral red examination [6]. Also in study conducted by park, et al. only 14 (46.6%)

samples were grown on culture among 27 neutral red positives [9].

Conclusion

This study conclude that isolation rate of fungus in patients with dermatophytic infection may show increased isolation after trypsinization and simultaneously it was found that neutral red staining is very useful technique to distinguish between viable and non viable fungal element.

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