JP

Journal of Advances in Medicine 3(1), 19-29, June 2014

DOI No. 10.5958/2319-4324.2014.01120.1

Evaluation of New Media for Dermatophytes and non Dermatophytes Fungal Infections in diabetic patients

Maysaa El Sayed Zaki* and Wafaa El emshaty

Clinical Pathology-Faculty of Medicine Mansoura University, Egypt

**Corresponding author: Maysaa El Sayed Zaki, may_s65@hotmail.com*

Abstract

Purpose. We studied the occurrence of dermatophytes and non-dermatophytes fungi from diabetic patients. In addition, we compared the newly developed dermatophyte identification medium (DIM) that has little cost with the established dermatophyte test medium (DTM).

Methods. The study was conducted on seventy one clinical samples from diabetic patients complaining of onychomycosis and foot mycosis. In addition to sixty six different fungi species used as positive internal control recovered in our laboratory. Samples were subjected to full mycological examinations.

Results. Using mycological diagnostic methods the commonest fungal isolates from onychomycosis were *Candida* species and *Aspergillus* (22.5% for each). For foot mycosis the commonest fungal isolates was *T. mentagrophytes* (7.5%). Comparable results were obtained for laboratory control fungal isolates and for clinical samples on DIM and DTM. The cost of one test with use of DTM was five dollars while for DIM it was tow dollars.

Conclusion. This study highlights the importance of non dermatophytes fungi as a causative fungal pathogen in onychomycosis and foot mycosis infections in diabetic patients. This finding highlights the importance of non dermatophytes as etiological pathogens rather than coincidence flora. The results of our study also clearly indicate that the use of new DIM is an inexpensive, rapid, and accurate means of presumptively identifying both dermatophytes and non dermatophytes fungi recovered from clinical specimens compared to DTM.

***Keywords**:* DIM, Dermatohytes, Non Dermatophytes fungi, Diabetes mellitus, onychomycosis

Introduction

Nail disfigurement is a common presentation in dermatologic practice, which manifests as a change in shape, surface, color or nail attachment. Fungal infection of the nails, the most common cause in adults, is often difficult to be

differentiated clinically from primary nail diseases such as psoriasis.

Dermatophytes are usually assumed as the principal causative agents of abnormal nails. However, dermatologists are frequently faced with treatment failure and microbiologists are frequently faced with failure to isolate dermatophytes in culture, this may be due to a possible infection by non-dermatophyte molds (NDM).

NDMs are filamentous fungi that are commonly found in nature as soil saprophytes and plant pathogens [1, 2]. It is not known whether infection with NDMs can occur as a primary infection on healthy nails or exist only as secondary invaders living saprophytically in nail plates already damaged by trauma, ischemia or other diseases especially dermatophyte infection. NDMs regularly identified in onychomycosis include *Scytalidium* species (spp.), *Scopulariopsis, Fusarium, Acremonium and Aspergillus* spp. [3].

Generally, diabetic patients are more susceptible to skin infections. Although the overall incidence of skin mycoses in diabetics is not higher as compared with healthy population, diabetics seem to suffer from certain types of mycoses more frequently like tinea pedis and onychomycosis [4].

For laboratory diagnosis culture is a valuable adjunct to direct microscopy and is essential to identify more dermatophytes [1]. The common used media is dermatophyte test medium. Dermatophyte test medium was developed during the Vietnam War for rapid identification of dermatophytes from soldiers with ringworm infections [5, 6].

It was also reported that non dermatophytic fungi caused a color change on DTM, thereby giving false-positive results[7, 8]. A new medium, termed dermatophyte identification medium (DIM), was developed that mainly used bromocresol purple (1.6 mg/ml) as an indicator of dermatophytes growth with production of alkaline metabolites turning the medium from light green into purple color [9]. Improvements introduced in DIM included incubation at 37°C and the use of an increased concentration of cycloheximide [10]. However, few reports evaluated DIM as primary isolating media from clinical samples [9, 10] and no reports evaluated it in diabetic patients.

The purpose of this study was to highlight the occurrence of non dermatophytes as causative fungal pathogen in onychomycosis and foot mycosis in diabetic patients. In addition, we assessed the utility of DIM for identifications for dermatophytes, yeasts and molds causing superficial fungal infections in diabetic patients.

Materials and Methods

Seventy one diabetic patients complaining of superficial fungal infections

attending out patients' clinic of Dermatology in Mansoura University were included in the present study. They were twenty six patients with foot mycosis and forty five patients with onychomycosis. Informed written consent was taken from each patient. The study was approved by ethical committee of Mansoura University. Sixty six different fungal isolates recovered in Mycological lab were included as control strains in the study.

Clinical Samples: Clinical examination was performed and specimens were collected from all patients in three separate occasions after disinfection with 70% alcohol and were kept in dry sterile containers. The suspiciously infected skin around infected toes was scraped and nail eclipses, scrapings or subangular curette were collected from suspected nails with onychomycosis.

The clinical specimens were subjected to direct microscopic examination using 10-20% KOH solution with methylene blue and culture on both DTM (ACU-DTM; Accuderm, Ft. Lauderdale, FL) and DIM media. Triplicate culture was performed for each sample on both media [9].

DIM Preparation DIM containing dextrose (20 mg/ml), neopeptone (10 mg/ml), cycloheximide (4 mg/ml), penicillin (20 U/ml), streptomycin (40 U/ml) and bromocresol purple (0.2gm/L) was prepared. Dextrose, neopeptone and agar were dissolved in water. Bromocresol purple solution was prepared and added to the above solution, which was then autoclaved. The medium was cooled and aseptically filter sterilized. Then, penicillin, streptomycin, and cycloheximide (4 mg/ml) were added, the pH level was adjusted to within a range of 5.5 to 5.7, and the medium was dispensed into tubes. Prepared media with no cyclohexamide were also poured to avoid inhibition of growth of other than *Candida albicans* species. The medium is light brown, and it can be stored at 4 to 8°C for 4 to 6 months. The growth of fungus turns the medium into purple color. *Trichophyton mentagrophytes* was used as positive control and uninoculated medium was used as negative control.

Fungal Culture

For Control strains

A portion of each original culture was grown for 5 to 7 days on Sabouraud dextrose agar (Difco) containing 40 µg of gentamicin per ml and 25 µg of chloramphenicol per ml (supplemented agar is referred to as SAB+ medium). The cultures were aseptically transferred onto two slants containing SAB+ medium and two slants containing DIM and DTM and these slants were incubated at 27°C for 5 to 7 days. These slants were observed daily for growth on SAB+ medium, for color change from light green to purple on DIM and color change of DTM to red color. The results were considered to be negative if either no growth was seen on SAB+ medium or no purple color was evident

on DIM and no red color on DTM.

For Clinical Samples

Microscopic examination was performed using 20% potassium hydroxide (KOH) preparation. The material was placed on a glass slide and 20% KOH was added. The addition of dimethyl sulfoxide (DMSO) to KOH solution was often helpful because of the thickness of the debris. The glass slide was heated for a minute or so under a cover glass then examined microscopically for the presence of fungal elements: spores and hyphae.

Duplicate cultures: Portions of the specimens were aseptically inoculated onto three DIM and three DTM. The slants were incubated at 27°C for four weeks. Examination of fungal colonies on different slants was done for growth morphology, colonial appearance, surface texture, shape, size, color, rate of growth, under surface and edge.

A wet mount preparation of the growth was stained by lactophenol cotton blue stain for easier visualization and examination of fungal elements and the identification of conidia morphology and arrangement. The set criteria for the diagnosis of onychomycosis due to non-dermatophytic molds were: (1) Observation of fungal elements in 15% KOH-preparations made from nail scrapings, (2) growth of the same mold in all three consecutive cultures of the specimens taken three times from the same patient with one-week intervals, (3) no growth of a dermatophyte or yeast in three consecutive cultures [10].

Phenotypic Identification of Fungal isolates

Culture characteristics such as surface texture, topography and pigmentation were reported for each isolate. Microscopic examination was then performed for detection of specific microconidia, macroconidia and chlamydospore.

If rapid microscopic examination was not conclusive more detailed examination of the preparation was performed in order to distinguish other structures that could assist in identification such as spiral and racquet hyphae, favic chandeliers and nodular bodies. It was sometimes useful to determine the effect of certain media and growth factors on the development of the colony potato dextrose agar is a reference medium especially for the production of pigment.as in example Trichophyton rubrum and it is usefull in differentiation of T.rubrum from T.mentagrophytes. Rice grains were used to differentiate Microsporum audouini and Microsporum canis as the former grow poorly on this media [9]. The morphologies of all Candida species were determined by microscopic examination and biochemical identification was performed through the use of API 20C (bioMerieux, Hazelwood, Mo.). Asperigyllus species were identified by characteristic sporing head seen on microscopic

examination and by strong colors and by marked color that it confers on the colony.

Results and Discussion

The study was carried on 71 diebetic patients with type II diabetes mellitus. They were 40 females and 31 males with age range 45-55 years. Clinical samples were 26 skin scales samples from patients with foot mycosis, 45 nails clips from patients with onychomycosis.

For Clinical Samples

Thirty five clinical samples had growth on DIM and forty samples had growth on DTM. The fungal isolates from the diabetic patients were *dermatophytes and non dermatophytes fungi with ratio* 52.5% and 47.5% respectively for DTM and 51.4% and 48.6% respectively, Table 1.

Table 1. Dermatophytes and Non Dermatophytes fungi isolated from Paetients

Type of fungal Isolate	DTM DIM No. % No. %
Dermatohytes	21 52.5 18 51.4%
NDF	19 47.5 17 48.6%
Total	40 100% 35 100%

For cases with foot mycosis dermatophytes had higher rate of isolation (15% & 14.3% for DTM and DIM respectively). While for onychomycosis the NDF had the higher proportion of isolation (40%, 37.1% on DTM & DIM respectively). The commonest dermatophyte associated with foot mycosis was *T. mentagrophytes*. The commonest NDF associated with onychomycosis were Candida albicans and Aspergillus (22.5%), Table 2.

Only ten samples (25%) had positive direct microscopic examination for fungus. Those samples had positive culture, data not shown.

Figure (1) Demonstrates *T. rubrum* growth on DIM.

Color change to violet was observed with all dermatophytes growth on DIM except with 2 isolates of *Trichophyton rubrum (T. rubrum*) & *T. tonsurans* while DTM turned red with all dermatophytes isolates. The mean growth durations on both media were comparable, table 3.

For fungal isolates used as control for both media 34/38 isolates were recovered on DIM and 4 isolates fail to grow. These isolates were *T. violaceum, T. tonsurans, T. rubrum and T. verrucosum* while DTM support

the growth of all 38 dermatophytes isolates. Color change to violet was observed with all dermatophytes growth on DIM except for two isolates *T. rubrum & T. tonsurans* while DTM turned red with all dermatophytes isolates. The mean growth durations on both media were comparable. Regarding non dermatophytes isolates subcultured on DIM & DTM both media support their growth with color change in 14/28 isolates on DTM and 16/28 isolates on DIM, Table 4.

Table 2. Isolated fungus from positive clinical samples.

	DTM (n=40)		DIM (n=35)	
	No	%	No	%
Skin scales	6/40	15%	5/35	14.3%
Dermatophytes	5/40	2.5%	5/35	14.3%
M. audouinii	1/40	7.5%	1/35	2.7%
T. mentagrophytes	3/40	2.5%	3/35	8.1%
M. canis	1/40	2.5%	1/35	2.7%
NDF	1/40	2.5%	0	0
Aspergillus.	1/40	85%	0	0
Nails	**34**/40	85%	30/35	85.7%
Dermatophytes	16/40	40%	13/35	37.1%
M. audouinii	1/40	2.5%	1/35	2.7%
T. mentagrophytes	7/40	17.5%	6/35	16.2%
T. schonlenii	1/40	2.5%	1/35	2.7%
E. floccosum	3/40	7.5%	3/35	8.1%
T.verrucosum	1/40	2.5%	0/35	0
T. tonsurans	2/40	5%	1/35	2.7%
T. rubrum	1/40	2.5%	1/35	2.7%
NDF	18/40	45%	17/35	48.6%
Candida albicans	9/40	22.5%	9/35	21.6%
Aspergillus	9/40	22.5%	8/35	21.6%

The mean time of isolation of fungus showed none statistically significant difference between both media for slowly growing fungi (P=0.3), intermediate growing fungi (P=0.26) and rapidly growing fungi (P=0.500), Table 5. The cost of one culture on DTM was about 5$ while that for DIM is about 2$(data not shown).

The sensitivity, specificity and accuracy of DIM as primary isolation media were 85%, 100% and 91.5% respectively, Table 6.

Superficial mycoses are limited to skin, hair, nails and mucous membranes. The most common etiological agents are dermatophytes and yeasts of Candida genus [11]. Culture is a valuable adjunct to direct microscopy and is essential to identify more dermatophytes. Sabauroud dextrose media is considered as media selective against most dermatophyte and non dermatophytic moulds. Many typical isolates of common dermatophytes can be identified directly from primary isolation media. Identification characters include: colony pigmentation, texture, morphological structure (macroconidia, microconidia, spirals, pectinate branches, etc) [1].

Table 3. Species, color changes and mean duration time of isolation for different dermatophytes and non dermatophytes from clinical samples on DIM & DTM

Isolates	DIM			DTM		
	No	**Color change**	**Duration (days) mean± SD**	**No**	**Color change**	**Duration (days) mean± SD**
Dermatophytes						
M. audouinii	2	2/2	13.75±1.79	2	2/2	14±2.0
T. mentagrophytes	9	9/9	7.11±0.99	10	10/10	7.9±1.04
T. schonelenii	1	1/1	15	1	1/1	14
E. floccosum	3	3/3	8.33±0.58	3	3/3	8.33±0.58
T.verrucosum	0	0	0	1	1/1	15
T. tonsurans	1	1/1	15	2	2/2	15±2.1
M. canis	1	1/1	8	1	1/1	9
T. rubrum	1	1/1	8	1	1/1	9
Non dermatophytes						
Candida albicans	9	2/9	2	9	2/9	2.11±0.33
Aspergillus	8	3/8	3.1±0.35	10	6/10	3.2±0.42
Total	35	23/35		40	29/40	

Onychomycosis, a condition that occurs with increased frequency in patients with diabetes [11, 12], is known to exacerbate diabetic foot problems [13, 14] and reduce patients' overall quality of life [15, 16]. The dermatophyte fungi *T. rubrum* and *T. mentagrophytes* are the predominant pathogens, causing 80–90% of all onychomycosis infections [17, 18]. However in the present study the most frequently isolated fungus species were *Candida*, *Aspergillus*. Similar results were reported by Himioglu *et al.,* (2005) [18] reported that (41%) of patients

with onychomycosis were due to Candida species, and Garg *et al.,* (2004) [19] found that Candida albicans, which was found in 22 patients (24.27%) and thirty-six (39.58%) non dermatophyte molds were isolated from 29 patients. Suggested predisposing factors for these results include immunosuppression, poor peripheral circulation, peripheral neuropathy and trauma.

Table 4. Comparison of culture, color change and mean growth duration of different dermatophytes and nondermatophytes on DTM and DIM (Sub cultured cases: 66).

Isolates	No	DTM			DIM		
		growth	color	Duration In days means± SD	growth	color	Duration In days means± SD
Dermatophytes							
T. mentagrophytes	12	12/12	12/12	6.4±0.7	12/12	12/12	6.08±0.67
M. canis	1	1/1	1/1	6.0±0.1	1/1	1/1	6.0±0.1
T. schonelenii	1	1/1	1/1	12±1.0	1/1	1/1	10± 2.0
E. floccosum	4	4/4	3/4	7.75±1.7	4/4	4/4	7.25±0.96
T. rubrum	4	4/4	4/4	8.5±1	3/4	2/4	8±1
M. audouinii	3	3/3	2/3	9.67±2.5	3/3	3/3	9.33±1.1
T. violaceum	4	4/4	4/4	10±0.8	3/4	3/4	10±2
M. gypsum	1	1/1	1/1	6.0±2.0	1/1	1/1	7.0±1.0
T. tonsurans	4	4/4	4/4	8.75±0.96	3/4	2/4	8.67±0.58
T. verrucosum	4	4/4	4/4	8.25±0.5	3/4	3/4	8.33±0.5
Total dermatophytes	38	38/38	37/38		34/38	32/38	
Non dermatophytes							
Aspergillus	16	16/16	8/16	2.50±0.52	16/16	8/16	2.56±0.51
Mucor	3	3/3	2/3	3.67±0.58	3/3	1/3	3.33±0.47
Candida albicans	9	9/9	4/9	2.0±0.1	9/9	7/9	2±.02
Total NDMs	28	28/28	14/28		28/28	16/28	
Total	66	66/66	51/66		62/66	48/66	

The genus *Aspergillus* was the most common species detected in our study. This may be explained by the presence of this species in some tropical and temperate areas as a predominant one.

It is difficult to determine the role of non dermatophyte fungi (NDF) as primary pathogens. Studies are required to evaluate their ability to invade intact healthy skin and nails, which is a known characteristic of dermatophytes. However, it is conceivable to speculate that NDF can colonize skin and nails that are damaged by trauma, or by other keratinophilic dermatophytes, thereby contributing to infection [18].

Table 5. Mean primary isolation duration

	DIM		DTM		
	No	Days Mean± SD	No	Days mean± SD	P value
Slowly growing fungi	5	13±3	7	13.8±2.6	0.3
Intermediate Growing fungi	13	8.07±2.3	14	8.57±1.7	0.26
Rapid Growing fungi	17	2.58±0.59	19	2.58±0.65	0.500
Total	35		40		

For laboratory diagnosis of mycotic infections direct microscopy using KOH preparation plays an important role in diagnosing nail fungal infections. In the present study all ten samples that were positive by microscopic examination were also positive by culture.

Table 6. Evaluation of DIM as medium for primary isolation of fungi in comparison to DTM

DIM	DTM		
	Positive	Negative	Total
Positive	34	1	35
Negative	6	30	36
Total	40	31	71

Sensitivity: 85% Specificity: 100% Accuracy : 91.5%

Furthermore, nails that are positive by microscopic examination may sometimes yield negative cultures. A possible reason for this discrepancy is that fungi seen on KOH examination may not be viable by the time they are inoculated in vitro and hence do not grow as expected. Also, sampling the nail beyond the distal tip may not yield positive cultures because the infection advances proximally and the fungal elements at the distal end of the nail are less likely to be viable.

Less frequent isolated dermatophytes from nails such as T. schonolini and T, verrucosum were isolated Sebacaree *et al.,* 2008 [20] reported that in Southern Europe and Arabic countries, zoophilic dermatophytes, such as Microsporum canis or Trichophyton verrucosum may cause onychomycosis. The isolation of T.verrucosum may be due to infection of scalp of this patient with contamination of defect in nail.

Valuable finding in our study was that both media used DTM and DIM, when used as primary isolation media or as sub culture media for control fungal isolates, had similar results. However, five isolates failed to grow one isolate of *T. mentagrophytes*, one isolate *T. verrucosum*, one *T. tonsurans* and two *Aspergillus* species. Similar results were obtained for the control strains.

It is relevant to recall that the concentration of cycloheximide in DIM (4 mg/ml) is eight times higher than that in DTM (0.5 mg/ml). A previous report documented that a higher concentration of cycloheximide (5 mg/ml) inhibited the growth of *T. rubrum* and *H. capsulatum*, while these organisms grew well at a lower concentration. It was also reported that the organisms' responses to cycloheximide were variable and depended upon various factors like the concentration of cycloheximide, the duration of exposure of the organism to the drug, species specificity and the development of resistance during exposure to cycloheximide. Interestingly, cycloheximide seems to affect also the growth of other species such as *T. rubrum, T. violaceum and T.verrucosum*.

Regarding non dermatophytes isolates *Candida albicans* was able to grow and induce a color change in the medium, about 100% did grow and approximately 40% of the isolates grew and induced a color change in the medium of both clinical samples and isolates. The lack of a color change in DIM associated with the development of *C. albicans* isolates is attributable to their acidification of the growth medium.

The sensitivity of DIM in indicating the development of both dermatophytes and non dermatophytes species exceeded 80%; with specificity 100%. There was also no difference in the mean time of isolation of different species of fungus. These results are comparable with previous reports [9, 10].

This study highlights the importance of non dermatophytes as a causative fungal pathogen in onychomycosis and tinea pedis infections in diabetic patients. The results of our study also clearly indicate that the use of new DIM is an inexpensive, rapid and accurate means of presumptively identifying both dermatophytes and non dermatophytes recovered from clinical specimens.

References

1 Tampieri MP. 2004. Update on the diagnosis of dermatomycosis. *Parassitologia*; **46** (2): 183-6.

2 Tosti A, 2000. Piraccini BM, Lorenzi S. Onychomycosis caused by non-dermatophytic molds. *J Am Acad Dermatol*; **42**(4):217-20.

3 Gupta AK, Horgan-Bell CB and Summerbell RC. 1998. Onychomycosis associated with *Onychocola canadienesis*: Ten cases reports and a review of the literature. *J Am Acad Dermatol*; **39**(3): 410-15.

4- korepová M.. Mycoses and diabetes. . *Vnitr Lek*; **52**: 2006, 470-473.

5 Taplin D, Zaias N, Rebell G, Blank H. 1969. Isolation and recognition of dermatophytes on a new medium (DTM). *Arch Dermatol*; **99**(3): 203-9.

6 Taplin D. 1972. Dermatophyte test medium. *J Investig Dermatol*.; **19**(1): 23-8.

7 Elewski BE, Leyden J, Rinaldi MG, Atillasoy E. 2002. Office practice-based confirmation of onychomycosis: a US nationwide prospective survey. *Arch Intern Med*; **162**(2): 2133-38

8 Sinski JT, Swanson JR, Kelley LM. 1972. Dermatophyte test medium: clinical and quantitative appraisal. *J. Investig Dermatol*; **58**(1): 405-11.

9 Kane J, Summerbell RC. 1999. *Trichophyton*, *Microsporum*, *Epidermophyton* and agents of superficial mycoses. Murray PR, Baron E J, Pfaller MA, Tenover FC, Yolken RH ed. Manual of Clinical Microbiology, 7th ed. American Society for Microbiology, Washington, D.C; **23**(2): 1275-98.

10 Milne LJR. Fungi. Mackie and McCartney eds. 2001. *Practical Medical Microbiology*, 14th ed Churchill Livingstone; **23**(1) 695-17.

11 Nardin ME, Pelegri DG, Manias VG and Mendez Ede L. 2006. Etiological agents of dermatomycoses isolated in a hospital of Santa Fe City, Argentina. *Rev Argent Microbiol*; **38**(2): 25-7.

12 Gupta AK, Konnikov N, MacDonald P. 1998. Prevalence and epidemiology of toenail onychomycosis in diabetic subjects: a multicentre survey. *Br J Dermatol*; **139**(2): 665–71.

13 Boyko WL, Doyle JJ, Ryu S, Gause D. 1999. Onychomycosis and its impact on secondary infection development in the diabetic population (Poster). Presented at the 4th annual meeting of the International Society for Pharmacoeconomics and Outcomes Research (ISPOR), Arlington; 23–6.

14 Greene RA, Scher RK. 1987. Nail changes associated with diabetes mellitus. *J Am Acad Dermatol*; **16**(2): 1015–21.

15 RichP, HareA. 1999. Onychomycosis in a special patient population: focus on the diabetic. *Int J Dermatol*; **38**(2): 17–9.

16 Drake LA, Scher RK, Smith EB, Faich GA, Smith SL, Hong JJ, Stiller MJ. Effect of onychomycosis on quality of life. *J Am Acad Dermatol* 1998; **38**(4): 702–4.

17 Kemna ME, Elewski BE. 2000. A U.S. Epidemiologic survey of superficial fungal diseases. *J Am Acad Dermatol*; **35**(2): 539–42.

18 Himioglu PS, Metin DY, Inci R, *et al.,* 2005. Non dermatophytic molds as agents of onychomycosis in Ismir, Turkey; a prospective study, *Mycopathologia*; **160**: 125-30.

19 Garg A, Venkatesh V, Singh M, *et al.,* 2004. Onychomycosis in central India: Clincoetiologic correlation. *Int J Dermatol*; **43**:498-502.

20 Seebacher C, Bouchara JP, Mignon B.. Updates on the Epidemiology of Dermatophyte Infections**.** Mycopathologia 2008; 166(6):335-52 .