folia medica

Folia Medica 64(6):922-931
DOI: 10.3897/folmed.64.e85486

Original Articles

In Search of Dermatophytes – Frequency and Etiology of Fungal Infections in Patients with and without Diabetes Mellitus

Gergana Z. Vazheva[1], Liliya G. Zisova[1], Elena A. Becheva[2], Veselin R. Chonov[3], Valentin D. Dichev[4], Tsonka M. Miteva-Katrandzhieva[5], Zoya V. Rachkovska[6], Maria M. Orbetzova[7,8], Veselin T. Belovezhdov[9]

[1] *Department of Dermatology and Venereology, Medical University of Plovdiv, Plovdiv, Bulgaria*
[2] *Department of Endocrinology and Metabolic Diseases, Medical University of Plovdiv, Plovdiv, Bulgaria*
[3] *Department of General and Clinical Pathology, Medical University of Plovdiv, Plovdiv, Bulgaria*
[4] *Department of Medical Biology, Medical University of Plovdiv, Plovdiv, Bulgaria*
[5] *Department of Medical Informatics, Biostatistics and E-learning, Medical University of Plovdiv, Plovdiv, Bulgaria*
[6] *Department of Microbiology and Immunology, Medical University of Plovdiv, Plovdiv, Bulgaria*
[7] *Clinic of Endocrinology and Metabolic Diseases, St George University Hospital, Plovdiv, Bulgaria*
[8] *Department of Endocrinology, Medical University of Plovdiv, Plovdiv, Bulgaria*
[9] *Department of General and Clinical Pathology, Medical University of Plovdiv, Plovdiv, Bulgaria*

Corresponding author: Gergana Z. Vazheva, Department of Dermatology and Venereology, Medical University of Plovdiv, 15A Vassil Aprilov Blvd., Plovdiv, Bulgaria; Email: g.vazheva@gmail.com; Tel.: +359 886 962 602

Received: 21 Apr 2022 ♦ **Accepted:** 12 July 2022 ♦ **Published:** 31 Dec 2022

Citation: Vazheva GZ, Zisova LG, Becheva EA, Chonov VR, Dichev VD, Miteva-Katrandzhieva TM, Rachkovska ZV, Orbetzova MM, Belovezhdov VT. In search of dermatophytes – frequency and etiology of fungal infections in patients with and without diabetes mellitus. Folia Med (Plovdiv) 2022;64(6):922-931. doi: 10.3897/folmed.64.e85486.

Abstract

Introduction: Onychomycosis is a frequent nail disorder, accounting for up to 50% of all nail problems. Treatment of onychomycosis is expensive and requires a long time of antifungal medications. Consequently, a proper and faster diagnosis is necessary. Especially for those patients with diabetes mellitus, where onychomycosis is among the most significant predictors of foot ulcer and possible severe complications.

Aim: To compare the sensitivity, specificity, and turnaround time between direct microscopy, culture, histology, and real-time PCR. In addition, to compare the frequency and etiology of onychomycosis in patients with and without DM.

Materials and methods: This study included 102 patients, divided into two groups. One group consisted of patients with diabetes mellitus and the other – without diabetes. Nail samples were collected and examined by direct KOH microscopic examination, culture, histology, and real-time PCR.

Results: From the 102 patients with clinical onychomycosis, positive KOH was found in 38 (37.3%). Culture – 82 out of 102 samples (80.4%) were positive for dermatophytes, yeasts, and/or NDM. Positive histology samples were 32 (41.6%). The PCR was positive in 57 (55.9%) out of the 102. We discovered that there is no significant statistical difference in the etiology of the fungal infections between the two groups.

Conclusions: All mycological investigations have their place in the diagnosis of onychomycosis. Direct microscopy, culture, and histology are useful methods for clinicians to diagnose and follow up the post-treatment period. The advantages of RT-PCR include obtaining results faster and accurately identifying fungi, thus becoming more valued in the diagnosis of OM.

Keywords

dermatophytosis, histology, onychomycosis, real-time PCR, Trichophyton rubrum

INTRODUCTION

Onychomycosis (OM) is the most common cause of nail infections, representing up to 90% of toenail and at least 50% of fingernail infections.[1] It is present in 2-13% of the general population increasing up to 48% at 70 years of age.[2] OM is caused by three groups of fungi – dermatophytes, yeasts, and non-dermatophyte molds (NDM). The term "dermatophytosis" describes infections by members of the genera *Trichophyton, Microsporum*, and *Epidermophyton*. It is considered that over 90% of onychomycoses are caused by two dermatophytes – *Trichophyton rubrum* and *Trichophyton mentagrophytes*. Yeasts and NDM are the pathogens in about 7% of fungal nail infections.[3] The yeasts causing OM are namely *Candida spp*. with its most frequent representative *C. albicans*, but also *C. krusei, C. glabrata, C. parapsilosis*, and *C. tropicalis*. As far as non-dermatophyte molds are concerned – *Alternaria spp., Aspergillus spp., Acremonium spp*. are among the most common species.[4]

Fungal nail infections are frequently recurrent and evolve into chronic conditions. This not only leads to nail thickening, discoloration, and onycholysis, but also causes discomfort, embarrassment, and in some cases, physical pain.[5] For some patients with concomitant diseases, such as diabetes mellitus, mycotic infection of the nails is a risk factor that could lead to severe possible complications. It is well known that diabetic patients often have problems with their feet, generally due to neuropathy and arterial insufficiency. Traumatic ulcerations, fissures, and following secondary infection lead to an increased risk of toe or lower leg amputation.[6] Infections are a common problem among diabetic patients. Fungal infections are estimated to exceed 50% of all infections in diabetic patients. They are twice as likely to suffer from onychomycosis compared to diabetes-free individuals.[7]

Diabetes mellitus has emerged as one of the most serious and common chronic diseases of our times, causing life-threatening, disabling, costly complications, and reducing life expectancy.[8] The global prevalence of diabetes had reached pandemic proportions with the 10th edition of the IDF reporting a prevalence of 537 million adults (20-79 years) living with diabetes. This number is predicted to rise to 643 million by 2030 and 783 million by 2045.[9]

AIM

The aim of the study was to compare the sensitivity, specificity, and turnaround time between four different diagnostic methods – direct microscopy, culture, histology, and real-time PCR. Also, to compare the frequency and etiology of onychomycosis in patients with and without diabetes mellitus, using these four methods.

MATERIALS AND METHODS

This prospective, comparative study was performed at the Department of Dermatology and Venereology in the Medical University of Plovdiv, Bulgaria, in the period from September 2020 to January 2022. The University Ethics Committee approved the study and all participants signed an informed consent (in Bulgarian) before participation. One hundred and two patients were enrolled in this study with clinically suggestive symptoms and signs of onychomycosis (discoloration, thickening, subungual keratosis, onycholysis, longitudinal and transverse grooves, and dystrophic nail). Patients were divided into two groups – group I – patients with diabetes mellitus (DM) and suspected onychomycosis, and group II – patients without diabetes mellitus, but with suspected onychomycosis (control group). The patients from group I (51 patients) were referred from the Department of Endocrinology, Medical University of Plovdiv and the Clinic of Endocrinology, St. George University Hospital, Plovdiv. They included patients with DM type 1 and type 2 and older than 18 years. Patients from the control group included the same number of nondiabetic patients presenting to the Clinic of Dermatology and Venereology, St. George University Hospital, Plovdiv. None of the 102 subjects suffered from skin disorders known to alter the nail aspect, nor a dermatosis with the potential to involve the nails. Each patient underwent the following examination: personal medical history, complaint – present history including the onset, course and duration of the lesion and associated nail changes, and past – history of fungal infections, previous trauma, previous treatment, diabetes mellitus (and possible complications such as peripheral neuropathy, retinopathy, nephropathy, etc.), and history of fungal infection in the family. All patients were generally examined for associated conditions and lesions predisposing to and suspecting fungal infection as diabetic foot, peripheral vascular disease and concomitant infections e.g. tinea pedis), and also local examinations of the nails was performed – mycological investigations and molecular detection of fungal DNA by real-time PCR.

If a local treatment had been applied, patients were advised to stop using it for at least three days before the sample was taken, so that it wouldn't compromise the results of the performed investigations. No patients taking systemic treatment at the time of the sample taking were enrolled in the study.

Patients were classified according to the following most frequent clinical presentations of onychomycosis – distal subungual (DSO), distolateral subungual (DLSO), superficial white (SWO), proximal (PO) and total dystrophic onychomycosis (TDO). The number of nails involved was evaluated as follows: mild cases (≤4 nails involved), moderate (5-8 nails involved), and severe (≥9 nails involved).[10]

Patients' nails were cleaned with 70% alcohol to remove contaminants and nail scrapings (taken with sterile scalpel

blade) and clippings (taken with small clippers) were collected at the Mycology Laboratory, Department of Dermatology and Venereology in the Medical University of Plovdiv.

The collected specimens were divided into four portions for most of the patients. For some of them, it was only possible to collect enough material for three portions, and histology for those was not possible. The first portion of the specimens was examined microscopically after incubation in 10% KOH. The second portion was cultured on two sets of media: Sabouraud Dextrose Agar (SDA) containing chloramphenicol with and without cycloheximide. The third portion was used to perform real-time PCR and the fourth one was used to perform histology using PAS stain technique.

Direct microscopic examination – the sample to be examined was placed on a clean glass slide. A drop of 10% KOH reagent was added, mixed with the sample, and incubated for 1 hour. The softened nail material was examined under both low (10×) and high (40×) power fields of the microscope for the presence of fungal elements – hyphae, spores, and pseudo-hyphae.

Cultures were carried out on all nail specimens obtained from patients from both diabetic and non-diabetic groups. Samples were inoculated on two sets of media – SDA with chloramphenicol with and without cycloheximide and incubated at 28°C – 36°C and examined regularly for 4 weeks, for any growth. If there was no growth after 4 weeks, the result was reported as negative. Identification of obtained growth was done by macroscopic examination of the culture – observing morphological characteristics of the colony including size, shape, consistency, margins, color both in recto and verso sides, type of the growth whether fluffy, cottony or creamy, and the presence or absence of diffusible pigments **(Figs 1, 2)**. Microscopic examination of the cultures was also performed by tease mount which is the most common technique used for rapid mounting of fungi. In the cases where *Candida spp* culture was obtained, an additional Chrom agar test was performed to differentiate the most common species of *Candida* – *C. albicans*, *C. glabrata*, *C. krusei*, and *C. tropicalis*. The other species were indicated as *C. non-albicans*.

Histological examination – nail clippings were sent for histopathologic examination in a 10% buffered formalin container. The stain chosen in this study was the Periodic Acid-Schiff (PAS). Then each material was observed under a microscope in low (10×) and high (40×) power fields for the presence of fungal elements in a magenta-red color – hyphae, spores, pseudo-hyphae **(Figs 3, 4)**.

Real-time PCR (RT-PCR) was performed using DermaGenius® 2.0 Complete multiplex real-time PCR kit (PathoNostics B.V., The Netherlands) which can detect 12 pathogens, including *Candida albicans*, *Trichophyton rubrum, Trichophyton interdigitale*, *Trichophyton tonsurans*, *Trichophyton mentagrophytes*, *Trichophyton soudanense*, *Trichophyton violaceum*, *Trichophyton benhamiae*, *Trichophyton verrucosum*, *Microsporum canis*, *Microsporum audouinii,* and *Epidermophyton floccosum*. RT-PCR was performed according to the manufacturer's instructions. First, the DNA extraction was carried out and then 5 µl of DNA extract was added both to the PCR mix 1 and PCR mix 2, and a DTprime Real-time Detection Thermal Cycler was used for amplification and melting curve analysis.

Statistical analysis

Descriptive and nonparametric (Fisher's test and McNemar) analyses were performed (SPSS Program, version 19). Some of the results are presented as frequency tables. The sensitivity, specificity, and efficacy of each test are presented and these diagnostic parameters were compared using the

Figure 1. Colony of *Trichophyton interdigitale* on Sabouraud Dextrose Agar.

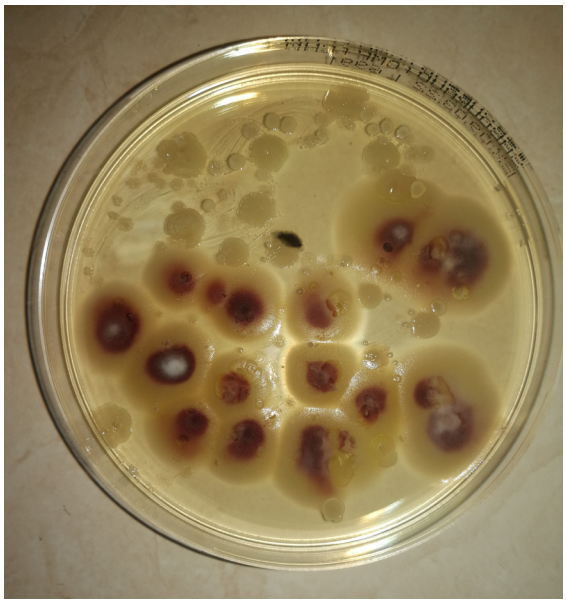

Figure 2. Colonies of *Trichophyton rubrum* on Sabouraud Dextrose Agar.

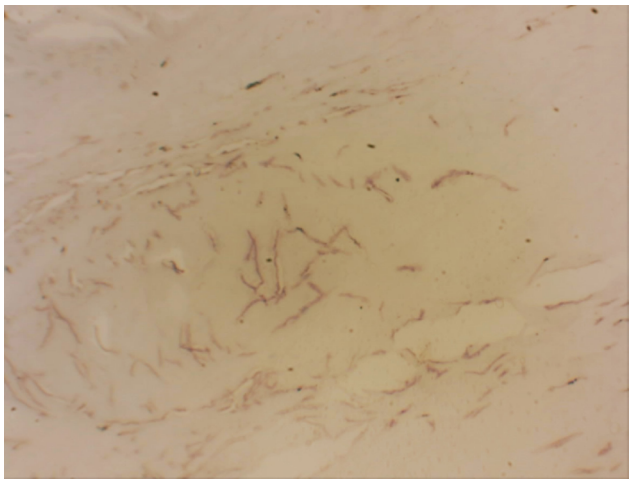

Figure 3. Hyphae seen on histological investigation - ×10 magnification.

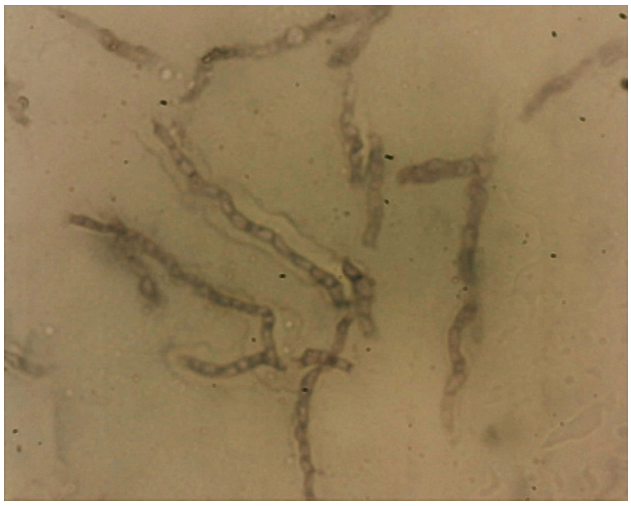

Figure 4. Hyphae seen on histological investigation – ×40 magnification.

McNemar nonparametric test. Values of $p<0.05$ were considered to be significant.

Onychomycosis

To define a true case of onychomycosis in this study, we have taken into account the combination of clinical symptoms of nail disorder with positive histology, and/or a positive culture for a true pathogen (dermatophyte or *Candida spp.*), and/or positive RT-PCR result.

RESULTS

One hundred and two patients were included in this study. Fifty-five of them were women (53.9%) and the rest 47 were men (46.1%). The youngest patient was 20 years of age, whereas the oldest patient was 89 years old. The age mode of patients was 55 with a SD of 16 years. Most of the patients had nail changes in their toenails – 98 (96.1%), and only 4 of them (3.9%) had problems with their fingernails. The severity of the onychomycosis was divided into categories, depending on the number of nails affected – mild (4 or fewer nails affected), moderate (5 to 8 nails affected), severe (≥9 nails involved)[10], and the results were respectively 70 (68.6%), 4 (3.9%), and 28 (27.5%) **(Table 1)**.

Most commonly, both big toes of the patients were affected – 55 cases (53.9%), secondly, all of the toenails – 24 cases (23.5%). According to the clinical presentation of the nail changes, the most frequent type of onychomycosis was the distal subungual, representing 93 (91.2%) of patients. Second in frequency was the TDO with 6 cases (5.9%), and then 2 cases for DLSO, and 1 case of SWO.

The duration of the nail changes varied from 3 months to 30 years. The following scale was introduced to categorize them – less than 1 year, 1 to 5 years, 6 to 10 years, and more than 10 years. The majority of patients had started having nail changes and symptoms between 1 and 5 years before the examination – 50 (49%), then 25 of them (24.5%) had had nail changes for 6 to 10 years. Nineteen (18.6%) patients had experienced nail changes for less than a year, and 8 (7.8%) – for more than 10 years **(Table 2)**.

Table 1. Level of severity of nail involvement

Level of severity of nail involvement	N	%
Mild	70	68.6
Moderate	4	3.9
Severe	28	27.5
Total	**102**	**100.0**

As far as the previous treatment is concerned, 70 patients (68.6%) did not treat their nails in any way before their consultation, whereas 32 (31.4%) used either local or systemic treatment. Out of those 32, 26 had local treatment (81.25%), and the rest 6 patients (18.75%) had systemic treatment. As previously established, patients were advised to stop applying local medication on their nails at least 3

Table 2. Duration of nail changes

Duration of nail changes	N	%
≤ 1 year	19	18.6
1-5 years	50	49.0
6-10 years	25	24.5
≥ 10 years	8	7.8
Total	**102**	**100.0**

days before the sample taking, and none of them had been taking systemic treatment for at least a month before it.

The group of patients with diabetes mellitus included 51 patients with controlled DM. Their disease was established 1 to 5 years ago in 18 cases (35.29%), 6-10 years ago in 18 (35.29%), and over 10 years ago in 15 patients (29.41%) **(Table 3)**.

Table 3. Duration of nail changes and duration of DM

			Duration of nail changes (years)				Total
			<1	1-5	6-10	>10	
Duration of DM (years)	1-5	N	3	10	4	1	18
		% duration of DM	16.7%	55.6%	22.2%	5.6%	100.0%
	6-10	N	4	8	4	2	18
		% duration of DM	22.2%	44.4%	22.2%	11.1%	100.0%
	>10	N	0	7	6	2	15
		% duration of DM	0.0%	46.7%	40.0%	13.3%	100.0%
Total		N	7	25	14	5	51
		% duration of DM	13.7%	49.0%	27.5%	9.8%	100.0%

Out of the total of 18 patients with DM from 1 to 5 years, 3 (16.7%) have had nail changes for less than one year. For the group of patients with a duration of DM of 6 to 10 years, results were as follows – 18 cases in total, 12 of which (66.7%) with nail changes for less than a year, or between 1 and 5 years. For the group of patients with diabetes mellitus longer than 10 years – 15 patients, 13 (86.7%) of them have experienced nail changes for less than one year, between 1 and 5 years, and between 6 and 10 years.

Direct microscopy

Of the 102 patients with clinical onychomycosis, positive KOH was found in 38 (37.3%), where 5 (4.9%) with positive KOH alone, and the rest 33 (32.4%) positive for both KOH and culture. The sensitivity of the direct microscopy was 47.4%, and the specificity – 95.8% (p<0.001, McNemar test).

Culture

In total, 82 out of 102 samples (80.4%) were positive for dermatophytes, yeasts, and/or NDM. Among them, 33 were dermatophytes, 29 were yeasts including 5 *C. albicans*, and 45 (20 of which found in mixed cultures with yeasts and/or dermatophytes) were positive for non-dermatophyte molds including *Alternaria, Scopulariopsis brevicaulis, Aspergillus spp., Penicillium spp., Rhodotorula spp., and Cladosporium spp*.

Trichophyton rubrum was the most frequently detected species with 20 (60.6%), followed by *T. mentagrophytes* with 10 (30.3%). Twenty-four (29.3%) mixed cultures were obtained including a dermatophyte in 11 (13.4%) cases. The sensitivity of this method was 73.1%, and the specificity was 100% (p<0.001, McNemar test).

Histology

Histological examination was possible in 77 out of 102 patients (75.5%). The positive samples were 32 (41.6%). The estimated sensitivity was 55.2%, and specificity was 100% (p<0.001, McNemar test). Negative results were observed in 45 samples among which 18 samples had a positive culture – 12 *Candida albicans and non-albicans*, and 6 dermatophytes, 26.7%, and 13.3%, respectively. A total of 13 out of 45 histology negative samples (28.9%) were associated with real-time PCR positive results.

RT-PCR

The PCR was positive in 57 (55.9%) out of the 102 patients included in the study, among which 28 (49.1%) had positive histologic results. The kit detected a majority of dermatophytes – 55 (96.5%), and 3 *C. albicans* (5.3%). There were 3 mixed infections detected among the 57 positive results that were associated with double signals – 2 samples – *T. rubrum* and *T. interdigitale*, and 1 – *T. rubrum* and *C. albicans*. The most frequent dermatophyte was T. rubrum with 37 cases (64.9%), followed by *T. interdigitale* with 20 cases (35.1%). The PCR was positive in spite of the presence of molds (19 cases) or yeasts other than *C. albicans* (8 cases), which could have been a reason for PCR inhibition. PCR was positive in 13 out of 28 (46.4%) samples with negative culture for a dermatophyte or *C. albicans* but with positive

histologic result, showing its ability to identify non-growing fungal agents. In total, RT-PCR was positive in 57 out of 78 cases of onychomycosis (73.1%). In addition, the test could detect the presence of non-growing dermatophytes in 11 cases (19.2%) and 2 cases of *C. albicans* where histologic result was negative. Real-time PCR had a sensitivity of 73.1%, specificity of 100%, and efficacy of 79.4% ($p<0.001$, McNemar test) **(Table 4)**.

(53.3%) from a total of 45 negative results from both groups. One patient from each group was diagnosed with *Candida albicans*. For the dermatophytes – 9 of each group had a T. interdigitale pathogen isolated and one of each group were with mixed infections consisting of T. *interdigitale* and *T. rubrum*. *Trichophyton rubrum* was the most frequent fungal pathogen. Only one patient from the non-diabetic group had a mixed infection with *T. rubrum* and *C. albicans* **(Table 5)**.

Table 4. Analysis of results of direct KOH microscopy, culture and real-time PCR

Type of examination	Sensitivity (%) N=78*	Specificity (%) N=24*	Efficacy (%) N=102	McNemar test
Direct KOH microscopy	N=37 (47.4%)**	N=23 (95.8%)	N=60 (58.8%)	<0.001
Culture	N=57 (73.1%)**	N=24 (100.0%)	N=81 (79.4%)	<0.001
Real-time PCR	N=57 (73.1%)	N=24 (100.0%)	N=81 (79.4%)	<0.001

* True positive cases of OM (78), true negative cases of OM (24) – according to the set standard; ** Positive cases of direct microscopy and culture after the standard for a true OM is considered.

Eventually, according to the set definition of a true case of OM, the number of the positive cases in our study was 78 and the number of the negative ones was 24.

RT-PCR discrepancies with culture results

In two cases, real-time PCR missed the detection of the dermatophyte *M. ferrugineum* because it was not included in the original kit. In four cases, PCR missed the detection of dermatophytes included in the kit that were detected by culture. In three cases, culture was positive for *T. rubrum*, whereas PCR showed *T. interdigitale*. Discordance with culture results regarding *T. mentagrophytes* (on culture) and T. *interdigitale* (on real-time PCR) was observed in eight cases, but these results could be positively confirmed in favor of PCR by ITS sequencing.

Combination of tests

The combination of histologic examination and real-time PCR had a better sensitivity, specificity, and efficacy than both tests separately with 77.6%, 100%, and 83.1%, respectively. ($p<0.001$, McNemar test).

Onychomycosis – diabetic and non-diabetic group

As far as the frequency of onychomycosis among the diabetic and the non-diabetic patients is concerned, and the etiology of the pathogens, we have found that there was no significant statistical difference between the two groups from RT-PCR ($p>0.05$, Fisher's exact test). The real-time PCR showed negative results in 21 patients of the diabetic group (46.7%) and 24 patients from the non-diabetic group

According to the culture investigation, our data show a slight difference in the etiology but still there was no significant statistical difference in the results between the two groups ($p>0.05$, Fisher's exact test). Out of a total of 20 negative results, 13 were in the diabetic group (65.0%) and 7 in the non-diabetic group (35.0%). Out of 3 *C. albicans* infections, one was in the diabetic group and the other 2 in the non-diabetic group. Whereas, the total number of *C. non-albicans* pathogens was 21, 11 for the diabetic group (52.4%) and 10 for the non-diabetic group (47.6%). For the dermatophytes – *T. mentagrophytes* was isolated in a total of 8 cases, 2 of them in the diabetic group and 6 in the non-diabetic group, 25% and 75%, respectively. There was only one *T. interdigitale* infection isolated in the non-diabetic group. All cases of *T. mentagrophytes* grown with culture were later confirmed by RT-PCR to be in fact *T. interdigitale*, hence the difference in the results. Due to their macroscopic and microscopic resemblance, and the fact that *T. interdigitale* was confirmed to be a variation of *T. mentagrophytes*[11], it is always a challenge to identify the two with cultural examination. *T. rubrum* was again the most frequent dermatophyte isolated with a total of 17 cases, with 7 (41.2%) and 10 (58.8%) for the diabetic and non-diabetic groups, respectively. A total of 5 mixed infections were detected – 2 consisting of *T. mentagrophytes* and *Candida*, 1 of *T. rubrum* and Candida in the diabetic group, and 2 cases of *T. rubrum* and *Candida* in the non-diabetic group.

DISCUSSION

Onychomycosis is a frequent nail infection caused by filamentous fungi. The fungal infections diagnosis based on traditional methods has its advantages but there are also

Table 5. Distribution of etiological agents, according to real-time PCR

Etiological agent, according to real-time PCR		Diabetes Mellitus		Total
		Yes	No	
Negative	N	21	24	45
	%	46.7%	53.3%	100.0%
Candida albicans	N	1	1	2
	%	50.0%	50.0%	100.0%
T. interdigitale	N	9	9	18
	%	50.0%	50.0%	100.0%
T. interdigitale + Tr. rubrum	N	1	1	2
	%	50.0%	50.0%	100.0%
T. rubrum	N	19	15	34
	%	55.9%	44.1%	100.0%
T. rubrum + Candida	N	0	1	1
	%	0.0%	100.0%	100.0%
Total	N	51	51	102
	%	50.0%	50.0%	100.0%

disadvantages that require a more accurate and fast method to facilitate the diagnostic process. In this study, we evaluated the use of four methods for fungal detection in 102 samples of fingernails (4 samples) and toenails (98 samples). As a positive result for onychomycosis, we have taken into account the combination of clinical symptoms of a nail disorder with positive histology, and/or a positive culture for a true pathogen (dermatophyte or *Candida spp*.), and/or positive RT-PCT result.

Other Candida species have been included in the positive results for culture because multiple studies have reported that they could also cause onychomycosis (*C. parapsilosis, C. krusei, C. glabrata, C. guilliermondii, C. zeylanides, and C. tropicalis*), especially in patients with immunosuppression and concomitant diseases such as diabetes mellitus, which is the other aspect of our study.[12-15] NDM were not included as positive results for culture, since it is difficult to determine whether they are concomitants or the real pathogen causing onychomycosis. To determine that, the culture should be repeated several times with the same non-dermatophyte mold as a result.[16] There are, though, some species of NMD, such as *Scopulariopsis brevicaulis* and *Fusarium spp.*, which can be pathogenic in some cases.[17]

The combination of direct microscopy and culture, despite the long turnaround time (TAT) of the culture, is still the most used one in the clinical practice to diagnose onychomycosis.[18] Direct microscopy is indeed the easiest, cheapest, and fastest method for the detection of OM showing hyphae, spores, and arthrospores that a skilled technician can spot in a matter of minutes. The downsides of this method are that it does not determine the etiology of the pathogens and has a low sensitivity and specificity, plus false negative or false positive results are possible. Our study evaluated that the sensitivity of this method was 47.4%, and specificity was 95.8%, which correlates with the results for the same method that Gupta et al. observed in their study – 48%.[18]

Although the PAS stain does not allow differentiation of dermatophyte from non-dermatophyte fungi, histologic examination allows the visualization of spores and hyphae, and can also determine the degree of invasiveness of the nail plate. It can also be used to differentiate fungal infections from psoriasis, lichen planus, or yellow nail syndrome.[19] This method also requires well-trained medical specialists. The specificity of PAS stain, however, is low because the morphology of hyphae and spores detected inside the tissue does not offer any indication as to the fungal genus or species.[20] The sensitivity of the PAS stain obtained in our study is 55.2%, which correlates with the one from a study carried out by Alkhayat et al. (60.9%).[21] For some studies, histologic investigation is a method that is often used and thus, a greater experience is gathered in its performance. The highest sensitivity of this method had been evaluated by a recent meta-analysis that reported a sensitivity of 98% obtained by Shenoy et al.[20,22]

Culture examination, still considered the gold standard, has its limitations that include long TAT and the microorganisms being alive. For studies that compare different diagnostic methods, the samples are divided into portions for each investigation. In our study, we divided the materials into four portions, which can additionally hinder the diagnostic process since unequal distribution of fungal elements in clinical specimens is quite likely. Thus, in some cases, false-negative results could happen. Moreover, the isolation of various NDM such as *Fusarium spp., Asper-*

gillus spp., and *Alternaria spp*. is frequent but their role in nail infection can only be suspected with some certainty in repeated isolations, which increases the time until diagnosis and may cover up a dermatophyte infection.[23,24] This has been observed in our study in 14 cases (13.7%) where real-time PCR isolated dermatophyte infections while culture was showing NDM in 11 cases, and NDM plus *Candida non-albicans* in 3 cases. The culture sensitivity of our study (73.1%) correlated with the one of Marie-Pierre Hayette et al. (71.6%).[17]

Molecular techniques, such as real-time PCR, allow faster (approximately 3 hours) and more sensitive diagnosis, with the advantage of detection of non-viable microorganisms.[25] In our study, we observed such cases where DermaGenius® 2.0 Complete multiplex real-time PCR showed positive results for 11 dermatophytes (10.8%), while cultures were negative. As expected, the two most frequent dermatophytes were *T. rubrum* in the first place, followed by *T. interdigitale*, which has been proven by many authors, such as Gupta et al.[18] The use of real-time PCR assays could positively redefine the role of potential causative agents like NDM and *C. non-albicans* by the detection of coinfections with dermatophytes previously not detected in culture.[17] Our study shows a 73.1% sensitivity, which is in concordance with the study of Marie-Pierre Hayette et al. (80%). The criteria for true onychomycosis chosen for this study explain the lower percentage of sensitivity of real-time PCR since in this study we have broadened them and accepted *C. non-albicans*, as well as other dermatophytes that are not included in the PCR kit, such as *M. ferrugineum*.

Other advantages of real-time PCR include giving clinicians the possibility to perform it in case patients have already started treatment without knowing the exact pathogen. Additionally, when combined with other methods, RT-PCT and histologic examination have even better results in sensitivity, specificity, and efficacy than both tests separately with 77.6%, 100%, and 83.1%, respectively (p<0.001, McNemar test).

Onychomycosis in diabetics is far from being just a cosmetic problem. On the contrary, it is a potentially dangerous disease, leading to complications that are even more dangerous. The morbidity of diabetic patients linked to OM, the growing size of the population with DM, and the high frequency of foot disorders in these patients present a substantial health issue.[6,10]

Our two groups of patients showed no significant statistical difference in the etiology of their fungal infection and infections rates (58.9% and 52.9%). Such conclusions have been reached by Buxton et al.[26] This is in contrast to studies by Pierard and Pierard-Franchimont[27] and Dogra et al.[10] In the latter, the prevalence of onychomycosis in diabetic patients was significantly higher than in controls (17% vs. 6.8%).

All these data do not change the fact that patients, whether with or without DM, still need to be diagnosed and treated for their onychomycosis timely, and thus prevent further possible complications.

CONCLUSIONS

In conclusion, the progress of mycological investigations and the statistical data obtained from the molecular techniques encourage considering new algorithms in the diagnosis of fungal infections, including real-time PCR. While it has still not been converted into the gold standard for fungal detection, it has demonstrated its advantages. It is also important to emphasize the fact that the combination of histologic examination and real-time PCR had a better sensitivity, specificity, and efficacy than both tests separately. Nevertheless, real-time PCR is a distinguished method to embrace, especially in cases with negative culture or NDM growth when a distinct clinical picture is observed.

Acknowledgments

This study was possible with the funding of Medical University of Plovdiv, Plovdiv, Bulgaria.

Conflict of Interest

The authors declare that there is no conflict of interest.

REFERENCES

1. Hafirassou AZ, Valero C, Gassem N, et al. Usefulness of techniques based on real-time PCR for the identification of onychomycosis-causing species. Mycoses 2017; 60(10):638–44.
2. Emam SM, Abd El-salam OH. Real-time PCR: A rapid and sensitive method for diagnosis of dermatophyte induced onychomycosis, a comparative study. Alexandria J Med [Internet] 2016; 52(1):83–90.
3. Summerbell RC, Kane J, Krajden S. Onychomycosis, tinea pedis and tinea manuum caused by non-dermatophytic filamentous fungi. Mycoses 1989; 32(8):609–19.
4. Farkas B, Paul C, Dobozy A, et al. Terbinafine (Lamisil®) treatment of toenail onychomycosis in patients with insulin-dependent and non-insulin-dependent diabetes mellitus: A multicentre trial. Br J Dermatol 2002; 146(2):254–60.
5. Vlahovic TC. Onychomycosis evaluation, treatment options, managing recurrence, and patient outcomes. Clin Pod Med Surg 2016; 33(3):305–18.
6. Al-Mutairi N, Eassa BI, Al-Rqobah DA. Clinical and mycologic characteristics of onychomycosis in diabetic patients. Acta Dermatovenerologica Croat 2010; 18(2):84–91.
7. Gupta AK, Konnikov N, MacDonald P, et al. Prevalence and epidemiology of toenail onychomycosis in diabetic subjects: A multicentre survey. Br J Dermatol 1998; 139(4):665–71.
8. Heald AH, Stedman M, Davies M, et al. Estimating life years lost to diabetes: outcomes from analysis of National Diabetes Audit and Office of National Statistics data. Cardiovasc Endocrinol Metab 2020; 9(4):183–5.
9. Sun H, Saeedi P, Karuranga S, et al. IDF Diabetes Atlas: Global, regional and country-level diabetes prevalence estimates for 2021 and projec-

tions for 2045. Diabetes Res Clin Pract [Internet] 2022; 183:109119. Available from: https://doi.org/10.1016/j.diabres.2021.109119
10. Dogra S, Kumar B, Bhansali A, et al. Epidemiology of onychomycosis in patients with diabetes mellitus in India. Int J Dermatol 2002; 41(10):647–51.
11. Gräser Y, Scott J, Summerbell R. The new species concept in dermatophytes – a polyphasic approach. Mycopathologia 2008; 166(5–6): 239–56.
12. Dorko E, Jautová J, Tkáčiková L. The frequency of Candida species in onychomycosis. Folia Microbiol (Praha) 2002; 47(6):727–31.
13. Ameen M, Lear JT, Madan V, et al. British association of dermatologists' guidelines for the management of onychomycosis 2014. Br J Dermatol 2014; 171(5):937–58.
14. Fich F, Abarzúa-Araya A, Pérez M, et al. Candida parapsilosis and Candida guillermondii: emerging pathogens in nail candidiasis. Indian J Dermatol 2014; 59(1):24–9.
15. Gautret P, Rodier MH, Kauffmann-Lacroix C, et al. Case report and review. Onychomycosis due to Candida parapsilosis. Mycoses 2000; 43(11-12):433–5.
16. Verrier J, Monod M. Diagnosis of dermatophytosis using molecular biology. Mycopathologia 2017; 182(1-2):193–202.
17. Hayette MP, Seidel L, Adjetey C, et al. Clinical evaluation of the DermaGenius® Nail real-time PCR assay for the detection of dermatophytes and Candida albicans in nails. Med Mycol 2019; 57(3):277–83.
18. Gupta AK, Versteeg SG, Shear NH. Onychomycosis in the 21st century: an update on diagnosis, epidemiology, and treatment. J Cutan Med Surg 2017; 21(6):525–39.
19. Nenoff P, Krüger C, Schaller J, et al. Mycology – an update part 2: dermatomycoses: clinical picture and diagnostics. J Ger Soc Dermatology 2014; 12(9):749–77.
20. Velasquez-Agudelo V, Cardona-Arias JA. Meta-analysis of the utility of culture, biopsy, and direct KOH examination for the diagnosis of onychomycosis. BMC Infect Dis 2017; 17(1):1–11.
21. Alkhayat H, Al-Sulaili N, O'Brien E, et al. The PAS stain for routine diagnosis of onychomycosis. Bahrain Medical Bull 2009; 31(2):1–7.
22. Shenoy MM, Teerthanath S, Karnaker VK, et al. Comparison of potassium hydroxide mount and mycological culture with histopathologic examination using periodic acid-Schiff staining of the nail clippings in the diagnosis of onychomycosis. Indian J Dermatol Venereol Leprol 2008; 74(3):226–9.
23. Cuchí-Burgos E, Rubio-Casino R, Ballestero-Téllez M, et al. Commercial real time PCR implementation for rapid diagnosis of onychomycosis: a new workflow in a clinical laboratory. Enfermedades Infecc y Microbiol Clin (English ed). 2021; 39(7):326–9.
24. Kupsch C, Ohst T, Pankewitz F, et al. The agony of choice in dermatophyte diagnostics – performance of different molecular tests and culture in the detection of Trichophyton rubrum and Trichophyton interdigitale. Clin Microbiol Infect 2016; 22(8):735-e11.
25. Spiliopoulou A, Bartzavali C, Jelastopulu E, et al. Evaluation of a commercial PCR test for the diagnosis of dermatophyte nail infections. J Med Microbiol 2015; 64(1):25–31.
26. Buxton PK, Milne LJ, Prescott RJ, et al. The prevalence of dermatophyte infection in well-controlled diabetics and the response to Trichophyton antigen. Br J Dermatol 1996; 134(5):900–3.
27. Piérard GE, Piérard-Franchimont C. The nail under fungal siege in patients with type II diabetes mellitus. Mycoses 2005; 48(5):339–42.

В поисках дерматофитов – частота и этиология грибковых инфекций у больных с сахарным диабетом и без него

Гергана З. Важева[1], Лилия Г. Зисова[1], Елена А. Бечева[2], Веселин Р. Чонов[3], Валентин Д. Дичев[4], Цонка М. Митева-Кантранджиева[5], Зоя В. Рачковска[6], Мария М. Орбецова[7,8], Веселин Т. Беловеждов[9]

[1] *Кафедра дерматологии и венерологии, Медицинский университет – Пловдив, Пловдив, Болгария*

[2] *Кафедра эндокринологии и болезней обмена веществ, Медицинский университет – Пловдив, Пловдив, Болгария*

[3] *Кафедра общей и клинической патологии, Медицинский университет – Пловдив, Пловдив, Болгария*

[4] *Кафедра медицинской биологии, Медицинский университет – Пловдив, Пловдив, Болгария*

[5] *Кафедра медицинской информатики, биостатистики и электронного обучения, Медицинский университет – Пловдив, Пловдив, Болгария*

[6] *Кафедра микробиологии и иммунологии, Медицинский университет – Пловдив, Пловдив, Болгария*

[7] *Клиника эндокринологии и и болезней обмена веществ, УМБАЛ „Св. Георги“, Пловдив, Болгария*

[8] *Кафедра эндокринологии, Медицинский университет – Пловдив, Пловдив, Болгария*

[9] *Кафедра общей и клинической патологии, Медицинский университет – Пловдив, Пловдив, Болгария*

Адрес для корреспонденции: Гергана З. Важева, Кафедра дерматологии и венерологии, Медицинский университет – Пловдив, бул. „Васил Априлов“ № 15А, Пловдив, Болгария; Email: g.vazheva@gmail.com; Тел.: +359 886 962 602

Дата получения: 21 апреля 2022 ♦ **Дата приемки:** 12 июля 2022 ♦ **Дата публикации:** 31 декабря 2022

Образец цитирования: Vazheva GZ, Zisova LG, Becheva EA, Chonov VR, Dichev VD, Miteva-Katrandzhieva TM, Rachkovska ZV, Orbetzova MM, Belovezhdov VT. In search of dermatophytes – frequency and etiology of fungal infections in patients with and without diabetes mellitus. Folia Med (Plovdiv) 2022;64(6):922-931. doi: 10.3897/folmed.64.e85486.

Резюме

Введение: Онихомикоз является частым заболеванием ногтей, на которое приходится до 50% всех проблем с ногтями. Лечение онихомикоза дорого и требует длительного приема противогрибковых препаратов. Следовательно, необходима правильная и более быстрая диагностика. Особенно для тех больных сахарным диабетом, у которых онихомикоз является одним из наиболее значимых предикторов язвы стопы и возможных тяжёлых осложнений.

Цель: Сравнить чувствительность, специфичность и время выполнения прямой микроскопии, посева, гистологии и ПЦР в реальном времени. Кроме того, сравнить частоту и этиологию онихомикоза у больных с СД и без него.

Материалы и методы: В исследование включено 102 пациента, разделённых на две группы. Одну группу составили пациенты с сахарным диабетом, другую – без диабета. Образцы ногтей собирали и исследовали с помощью прямого микроскопического исследования КОН, посева, гистологии и ПЦР в реальном времени.

Результаты: Из 102 пациентов с клиническим онихомикозом положительный КОН был обнаружен у 38 (37.3%). Культура – 82 из 102 образцов (80.4%) были положительными на дерматофиты, дрожжи и/или НДП (недерматофитные плесени). Положительные гистологические образцы были 32 (41.6%). RT-PCR была положительной у 57 (55.9%) из 102. Мы обнаружили, что нет существенной статистической разницы в этиологии грибковых инфекций между двумя группами.

Заключение: Все микологические исследования имеют своё место в диагностике онихомикоза. Прямая микроскопия, посев и гистология являются полезными методами для клиницистов для диагностики и наблюдения за периодом после лечения. К преимуществам RT-PCR можно отнести более быстрое получение результатов и точную идентификацию грибов, что делает её более ценной при диагностике ОМ.

Ключевые слова

дерматофития, гистология, онихомикоз, ПЦР в реальном времени, Trichophyton rubrum