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Prevalence of Non-Dermatophytic Molds in Toenails among Diabetic Patients in Homs, Syria

Lilian George Azar[1*], Walid Khaddam[1], Sundus Yaseen[2]
[1]*Department of Biochemistry and Microbiology, Faculty of Pharmacy, AL-Baath University, Homs, Syria*
[2]*Department of Biochemistry and Microbiology, Faculty of Pharmacy, University of Kalamoon, Damascus, Syria*

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Abstract

Diabetes Mellitus (DM) is a risk factor for fungal infections, including onychomycosis. The study aims to determine the prevalence of toenail onychomycosis in diabetic patients and its correlation with associated factors, identify the causative agents and compare the fungal culture findings with the real-time polymerase chain reaction (RT-PCR) findings of diagnosing 6 fungal isolates. A total of 126 diabetic patients were included between November 2020 to June 2021. Nail samples were subjected to potassium hydroxide 20%. Culture was done on Sabouraud dextrose agar medium (SDA), both with and without cycloheximide. Identification of non-dermatophytic molds was based on colony characteristics, colony reverse, cello-tape flag method and slide culture technique. Molecular analysis was done to make sure of the identification of 6 fungal isolates. Out of 126 cases, 106 (84.1%) had toenails onychomycosis. Non-dermatophytic molds (NDM) were the most common isolates 75 (70.8%), followed by yeasts 8 (7.5%) and mixed infections 23 (21.7%). Distal lateral subungual onychomycosis (DLSO) was the most frequent clinical type 90 (85%). Onychomycosis was found to correlate significantly with advancing age, type 2 diabetes, its duration and clinically abnormal nails. However, there was no significant correlation with gender and glycosylated hemoglobin (HbA1C). When the results obtained by RT-PCR were compared with the results obtained by fungal culture, a 100% match of the isolates was found.

Keywords: Onychomycosis, Diabetes mellitus, Toenails, Non-dermatophytic molds, RT-PCR

انتشار الأعفان من غير الفطريات الخيطية الجلدية في أظافر القدمين بين مرضى داء السكري في حمص، سوريا

ليليان جورج عازار[1*]، وليد خدّام[1]، سندس ياسين[2]
[1] قسم الكيمياء الحيوية والأحياء الدقيقة، كلية الصيدلة، جامعة البعث، حمص، سورية
[2]قسم الكيمياء الحيوية والأحياء الدقيقة، كلية الصيدلة، جامعة القلمون، دمشق، سورية

الخلاصة

يعدُّ داء السكري عامل خطر للإصابة بالإنتانات الفطرية، بما فيها فُطَار الأظافر. تهدف الدراسة إلى تحديد انتشار فطار أظافر القدمين لدى مرضى داء السكري وارتباطه مع عوامل تتعلق به، وتحديد العوامل

* Email: lilianazar020@gmail.com

المُسبِّبة للإصابة، ومقارنة نتائج الزرع الفطري مع نتائج تفاعل البوليميراز التسلسلي بالزمن الحقيقي Real time–polymerase chain reaction (RT–PCR) لتشخيص 6 عزلات فطرية. شملت الدراسة 126 مريضاً بداء السكري وامتدَّت بين تشرين الثاني 2020 وحزيران 2021. تم إخضاع عينات الأظافر بمحلول هيدروكسيد البوتاسوم 20%. أُجرِيَ الزرع على وسط سابورو دكستروز آغار (SDA) مع سيكلوهكزيميد وبدونه. حُدِّدَت هوية الأعفان من غير الفطريات الخيطية الجلدية (NDM) بالاعتماد على خصائص المستعمرات ومقلوب المستعمرة وطريقة الشريط اللاصق وتقنية الزرع على الشريحة. تمَّ إجراء التحليل الجزيئي من أجل التَّأكّد من هوية 6 عزلات فطرية. كان (84.12%) 106 مصابين بفطار أظافر القدمين من أصل 126 حالة. كانت الأعفان من غير الفطريات الخيطية الجلدية (NDM) هي الأكثر عزلاً (70.8%) 75 تبعتها الخمائر (7.5%) 8وإنتانات مختلطة (21.7%) 23. كان فطار الأظافر الجانبي القاصي هو النوع السريري الأكثر تواتراً 90 (85%). وجد أنَّ فطار الأظافر يرتبط مع التقدم بالعمر والنوع الثاني لداء السكري ومدة الإصابة بداء السكري والأظافر الشاذّة سريرياً. لكن لم يكن هناك ارتباط مع الجنس والخضاب السكري (HbA1C). وُجِدَ تطابق بنسبة 100% من العزلات فيما يتعلق بمقارنة النتائج التي حصلنا عليها باستخدام RT–PCR والنتائج التي حصلنا عليها باستخدام الزرع الفطري.

Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized as complete or relative insulin deficiency. The prevalence of diabetes was estimated to be over 246 million people in 2007 and has been predicted to rise to 380 million by 2025 [1]. Diabetes patients are more prone to develop fungal infections [2]. The greater frequency of infections in diabetics is caused by immunity deficiency associated with a hyperglycemic environment, peripheral vascular disease and peripheral neuropathy [3].

Diabetes mellitus is an important predisposing condition for onychomycosis [4], and is characterized as a chronic fungal disorder that can infect toe and finger nails [5]. Onychomycosis presence can be determined with discoloration of the nail, onycholysis and nail plate thickening, and it accounts for 90% of toenail infections globally [6].

The incidence of onychomycosis is up to 50% of all nail infections and 30% of fungal cutaneous infections [7]. There are five different morphological manifestations of this disease: distal lateral subungual onychomycosis (DLSO), proximal subungual onychomycosis (PSO), white superficial onychomycosis (WSO), endonyx onychomycosis (EO) and total dystrophic onychomycosis (TDO) [8]. Onychomycosis can be caused by dermatophytes, yeasts and non-dermatophyic molds (NDM) [9]. Treatment options include debridement, antifungal medications, laser therapy and surgical modalities [10].

At present, the laboratory diagnosis of onychomycosis depends on direct microscopy, culture and molecular techniques [11], like polymerase chain reaction (PCR) which can save time and provide high specificity [12]. However, PCR techniques are expensive and not easily available which reduces their use in general practice [13].

Onychomycosis is not life threatening but because of its high incidence, weak response to medications and its effect on the social and economic life of patients, it constitutes a significant health problem [14]. Onychomycosis can be a cause of diabetic foot ulcers due to inability of diabetics to clip their nails, hence causing them to hurt themselves [15], producing a portal of entry for bacteria, fungi and other organisms [16].

The Aim of the Study

The study aims to determine the prevalence of toenail onychomycosis in diabetic patients and its correlation with associated factors, identify the causative agents and compare the fungal culture findings with the real-time polymerase chain reaction (RT-PCR) findings of diagnosing 6 fungal isolates.

Materials and Methods

Patients

A total of 126 diabetic patients, with age ranging from 27 to 80 years, admitted in Al-Basel Hospital and the Military Hospital in Homs, Syria during November 2020 to June 2021, were enrolled for this study. General information such as age, gender, type and duration of diabetes, glycosylated hemoglobin (HbA1C) levels were recorded. However, participants with gestational diabetes, two feet amputation and those who had been on antifungal treatment during the preceding four weeks were excluded. Control group included 20 non-diabetic, non-immunocompromised, age and gender-matched persons present to outpatient clinics.

Sample Collection

Nail specimens from all patients were collected from both big toenails if all toenails looked clinically normal, otherwise samples were taken from the two toenails that were clinically most likely having onychomycosis if one or more of the toenails looked clinically abnormal [17]. The affected area was first cleaned with 70% ethanol and then the nail clippings were collected with a sterile nail clipper into sterile petri dishes [18] and were afterwards sent to the microbiology laboratory to be examined within 24 hours of specimen collection [19] at Pharmacy College, Al-Baath University. The samples were labelled with patient code, gender, age and the date of collection. In order to know whether there was contamination in the hood or not, the petri dish containing the culture medium without culturing samples in it was placed in the hood. Molecular analysis was done in the Department of Biology in the Atomic Energy Commission of Syria. All participants signed a written informed consent and the protocol was approved by the institute's ethics committee.

Processing Samples

Microscopy and Culture

Direct microscopy was done, where nail materials were incubated in 20% potassium hydroxide solution and then examined with ×10 and ×40 objectives for the presence of any fungal elements [20]. Specimens were inoculated onto Sabouraud dextrose agar (SDA) (Himedia, India), with and without cycloheximide, and were later incubated at 25°C for 6 weeks [21]. The plates were sealed with proprietary tape to prevent airborne contamination in the laboratory. The media were checked daily for growth. Sub-culturing on new SDA plates was carried out to get pure isolates. Identification of non-dermatophytic molds grown on culture plates was based on the colony morphology, reverse pigmentation on SDA, cello-tape flag method and slide culture technique [19].

Molecular Analysis

Molecular characterization was done to confirm the identification of six fungal isolates which were cultured in potato dextrose broth medium (PDB) and potato dextrose agar medium (PDA). Then incubated at 28˚C for 5 days. Genomic DNA was extracted from fungal isolates using an in-house protocol [22]. The extracted DNA concentration was measured by a NanoVue Plus spectrophotometer (Biochrom, Britain). Real time-polymerase chain reaction (RT-PCR) test was performed using Mastercycler Pro Vapo.protect (Eppendorf, Germany) and carried out by genes coding amplification of ribosomal RNA using specific primers (ITSF and ITSR)

(Table 1). All loci were amplified with 200 ng of genomic DNA (1 µL) in a 25 µL reaction volume that contained 1×PCR buffer (20 mM Tris HCl [pH 8.4], 50 mM KCl), 100 mM of each of the four deoxy-nucleoside triphosphates (dNTPs), 1.5 mM MgSO4, 2.5U of Taq DNA Polymerase and 10 µM of each primer. RT-PCR program consisted of a pre-incubation for 4 min at 94˚C for, followed by 34 cycles of denaturation 1 min at 94˚C, annealing for 30 s at 58˚C and extension for 30 s at 72˚C, with a final extension of 10 min at 72˚C. The results of the RT-PCR tests were visualized in a 1% agarose electrophoresis gel at 100V and then visualized under ultraviolet light. The bands corresponding to fungal DNA were cut and purified using QIAquick PCR purification kit (QIAGEN, Germany) and were later sent for nucleotide sequencing in the Department of Molecular Biology using Applied Biosystems (California, USA). The identification was obtained by comparing the sequences in the NCBI database using a Blast search.

Table 1: List of primers used in this study

	Name	Length	Tm	Primer sequence (5'-3')
1	ITSF	18	60	TCCGTAGGTGAACCTGCG
2	ITSR	20	60	GCATCGATGAAGAACGCAGC

Statistical Analysis

Statistical analysis was performed using the Statistical Package for Social Science (SPSS) version 22. Chi-square test was used for categorical data. $P<0.05$ was accepted as being statistically significant.

Results

A total of 126 diabetic patients were enrolled, 80 (63.5%) males and 46 (36.5%) females of which 28 (22.2%) had diabetes type 1 and 98 (77.8%) had diabetes type 2, aged between 27-80. 98 (77.8%) had type 2 diabetes mellitus. 110 (87.3%) had at least one clinically abnormal toenail. In diabetic group with clinical onychomycosis, DLSO was the most common clinical presentation recorded in 90 (85%), followed by WSO in 5 (4.7%) and TDO in 1 (0.9%) of patients, while the rest 10 (9.4%) had normal nails. The prevalence of clinical onychomycosis in diabetic and control groups was 84.1% (106 cases) and 15% (3 cases) respectively. Out of the 126 diabetics, only 14 (11.1%) cases had positive direct microscopy. The 57-71 years old age group most commonly affected. In our study non-dermatophytic molds were the most common group 75 (70.8%) followed by yeasts 8 (7.5%) and mixture of fungal organisms 23 (21.7%). *Penicillium* spp. was the most common isolate among NDM 46 (25.27%), followed by *Chaetomium* spp. 28 (15.38%). In control group, *Aspergillus Niger* was the only isolated fungus in 3 cases. The distribution of non-dermatophytic molds causing onychomycosis in diabetic group is shown in Table 2.

A positive correlation was noted between the presence of onychomycosis and advancing age, type 2 diabetes, its duration and clinically abnormal nails. However, there was no significant correlation with gender and glycosylated hemoglobin. The frequency of onychomycosis in diabetics was significantly higher as compared with control group. Characteristics associated with onychomycosis in diabetics have been summarized in Table 3. There was 100% match between the results of identification of the 6 fungal isolates by both methods (RT-PCR and classical culture) which indicates that the conventional culture techniques used for fungal diagnosis can be reliable to detect fungal infections. The result of molecular analysis is shown in Figure 1 and the fungal species identified using RT-PCR are shown in Table 4.

Table 2 - The distribution of non-dermatophytic molds causing onychomycosis in diabetic group.

Non-dermatophytic Molds Isolated	(%)Number
Acremonium strictum	2 (1.10)
Alternaria spp.	19 (10.43)
A. alternate	3 (1.64)
A. brassicae	2 (1.10)
Aphanoascus spp.	)0.55 (1
Aspergillus candidus	2 (1.10)
A. flavs	6 (3.30)
A. fumigatus	4 (2.20)
A. niger	8 (4.40)
A. versicolor	8 (4.40)
Chrysosporium spp.	2 (1.10)
Chaetomium spp.	28 (15.38)
Cladosporium cladosporoides	16 (8.79)
C. sphaerospermum	10 (5.49)
Fonsecaea spp.	7 (3.85)
F. pedrosoi	1 (0.55)
Monascus spp.	1 (0.55)
Mucor spp.	2 (1.10)
Penicillium spp.	46 (25.27)
Rhizopus arrhizus	6 (3.30)
Scopulariopsis brevicaulis	6 (3.30)
Scedosporium apiospermum	2 (1.10)
Total	182 (100)

Table 3: Characteristics associated with onychomycosis in diabetics.

Characteristics	Onychomycosis (%)		P-value
	Positive	Negative	
Age (years)			
27-41	9 (60)	6 (40)	
42-56	45 (91.8)	4 (8.2)	**0.028**
57-71	50 (83.3)	10 (16.7)	
≥72	2 (100)	0 (0)	
Gender			
Male	66 (82.5)	14 (17.5)	**0.51**
Female	40 (87)	6 (13)	
Clinically abnormal nails			**0.011**
Yes	96 (87.3)	14 (12.7)	
No	10 (62.5)	6 (37.5)	
Type of diabetes			**0.037**
Type 1	20 (71.4)	8 (28.6)	
Type 2	86 (87.8)	12 (12.2)	
glycosylated hemoglobin (HbA1C)			**0.338**
<6.5	20 (90.9)	2 (9.1)	
≥6.5	86 (82.7)	18 (17.3)	
Duration of diabetes (years)			
>5	7 (63.6)	4 (36.4)	
5-10	9 (64.3)	5 (35.7)	**0.007**
10-15	13 (76.5)	4 (23.5)	
15-20	28 (100)	0 (0)	
≥20	49 (87.5)	7 (12.5)	

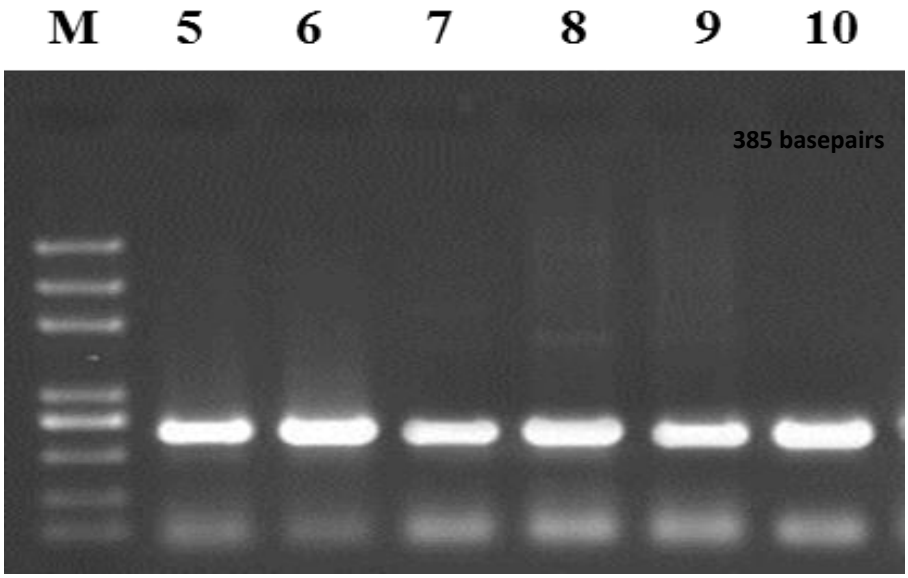

Figure 1: Analysis of the result of RT-PCR using agarose electrophoresis gel.

Table 4: The fungal species identified using RT-PCR.

Isolate N	Isolate Reference	Isolate Name	Identity (%)	NCBI Accession N
5	*Fonsecaea* sp.	*Fonsecaea pedrosoi*	100	**NR_130652**
6	*Alternaria* sp.	*A. alternata*	100	**KX118413**
7	*Alternaria* sp.	*A. brassicae*	100	**KX139152**
8	*Alternaria* sp.	*A. alternata*	100	**KX118413**
9	*Alternaria* sp.	*A. alternata*	100	**KX118417**
10	*Alternaria* sp.	*A. brassicae*	100	**KX139155**

Discussion

The prevalence of onychomycosis among diabetics confirmed by culture was 106 (84.1%), which is similar to the 81.5% obtained by a study in Malaysia [23]. Whereas it is higher than earlier studies [24, 25].

As per the other studies [18, 23], the most frequently isolated fungal element in this study was non-dermatophytic molds 75 (70.8%). However, another study [19] had shown dermatophytes as the most common pathogen isolated from diabetics with onychomycosis which is probably due to the result differences in humidity level, and in climatic and socio-economic factors in the different geographical areas.

In the present study, distal subungual onychomycosis (DLSO) was observed to be the most common clinical presentation which is in line with studies performed elsewhere [26, 27]. Similar to our study, a previous study [28] found a significant correlation with the duration of diabetes, but other ones did not detect any such association [29].

The prevalence of onychomycosis in both genders was also compared. Apparently, more males had onychomycosis than females. However, the difference was not significant which is in accordance with another study [21]. This result may be due to the similarity between the two genders in the lifestyle, occupational activities, exposure to nail trauma and use of close-toe shoes.

In our study, like two others [31, 32], advancing age was significantly associated with the presence of onychomycosis. In contrast, no such relation was observed in other studies [32]. This can be attributed to many reasons, including suboptimal immune status, inability of grooming the nails, increased exposure to causative fungi and nail trauma, poor circulation, slower nail growth and larger and distorted nail surfaces.

In this study, the glycemic control seems to have no statistically significant role in determining the incidence of fungal infection in toenails which is consistent with another study [23]. In contrast, another study has observed such relationship [27]. This could be due to the different cut-off values for HbA1C or different laboratory measures used in these studies.
The results of this study agreed with another study [23, which showed a positive correlation between the presence of onychomycosis and clinically abnormal nails.
Unlike previous investigation [32,] a significant relationship was found between type 2 DM and onychomycosis.

Comparing the results obtained by RT-PCR with those obtained by fungal cultures, there was a match in 100% of the isolates which is higher than what was reported in a previous work [33] (76.5%), where the fungal DNA was extracted using a small fragment of the same toenail sample. Although *A. brassicae* is pathogenic to plants, there was no relationship between the work of patients whose nails samples were taken from and their infection with onychomycosis by this species.

Conclusions

This study shows that the prevalence of onychomycosis was high among diabetes patients. Fungal nail infections are much more than a cosmetic problem and cause an important medical disorder in diabetics who have a greater risk of serious complications from this disease, including limb amputations. In order to minimize the possible problems associated with onychomycosis, regular examination and appropriate treatment are.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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