

ORIGINAL ARTICLE

Rapid Detection of Dermatophytes in Patients with Suspected Onychomycosis using a Multiplex Polymerase Chain Reaction (PCR)

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ABSTRACT

Key words:
Onychomycosis;
Dermatophytes;
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Background: Onychomycosis is a common nail disorder caused mostly by *Trichophyton rubrum* and *Trichophyton mentagrophytes*. As other conditions may resemble onychomycosis, the accurate detection and identification of the causal agent is mandatory. Conventional methods for detecting fungi in nail specimens are either nonspecific or insensitive and time consuming, especially in cases of previous antifungal therapy. Recently, PCR based assays have been introduced to improve onychomycosis diagnosis. **Objectives:** Evaluation of the performance of a multiplex PCR for detecting dermatophytes compared with the results of the conventional culture technique in patients with clinically suspected onychomycosis. **Methodology:** A multiplex PCR-based method was evaluated using 50 nail specimens collected from onychomycosis patients. After a rapid DNA extraction method, the multiplex PCR was performed using three sets of primers aimed at detecting dermatophytes generally, *T. rubrum* and *T. mentagrophytes*. Amplicon analysis was made using agarose gel electrophoresis. PCR results were compared with those of the direct microscopy and culture of the nails. **Results:** Among the 50 patients with onychomycosis, 70% (35/50) were positive for fungal elements microscopic examination. Positive fungal cultures were detected in 60% (30/50). The results of PCR showed that 58% (29/50) were positive by pan Derm PCR. Dermatophyte positive by PCR were 23 *T. rubrum*, 4 *T. mentagrophytes* and 2 other dermatophytes, while 20/50 (40%) of them yielded growth of dermatophytes in culture, 17 *T. rubrum* and 3 *T. mentagrophytes*. In ten samples where culture yielded growth of non-dermatophyte moulds and yeasts, PCR was positive for *T. rubrum* (one) while PCR was negative in 9 non-dermatophyte mould and yeast positive cultures. **Conclusion:** Multiplex-PCR method applied directly on nail specimens could be a promising diagnostic tool for the management of the patients with suspected onychomycosis.

INTRODUCTION

Tinea unguium, a dermatophyte infection of nails, also known as onychomycosis, is considered one of the most prevalent fungal infections in human affecting different ages and population. It can affect both fingernails and toenails representing about 90% of fingernail infections and up to 50% of toenail infections¹⁻⁴.

Predisposing factors for onychomycosis include increasing age, male gender, trauma, immunosuppression, diabetes mellitus, poor peripheral circulation, smoking and tinea pedis. In addition for fingernails persistent exposure to water, the use of artificial nails, and trauma induced by pushing back the cuticles and aggressive manicuring may also be predisposing factors⁵.

Trichophyton rubrum is the main pathogen implicated^{6,7} followed by *Trichophyton interdigitale* formerly *Trichophyton mentagrophytes* var. *interdigitale*^{8,9}. Less commonly associated species are *Epidermophyton floccosum*^{10,11} and *Trichophyton*

*verrucosum*¹². In addition to dermatophytes, *Candida* and non-dermatophyte moulds may be recovered from clinically affected nails; however, their clinical significance is controversial⁶.

Treatment options of tinea unguium depend mainly on proper diagnosis and identification of the causative agent whether it is dermatophytes or not¹³. Conventional diagnosis is based on detection of fungal elements by direct microscopy of clinical specimens followed by culture and morphological identification of the fungus. The whole procedure is time-consuming, requiring 10 to 15 days, sometimes up to 3 to 4 weeks, and accuracy depends on the expertise of the personnel. Introduction of PCR-based methodology could increase sensitivity, specificity and speed, and, potentially, even reduce cost in the diagnostic approach associated with additional visits to the clinician and additional sampling and diagnostic tests, and inconvenience for the patient due to the delay in appropriate treatment¹⁴.

The present study was performed to evaluate PCR technique which allows detection of pandermatophyte DNA and *T. rubrum* and *T. interdigitale* DNA in a

specimen, by comparing the detection rates with the conventional diagnostic methods of direct microscopy and fungal culture in patients with suspected onychomycosis.

METHODOLOGY

Study Design and Population

This study was performed on 50 patients, who had clinical features of various types of onychomycosis (distal and lateral subungual onychomycosis, superficial white onychomycosis, proximal subungual onychomycosis and total nail dystrophic onychomycosis) who attended the outpatient clinic of the Department of Dermatology, Ain Shams University Hospitals (ASUHs) in the period from April 2016 to May 2017. The study was approved by the ethics committee and informed consent was obtained from those who agreed to participate after explaining the study and its goals to them. Patients who received topical or systemic antifungal treatments four weeks before sampling were excluded from this study.

Identification of dermatophytes

The specimens were obtained from clinically abnormal nails, by a vigorous scrapping of the nail bed,

underside of the nail plate and the hyponychium, after cleaning the affected area with 80% ethanol.

All collected samples were divided into three portions. The first portion was examined microscopically, Specimens were placed on slides and one drop of 20% Potassium Hydroxide (KOH) was added to each slide. A microscopic examination was carried out for the presence of fungal elements after incubating the slides for two hours or until digestion of specimens occurred. The second was cultured on each of two isolation media 1) Sabouraud's dextrose agar without antibiotics and 2) Sabouraud's dextrose agar with 5% Chloramphenicol and Cycloheximide. Both media were used in duplicate and they were kept at 25°C and 37°C. They were examined daily for six weeks before they were declared as negative. The growth was noted for colony characteristics in the form of rate of growth, texture of growth, surface colour, and colour on reverse and diffusible pigments (figure 1). For microscopic morphology, tease mounts, cellophane tape mounts and slide cultures were done. The third portion of the nail specimen was used for PCR analysis.

Yeasts were identified on the basis of germ tube test and microscopic morphology on Sabouraud's dextrose agar.

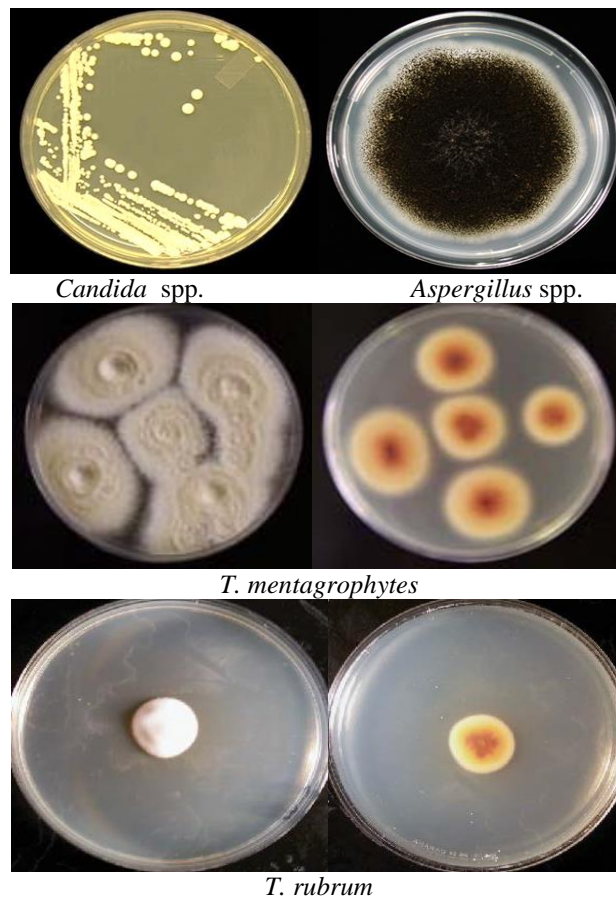


Fig. 1: The isolated fungi on Sabouraud's dextrose agar

DNA extraction

Prior to the extraction, relatively large nail fragments were cut into small pieces with a surgical blade.

Subsequently, nucleic acid extraction was performed according to the manufacturers instructions by using the QiaAmp DNA extraction Kit (Qiagen, Venlo (Pays Bas), Germany). At the end of the procedure, the DNA pellet was dissolved in 50–70 µl hydration solution, depending on the amount of the nail material used at the beginning. Extracted DNA was kept at -20 °C until use. A quantity of 2 µl of DNA was added in PCR mixture.

Primers design

The nucleotide sequences of the different dermatophytes were selected from the NCBI (*National Center for Biotechnology Information*) nucleotide database. The selected primers and their PCR product size are shown in table (1). The primers consisted of the following: Derm primers that amplify all dermatophyte species, TR primer and TM primer that specifically amplify *T. rubrum* and *T. mentagrophytes* respectively. A Multiplex (MX) PCR using the three primers in the same reaction was performed.

Table 1: Primer sequences, priming regions and target amplicon size for multiplex PCR.

Primer name	Nucleotide sequence 5'→3'	Gene region	PCR product (bp)
Derm F	GAA GCC TGG AAG AAG ATT GTC G	Chitine synthase gene 1	432
Derm R	CCT TGA TTT CAC CGC AGG CAC		
TRF TRR	CCC CCC ACG ATA GGG ACCG GAC TGA CAG CTC TTC AGA GAA TT	ITS gene	214
TMF TMR	GCC CCC CAC GAT AGG GCC AA CTC GCC GAA CGG CTC TCC TG	ITS gene	132

TR: *Trichophyton rubrum*, TM: *Trichophyton mentagrophytes*, ITS: internal transcribed spacer. Derm F and Derm R amplify all dermatophyte species¹⁵.

Multiplex PCR amplification

Multiplex PCR was performed on DNA extracts from all samples under the following conditions: the amplification reaction was performed in a total volume of 50 µl; the PCR mixture contained 10 µl of 5X reaction buffer (GoTaq DNA buffer; Promega, Madison, WI, USA), 0.5 µl of 25 mmol/L desoxynucleoside triphosphates containing an equimolar mixture of dATP, dCTP, dGTP and dTTP (Promega), 1 µl (30 µmol/L) of each primer, 1.25 unit of GoTaq DNA polymerase (Promega) and 50 ng of template DNA.

Samples were amplified through 30 cycles in a thermocycler (Thermolyne Amplitron II Series 1091, Barnstead Thermolyne Corporation, Dubuque, IA, USA) as follows: initial denaturation for 5 min at 95 °C, denaturation for 30 s at 94 °C, annealing for 30 s at 60 °C and extension for 30 s at 72 °C. This was followed by a final extension step for 10 min at 72 °C. PCR products were separated on 2% agarose gel, stained with ethidium bromide and visualized under an UV illumination (figure

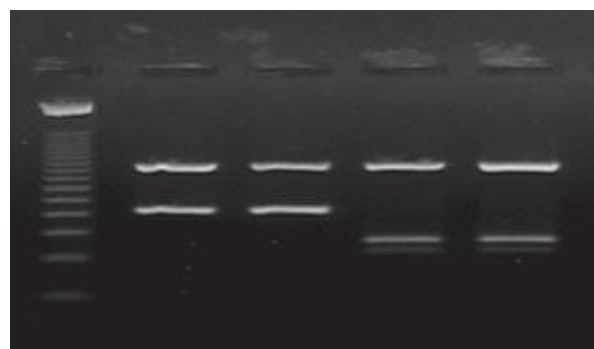


Fig. 2: Results of multiplex PCR. M, size markers (50 bp DNA ladder); lane 1 and 2 specimens from *T. rubrum* infection; lanes 3 and 4, specimens from *T. mentagrophytes* infection; lane 5 negative control.

Statistical Analysis

The collected quantitative data, the mean and standard deviation were calculated. For qualitative data, description of qualitative variables was done in the form of number and percentage. The sensitivities, specificities, positive predictive values (PPV) and negative predictive values (NPV) of the Pan Derm PCR using culture as the gold standard were calculated as described by Ilstrup, 1990¹⁶.

RESULTS

The age of the study population ranged between 18 and 60 years Mean±SD 31.1±10.8. The study population comprised from 32 females and 18 males. Of the 50 patients with clinically suspected cases of onychomycosis, 70% (35/50) were positive for fungal elements by KOH microscopy. Positive fungal cultures were detected in 60% (30/50). PCR giving positive

results for dermatophytes in 58% (29/50). *T. rubrum* was detected in 46% (23/50), *T. mentagrophytes* was detected in 8% (4/50) while other dermatophytes were detected in 4% (2/50) as shown in table (2). As regards the positive fungal cultures, dermatophytes were detected in 40% (20/50) (17 *T. rubrum* and 3 *T. mentagrophytes*), non-dermatophytic molds (NDMs) were detected in 6% (3/50) while yeast were detected in 14% (7/50) as shown in table (3).

Table 2: Comparison of different methods used for diagnosis of onychomycosis among the study group.

Test		N	%	
Direct KOH microscopy		+ve	35	70%
		-ve	15	30%
Fungal culture		+ve	30	60%
		-ve	20	40%
PCR	Pan-dermatophyte PCR	+ve	29	58%
		-ve	21	42%
	<i>T. rubrum</i>	+ve	23	46%
		-ve	27	54%
	<i>T. mentagrophytes</i>	+ve	4	8%
		-ve	46	92%
	Other Dermatophytes	+ve	2	4%
		-ve	48	96%

Table 3: Distribution of dermatophytes and non dermatophytic isolates among subjects.

Microorganism		No of isolates	%
Dermatophytes	<i>T. rubrum</i>	17	40%
	<i>T. mentagrophyte</i>	3	
Non dermatophytic moulds	<i>Aspergillus</i> spp.	3	6%
Yeasts	<i>Candida</i> spp.	7	14%
Total		30/50	60%

Results of culture and PCR among positively samples were shown in tables (4). Among 35 microscopy positive samples, *T. rubrum* was detected by culture but not detected by PCR in 2 samples possibly due to the small quantity of the examined

material. While 7 samples were positive by PCR for dermatophytes but negative by culture (5 *T. rubrum*, 1 *T. Mentagrophyte* and 1 other dermatophyte). One sample was both negative by culture and PCR.

Table 4: Results of culture and PCR among positively microscopic samples

Results	<i>T. rubrum</i>	<i>T. Mentagrophytes</i>	Other dermatophytes	<i>Aspergillus spp</i>	<i>Candida albicans</i>	Other micro-organism
+ve culture -ve PCR	2	0	0	2	6	0
-ve culture +ve PCR	5	1	1	0	0	0
+ve in both	15	2	0	0	0	0
-ve in both	0	0	0	0	0	1
Total (35)	22	3	1	2	6	1

Among 15 microscopy negative samples, 3 were positive by culture (1 *T. mentagrophytes*, one non dermatophyte mould, 1 yeast). Whereas, among 12 samples negative by both conventional methods, 3 were PCR positive (2 for *T. rubrum* and 1 for other dermatophytes). *T. rubrum* was detected by PCR as mixed infection in one sample in which culture also detected growth of non-dermatophyte moulds.

The sensitivity, specificity, NPV, PPV for Pan Derm PCR using culture as the gold standard, PCR demonstrated sensitivity up to 90%, whereas specificity was 63.33%. The NPV was excellent (90.47%) and PPV was 62.07%.

DISCUSSION

Results indicate that incorporation of multiplex PCR techniques in routine laboratory processing of nail scrapings not only augments detection of dermatophytes, but also, in the vast majority of cases, identifies the causative agent.

In the current study occurrence of onychomycosis was detected in patients ageing between 18 and 60 years. This is in accordance with that reported by Bokhari et al.¹⁷ and Garg et al.¹⁸. The increased prevalence of nail lesions by fungi in adults can be attributed to increase the possibility of nail trauma and slow rate of nail growth¹⁹.

Our study included more female patients 64% affected by onychomycosis. This goes in agreement with Tasic et al.²⁰ who revealed that (67%) of onychomycosis patients were female. Also, Brilhante et al.²¹ and Bonifaze et al.²² were in line with our result as they found that male to female ratio was 1:1.6 and 1:3 respectively. On the other hand, Ghannoum et al.¹ and Saunte et al.⁴ detected that onychomycosis was twice or three times more in male patients than female patients. Veer et al.²³ found that higher incidence 65% was noted amongst males, with a male to female ratio 1.8:1. This attributed to the suggestion that men exercise more. This higher incidence was observed also in other studies^{24,25}. However, Roberts found that incidence was the same in both sexes²⁶.

According to Muge et al.²⁷ dermatophytes, yeast and non-dermatophytic moulds (NDMs) may cause onychomycosis. Dermatophytes appear to be the chief organisms capable of primary attack of the nail and consequently the majority of cases were clearly caused by dermatophytes. They reported that dermatophytes mainly *Trichophyton* represented the most commonly isolated agent, followed by *Candida*. Also, Gupta and Ricci²⁸ showed that *T. rubrum* and *T. mentagrophytes* were the main causative agents in all cases of onychomycosis. The results of both studies are consistent with results of our study. On the other hand, Khafagy et al.²⁹ isolated high percentage of NDMs in

onychomycosis in Egypt. Also, El-Batawi et al.³⁰ showed that most cases were caused by *Aspergillus* infection.

The difference in the results between the studies may be due to the difference in the criteria and mycological methods used for diagnosis of fungal infection.

In the present study, positive samples for fungi represent (70%) microscopic examination which is in accordance with Pontes et al.³¹ and Brilhante et al.²¹ who observed positive microscopic examination in (68.4%) and (48%) of the examined samples respectively. On the other hand, Kam et al.³² and El-Batawi et al.³⁰ found low percentage (14.3%) and (21.8%) respectively. We also found that fungal culture on SDA was positive in 30 specimens (60%) which is in accordance with that detected by Chandran et al.³³ and Lopes et al.³⁴ who observed positive culture in (53%) and (56.6%) of the examined samples respectively. Furthermore, the percentage of positive samples for fungi by culture found by Pontes et al.³¹ (66.5%), and El-Batawi et al.³⁰ (68.7%).

High positive rate of detection by direct KOH may be attributed in part to high detection of septate hyphae that could be due to non-dermatophyte filamentous fungi as their detection in nail specimens may be attributed to contamination, transient colonization and infection of a traumatized or otherwise diseased nail, mixed infection and persistence after cure of the dermatophyte or even contamination in the laboratory; thus, repeated recovery is often required before a pathogenic role is considered^{35,36}.

The KOH positive/culture negative results may be due to the presence of artifacts which may yield false positive results.

Overall 29/50 (58%) of the samples were dermatophyte positive by PCR (23 for *T. rubrum*, 4 for *T. mentagrophytes* and 2 dermatophytes), while 20/50 (40%) of them yielded growth of dermatophytes in culture (17 *T. rubrum* and 3 *T. mentagrophytes*) (increase in species-specific identification by PCR-method: 16%). Also, PCR picked up 3 specimens missed by microscope. Positive *T. rubrum* culture but negative PCR was found in 4% of samples. This goes in agreement with that of Luk et al.³⁷.

Furthermore, Brasch et al.³⁸ found that employment of PCR increased detection of *T. rubrum* in nail scrapings by approximately 20 %, whereas positive *T. rubrum* culture but negative PCR was found in 3% of samples. Also, a study performed by Spiliopoulou et al.³⁹ detected negative PCR results despite a positive culture in 1.4% of nail samples. This may be explained by an imbalanced distribution of fungal elements within different parts of a sample leading to an insufficient amount of DNA within the material used⁴⁰.

The sensitivity, specificity, NPV, PPV for Pan Derm PCR using culture as the gold standard, PCR demonstrated sensitivity up to 90%, whereas specificity was 63.33%. The NPV was excellent (90.47%) and PPV was 62.07%.

Lower PCR diagnostic indices such as PPV and specificity, at a lesser extent, by the use of culture as the gold standard can be explained by the lower number of onychomycosis cases identified by culture. The only way to increase the positivity rate is to increase the number of analysed samples ⁴⁰.

An inherent trait of PCR is that non-viable cells with intact nucleic acid will be detected rendering a more sensitive test; the PCR-test is therefore less vulnerable to poor sample quality than culturing.

CONCLUSION

This easy and rapid multiplex-PCR method applied directly on nail specimens could be a promising diagnostic tool to conventional methods for the management of the patients with suspected onychomycosis. This study demonstrates that there is increase in species-specific identification by PCR-method. So, we suggest that PCR should be used as a complementary method for confirmation of clinically suspected onychomycosis.

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