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The role of PCR in the diagnosis of dermatophytes in onychomycosis

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Abstract

Background: Onychomycosis is a critical medical problem. This fungal infection of the nails leads to disfigurement, pain and impaired quality of life. Given that it requires long-term expensive therapy; a proper diagnosis of this infection is greatly demanded.

Aim: To address onychomycosis regarding clinical and laboratory findings, and investigate different laboratory methods used in the identification of dermatophytes implicated in onychomycosis; including conventional laboratory methods (KOH preparation), culture and multiplex polymerase chain reaction (PCR).

Methods: This cross-section study included sixty-one (61) patients (82% females and 18% males). All collected samples were investigated using microscopic examination and cultivation of samples. In addition, DNA extraction and PCR amplification were evaluated.

Results: After mycological study of 61 cases, we found that distal lateral subungual onychomycosis (DLSO) was the most common clinical type in our study. Also, the dermatophytes appeared to be the chief causative agent in onychomycosis (61%). In addition, positive results were identified in 67.2%, 60.7% and 73.8% of the studied group using PCR, culture and, KOH direct microscopy, respectively. With respects to the results of PCR in the study, the sensitivity, specificity and accuracy were, 100%, 83.3% and 93.4% respectively.

Conclusion: PCR was a highly sensitive method for diagnosis of onychomycosis. The application of PCR technology directly to the clinical specimens will permit early and accurate diagnosis of onychomycosis, and leading to prompt initiation of specific antifungal therapy.

Keywords
Onychomycosis, Dermatophytes, PCR diagnosis, Egyptian Patients.

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Introduction

Onychomycosis refers to a fungal infection that affects the toenails or the fingernails [1]. In clinical practice, it is the most commonly encountered nail disorder. Onychomycosis of the finger and toe nails affects approximately 5.5% of the world's population. It results in local pain,paraesthesia, diminished social interactions and quality of life [2]. Treatment of onychomycosis requires expensive and long-term therapeutic regimens with low cure rates and high relapse rates potential side effects. Therefore, a proper diagnosis of infection is needed [3-4].

Onychomycosis is most commonly caused by dermatophytes. These hyaline septated moulds such as *Tinea rubrum* and *Tinea mentagrophytes* are the main causative pathogens responsible for 80-90% of cases (about 90% of cases of toe nail onychomycosis and at least 50% of finger nail onychomycosis). However, *Candida* species and Non-Dermatophyte Moulds (NDMs) have also been implicated. Dermatophytes account for [5]. NDMs such as *Acremonium* species, *Alternaria* species, *Aspergillus* species, *Fusarium* species, *Scytalidium* species and *Scopulariopsis* species have been found to be involved in 2-11% of the onychomycosis cases reported. Yeasts, including *Candida* species, account for 2-10% of fungal nail infections [6].

Clinically, Onychomycosis is commonly classified as distal lateral subungual onychomycosis (DLSO), superficial white onychomycosis (SWO), proximal subungual onychomycosis (PSO) and total dystrophy (TD) based on the pattern and the site of infection involving the nail complex [7]. In DLSO, the invasion begins at the hyponychium and disturbs the distal nail bed; and spreads gradually to the matrix from distal to proximal with subungual hyperkeratosis and yellowish discoloration. However, in PSO, it affects the skin of the proximal nail fold, the cuticle and progressing along the eponychium (epithelium of the underside of the proximal nail fold). In this type, the fungi invade the nail plate from the matrix and proliferate distally within the nail plate. Other clinical forms include (SWO), where the upper surface of the nail plate is first attacked; (TD), which describes total nail plate involvement and surrounding periungual tissue; and endoynx, which describes distal nail plate attack resulting in a deeper penetration of hyphae [8-9].

Laboratory diagnosis of onychomycosis relies on proper sampling of the nail and demonstration of hyphae by direct microscopic examination after treatment with KOH, followed by culture and species identification [10]. Direct microscopic examination of nail material is often adequate for the diagnosis of a fungal infection. It is considered as simple, rapid and cost-effective method. However, it does not provide genus or species identification and also it may give false-negative results [11].

Conventionally, a definitive diagnosis depends on culture isolation. Nevertheless, the culture is negative in up to 20% of the microscopy-positive cases and is time-consuming due to the slow growth (3 to 4 weeks) and sporulation of the causative organisms and the need for additional physiological tests [10-12].

Traditionally, KOH direct smear and fungal culture have been the preferred methods for the diagnosis of onychomycosis. Culture is considered the gold standard, while histopathology is often performed to diagnose and differentiate onychomycosis from other nail disorders such as psoriasis and lichen planus [9, 13].

Molecular tests such as PCR and Real-time PCR can be used for diagnosis of various microorganisms including fungal pathogens [14, 15]. The application of a two-step, 15-minute procedure for extraction of DNA directly from nail specimens and a multiplex PCR-based diagnosis of any dermatophyte and/or tinea rubrum with increased sensitivity compared to conventional diagnostic procedures allow integration of a molecular biology-based method into the routine examination of nail dermatophytosis and also for diagnostic laboratories receiving specimens on a larger scale [9, 16].

This study aimed to address onychomycosis regarding clinical and laboratory findings. And, it aimed to investigate different laboratory methods used in the identification of dermatophytes implicated in onychomycosis; including conventional laboratory methods (KOH preparation), culture and PCR.

Materials and Methods

Study design

This cross -section study included 61 patients with abnormal nails; for more than 12 months. This work was carried out in outpatient Dermatology and Andrology clinic and Microbiology and Immunology department; Faculty of Medicine in Zagazig university. The followings were the inclusion criteria after getting their consent agreement: patients more than 10 years old, having symptoms and signs suggesting onychomycosis and visiting outpatient Dermatology and Andrology clinic in Zagazig University. Diagnosis of onychomycosis caused by dermatophytes clinically; Primary criteria as white/yellow or orange/brown patches or streaks and secondary criteria as onycholysis, subungual hyperkeratosis/debris, nail-plate thickening. The exclusive criteria included those who received topical or systemic antifungal treatment for at least four weeks, before sampling.

Sample Collection and processing

Ethyl alcohol (70%) was applied to the nails of the subjects before the sample collection. Then, the finger nail clippings/scrapings/fragments were collected in a black envelope and they were sent to the laboratory for testing. The collected specimens were divided in to three portion [11].

The first portion of specimens were examined microscopically using 20% KOH. The specimen was mounted in a solution of 20 KOH mixed with 5% glycerol, heated to emulsify lipids (1 h at 51 to 54°C), and examined under ×40 magnification [11]. In this first evaluation, negative samples were stored *overnight* in a humid chamber and examined again on the following day to confirm the result. The nail is examined for fungal hyphae, arthrospore or yeast forms. The second portion of nail specimen was crushed thoroughly to ensure fungal growth and cultured on: Sabouraud's dextrose agar (0.5%) with dermasel supplement followed by microscopic examination of isolates [11, 17, 18]. Specimens were incubated at 25-30°C. All plates were kept for a minimum of 2 weeks and absence of growth after 3-6 weeks was interpreted as negative. All the culture growths were identified on the basis of colony morphology and microscopical examination of lacto-phenol blue mounts [11].

The third portion of specimen was used in molecular detection of fungal DNA by PCR. Genomic DNA extraction form nail samples using fungal DNA preparation kit (i- genomic BYF DNA Extraction Mini kit, ThermoFisher Scientific, USA)[19]. Multiplex PCR was preformed using two pairs of pan dermatophyte primers targets the chitin synthase – encoding gene (chitin synthase 1- chs1) and served for detection of dermatophytes in general.; panDermal 1 5' - GAAGAA-GATTGPCG TTT GCATCGTCTC-3' and pan Dermal 2 5' - CTCGAGG-TCAAAAGCACGC-CAGAG-3'). In addition, T. rubrum – specific primer that targets internal transcribed spacer gene2 for the specific detection of T. rubrum was also used (T. rubrum – for 5'- TCTTTGAACGCA-CATTGCGCC-3'and T. rubrum – rev 5'– CGGTCCTGAGGGCGCTG-AA-3') [20].

Agarose gel electrophoresis was done by conventional agarose gel electrophoresis [21]. The gel was then examined under the UV illuminator. Visualization of the bands between 300 and 400 bp in length (approximately 366 bp) recognizes pan-dermatophyte amplification product. In *trichophyton*-rubrum specific PCR, amplification products were detected by visualization of the band approximately at 206 bp length.

Ethical consideration

The study was approved by the Scientific Ethical Committee at the Faculty of Medicine, Zagazig University Hospital andan informed consent from each patient was obtained.

Statistical Analysis

Data were checked, entered and analyzed using SPSS version 19 for data processing. The following statistical methods were used for analysis of results of the present study. Data were expressed as number and percentage for qualitative variables. Chi-square test (X2) was used to find the association between row and column variables. The agreement between different laboratory methods in measuring the same variable was estimated by Cohen's kappa test (K). Sensitivity, Specificity, Positive predictive value, Negative predictive value, Accuracy of different laboratory methods were determined. For all statistical tests done, the threshold of significance was fixed at 5% level (P-value).

Results

Sixty-one patients were enrolled in this study; all showed nail abnormalities clinically suggestive of onychomycosis. Regarding demographic data as shown in **Table 1**; their age ranged from 10 to 50 years and 54.1% of patients were from 30 to 49 years. Of these 61 patients, 50 (82%) were females and 11 (18%) were males. In addition, 65.6% of patients were from rural residents and 50.8% were house wives.

Based on clinical examination, there were 46 cases of fingernail abnormalities, 4 cases of toenail abnormalities and 11 cases showed combined abnormalities of fingernail and toenail abnormalities. Distal and lateral subungual onychomycosis (DLSO) was the most common clinical types of onychomycosis represented 70 % of total cases as shown in **Table 2**.

In the current study, mycological culture was chosen as the reference method (gold standard) to assess

Table 1. Demographic data of the studied group.

Variable	(n=61)	
	No	%
Age		
10y	1	1.6
10 – 29y	18	29.5
30 – 49y	33	54.1
≥ 50y	9	14.8
Sex		
Male	11	18
Female	50	82
Residence		
Rural	40	65.6
Urban	21	34.4
Occupation		
House wife	31	50.8
Student	6	9.8
Farmer	2	3.3
Clark	12	19.7
Drivers	1	1.6
Lab Tech	1	1.6
Manual Worker	2	3.3
Specialist	6	9.8

Table 2. Clinical findings among the studied group.

Variable	(n=61)	
	No	%
Site		
Finger Only	47	77
Toe Only	4	6.6
Both	10	16.4
Type		
DLSO	43	70.5
TDO	15	24.6
WSD	2	2.3
PSO	1	1.6

the performance of each test. Diagnosis of onychomycosis was based on at least one test was positive. Fungal culture demonstrated that 41 (67.2%) cases were dermatophyte positive, while 20 (32.8%) was-dermatophyte negative. As regard direct microscopy by KOH examination; 45 (73.8) nail samples were positive. Culture was positive only in 37 (60.71%) of nail samples revealing different fungi. Pan-dermatophyte PCR was positive in 41 (67.2%) nail samples, while *T. Rubrum*-specific PCR was positive in only 14 (12.9%) nail samples as shown in **Table 3**. With respects to the results of PCR in the study; the sensitivity, specificity and accuracy were, 100%, 83.3% and 93.4% respectively as shown in **Table 4**.

Table 3. Demographic data of the studied group.

PCR	Culture		Total	Kappa	P
	+ve	-ve			
+ve	37	4	41	0.86	<0.001**
-ve	0	20	20		
Total	37	24	61		

**: highly significance. Sensetivity100%; NPV100%; Specific83.3%; Accuracy 93.4%; PPV 90.2%.

Table 4. Validity of PCR in diagnosis of onychomycosis in comparison to culture as gold standard.

Variable	(n=61)	
	No	%
PCR		
-ve	20	32.8
+ve	41	67.2
Culture		
-ve	16	26.2
+ve	45	73.8
Direct smear		
-ve	16	26.2
+ve	45	73.8

Discussion

Onychomycosis is a major widespread problem in dermatology. It is considered a disease of civilization. And, it is characterized by extreme chronicity and resistance to therapy [2, 22-23]. As regards age, in our study, onychomycosis incidence rates were the highest between 20 and 50 years and decreased in patients over the age of 60 years. The suggested predisposing factors of onychomycosis include increasing age, immuno-suppression, poor peripheral circulation, and trauma [24]. Based on literature, subject 's age was a strong risk factor for development of onychomycosis. These studies reported its occurrence in 20% of elderly persons (> 60 years old), and in 50% of those (> 70 years old) [24-26].

Our study included more female patients affected by onychomycosis. This may be since females are of high risk to develop onychomycosis due to frequent immersion of their hands in water, exposure to chemicals and other household activities. This could be furtherly explained if we consider that over 50% of subjects were house wives. The low incidence of onychomycosis in men in our study may be due to lower rate of patients visited our hospital [27]. Also in line with our results,, Brilhante et al., and Morales et al., found that male to female ratio was 1:1.6 and 1:3 respectively [28, 29]. However, in other studies conducted in North America and India, onychomycosis infection was higher in males rather than the females which may be attributed to more exposing activities practicing by men. Such differences in results may be also attributed to the different geographical areas as well [23, 24].

The current study demonstrated a greater involvement of fingernails compared to toenails. This finding is comparable with studies conducted by Sen *et al.,* and Souza *et al.*, [23, 30]. The reason for the higher frequency of fungal infection of fingernails is probably because of the frequent immersion of the hand, in water or exposure to chemicals and trauma [31]. Also, the toe nail onychomycosis has lower incidence in Egypt than those in studies conducted

in India. This may be attributed to open foot wear and lesser concern for appearance of feet and toe nails [32].

DLSO was the most common clinical type in our study, and the second common clinical presentation was TDO type which is comparable to the findings of Wang and Ching [33] and Aagrwalla *et al.,* [34]. Other authors like Romano *et al*., [35] and Bonnifaz *et al*. [36] also reported DLSO as the predominant clinical form.

With respects to our results based on laboratory diagnosis of onychomycosis in this study. The number of positive samples for fungi by 20X KOH microscopic examination were 45 (73.8%) samples which was also in accordance with the results of Pontes *et al*. [37]. In addition, the percentage of positive samples by culture on SDA (60.7%). was likeresultsdetected by Munir et al. [38], exceeding culture results detected by Brilhante *et al*., in Brazil [28]. There were 8 cases positive by KOH and negative by culture. The direct microscopic examination using KOH is considered efficient and quick test to detect fungal infection especially when conducted by skilled professionals. However, it cannot identify the specific pathogen and may give false positive results.

Only four samples were positive by PCR and negative by culture in our study. This may be due to insufficiency amount of DNA samples [28, 38]. It might be relevant that PCR method can detect the genome of the dead fungal cells which could not be grown by culture. In addition, four samples were PCR negative but positive by KOH examination. Also, it is important to include significant amount of samples in PCR assays despite the fact that the available nail samples may be scarce [39]. Therefore, false negative result of PCR could be due to this factor. In agreement with our study, Lawry *et al*., examined 69 collected nail specimens by PCR and culture. They found that PCR detected dermatophytes in 35 and culture in 28 of 38 samples that were classified as positive and the sensitivity of PCR test was (92%) higher than in culture (73%) [40].

In this study, the dermatophytes appeared to be the chief causative agent in onychomycosis (61%) and *T. rubrum* was the most common organism in dermatophyte onychomycosis in 14 (38%) of positive samples. According to Mugge *et al*., dermatophyte, yeast and NDM may cause onychomycosis [22]. However, dermatophytes appeared to be the main organisms capable of attaching to the nails and producing infection. They examined 5077 nail samples from 4177 patients and found that the majority of causative agents were dermatophytes (68%) and *Trichophyton* species were associated with the most cases [22].

This study shows that PCR had 100% sensitivity, 83.3% specificity, 93.4% accuracy and 90.2% PPV, whereas KOH had 83.8% sensitivity, 41.7% specificity, 67.2% accuracy and 68.9% PPV. These results results are similar to the results of Wisselinke *et al.,* who has concluded that the sensitivity of the PCR was 97%, and it has showed a significant increase in detection rate of dermatophytes in clinical samples compared to culture [41].

Several molecular methods for the detection and identification of dermatophytes from clinical samples have been developed. Major difficulties of PCR methods are that it required training, sophisticated equipment and standardization and it is also expensive [42]. On the other hand, it is not only sensitive, but also has the potential to decrease the time taken for the laboratory identification of pathogens that grow slowly or are difficult to culture. One of the important advantages of PCR technology is direct detection of fungi in the clinical specimens that would allow early and accurate identification of causative agents of onychomycosis. This would permit prompt and targeted initiation of antifungal therapy. The use of PCR is reliable and rapid method which can be done within 24 hours in contrast to the 21 days of incubation required for the isolation of dermatophytes by culture [43, 44].

Conclusion

This study found -among the studied subjects- DLSO was the most common clinical type of onychomycosis, and the dermatophytes appeared to be the chief causative agent of onychomycosis (61%). The application of PCR technology directly to the clinical specimens allowed early and accurate diagnosis of onychomycosis, leading to prompt initiation of antifungal therapy.

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