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Comparative Study between Polymerase Chain Reaction and Conventional Methods Used for Diagnosis of Clinically Suspected Onychomycosis

M. A. Abd El-Wahab[1*], W. S. El Naghy[1] and R. A. El Tatawy[2]

[1]Department of Medical Microbiology and Immunology, Faculty of Medicine, Tanta University, Egypt.
[2]Department of Dermatology and Andrology, Faculty of Medicine, Tanta University, Egypt.

Authors' contributions

This work was carried out in collaboration between all authors. Authors MAAEW and WSEN designed the study and carried out the research, performed the statistical analysis, wrote the protocol. Author MAAEW wrote the first draft of the manuscript and managed literature searches. Authors MAAEW, WSEN and RAET managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background: Proper treatment of onychomycosis depends mainly on accurate diagnosis. Diagnosis using conventional methods (direct microscopy and culture) is still insensitive and time consuming so we aimed to evaluate the commercially available PCR as a rapid method used for detection of fungal elements especially *Trichophyton rubrum* in nail specimens with clinically suspected onychomycosis and compare the results with conventional diagnostic methods.

Methodology: This study included 50 patients with clinically suspected onychomycosis of nails. Nail specimens were obtained by scraping of the diseased nail(s) with a sterile scalpel blade and collected in a sterile container. Each specimen was divided into 3 portions and processed immediately or kept at room temperature until use. The first part was examined by direct microscopy with 20% KOH, the second part was cultured on Sabouraud dextrose agar (SDA) and the third part was subjected to pan-fungal PCR for detection of fungal infection followed by specific

*Corresponding author: E-mail: drmarwa78@yahoo.com;

PCR for identification of *T. rubrum.*
Results: Regarding all fungal isolates KOH microscopy, culture and pan-fungal PCR respectively yielded positive rates of 27 (54%), 26 (52%) and 37 (74%). Pan-fungal PCR picked up 11 specimens missed by culture. Regarding *T. rubrum*, KOH microscopy, culture and *T. rubrum* specific PCR respectively yielded positive rates of 3 (6%), 11 (22%) and 20 (40%). *T. rubrum* specific PCR picked up 9 specimens missed by culture. Of the 26 specimens that were culture positive, 14 grew dermatophytes, 9 *Candida spp* and 3 non-dermatophytic molds. These results emphasize the superiority of PCR over conventional methods.
Conclusion: This study demonstrates that both pan-fungal and *T. rubrum* specific PCR have higher positive rates for detection of fungal infections of the nails compared with KOH microscopy or culture. We suggest that PCR should be used as a complementary method for confirmation of clinically suspected onychomycosis.

Keywords: Nail; dermatophytes; fungal culture; fungal microscopy; PCR.

1. INTRODUCTION

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 infections [1-4]. It is caused mainly by dermatophytes specifically *Trichophyton rubrum* and *Trichophyton mentagrophytes* [5,6]. However, other fungi can cause such infection including yeasts and non dermatophyte moulds but less frequently [7].

Treatment options of such cases depend mainly on proper diagnosis and identification of the causative agent whether it is dermatophytes or not [7].

Onychomycosis is routinely diagnosed by conventional mycological methods including direct microscopy and fungal culture. Direct microscopic examination of the infected nail materials may show false positive results as it neither differentiates between dermatophytes and other filamentous fungi nor provides genus or species differentiation [8,9]. Although histopathological examination is recently considered as a new gold standard for diagnosis, it cannot be routinely used due to its high cost. Fungal culture is still considered the gold standard for diagnosis in many centers. However, fungal culture is time consuming, requires well trained personnel and sometimes associated with contaminants. Furthermore, it may give false negative results in up to 40% of cases [10-12]. Also, the phenotypic characters are affected by changes in temperature and type of medium used [9].

However, the isolation rate of the pathogens by the conventional methods is considered low [13]; a more accurate method is needed for detection of the infectious agents [14].

Recently, molecular biological techniques are promising and have been used successfully to identify both dermatophytes and non dermatophytes rapidly and accurately [15,16].

The present study was performed to evaluate PCR technique which allows detection of panfungal DNA and *T. rubrum* 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.

2. MATERIALS AND METHODS

2.1 Study Design and Population

This study was a prospective longitudinal study carried out 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 Dermatology and Andrology at Tanta University Hospital, Egypt from July 2015 to November 2015. The study was approved by the ethics committee of our institution and informed consent was obtained from the participants. Patients who received topical or systemic antifungal treatments four weeks before sampling were excluded from this study.

2.2 Sample Collection and Processing

The suspected nails were cleaned with 70% alcohol to remove contaminants. Clippings and

scrapings of the diseased nail(s) were taken with a sterile scalpel blade and collected in a sterile container. The scrapings were taken from the junctions between healthy and diseased nails. The friable subungual debris was also collected as well as the nail bed. In case where both finger and toe nails were involved, the samples were taken from both sites and tested separately.

All collected samples were divided into three portions. The first portion was examined microscopically in 20% KOH for the presence of fungal elements.The second was cultured on SDA supplemented with chloramphenicol (50 mg/l) and/or cyclohexemide (500 mg/l) at 27℃ for up to 4 weeks. The third portion of the nail specimen was used for PCR analysis.

2.3 Microscopy and Culture

Nail material was digested in 20% KOH directly on a glass slide. The slide was heated gently for rapid dissolution of the keratinous material. The softened nail material was examined under both low (10x) and high (40x) power fields of the microscope for the presence of fungal elements. The details regarding the hyphae, spores, budding cells and pseudohyphae were noted.

The fungal culture for the nail specimens was performed by inoculation into SDA plates, with one containing cycloheximide to inhibit the growth of saprophytic moulds, and one without. The date of culture and patient's number were clearly labelled on each plate. Plates were read daily for the first week then twice weekly for the next 3 weeks. Negative cultures were discarded after 30 days. All fungal isolates were identified by morphology (including size, shape, consistency, margins, colour of the colony both in recto and verso sides, type of the growth whether fluffy, cottony or creamy and the presence or absence of diffusible pigments) and in lactophenol cotton blue stain [17].

2.4 Molecular Detection of Fungal DNA by PCR

2.4.1 DNA extraction

DNA extraction from nail scrapings was performed using DNA extraction kit (Biotech, Germany) with protocol provided by the manufacturer. Prior to the extraction, relatively large nail fragments were cut into small pieces with a surgical blade.

2.4.2 PCR amplification

For each sample, primers (Bioneer) targeting the Pan-fungal DNA [18] and *T. Rubrum* DNA [19] were used in 2 sets of single PCR assays (Table 1).

2.4.2.1 For Pan-fungal PCR

The PCR reaction was performed in a 25µl reaction volume containing 12.5 µl Taq PCR Master Mix (Dream Taq Green, Fermentas™, Germany), 1 µl of each primer (sense and antisense), 2 µl template DNA and 9.5 µl nuclease free water. The samples were gently vortexed and briefly centrifuged to collect all drops to the bottom of the tube. The samples were overlaid with mineral oil (Biomerieux) and placed in the thermal cycler (Applied Biosystems at Life Technologies, Foster City, CA). After an initial denaturation at 94℃ for 5 min, the cycling conditions were 35 cycles of 94℃ for 30s, 52℃ for 1 min, and 72℃ for 2 min, followed by a final elongation at 72℃ for 7 min. Fifteen microliters of each PCR product was electrophoresed through a 2% agarose gel stained with ethidium bromide and visualised under an UV illumination. Amplicon size as shown in Table 1 and Fig. 1.

2.4.2.2 For T. rubrum specific PCR

Each reaction was performed in a volume of 20 µl by the addition of 4 µl of DNA, 0.2 µl of each primer (at 100 µM), and 10 µl of Taq PCR Master Mix (Dream Taq Green, Fermentas™, Germany). The amplification was performed in a thermal cycler (Applied Biosystems at Life Technologies, Foster City, CA) and consisted of one initial cycle of denaturation for 5 min at 94℃ and 45 cycles of 30 s at 94℃, 30 s at 60℃, and 30 s of extension at 72℃. PCR products were separated on 2% agarose gel, stained with ethidium bromide and visualised under an UV illumination. Appropriate positive and negative controls were included in each amplification. Amplicon size is shown in Table 1 and Fig. 2.

2.5 Statistical Analysis

The collected data were analyzed using SPSS statistical software package version 21. For quantitative data, the mean and standard deviation were calculated. For qualitative data, relation was done using Chi-square test (X^2). A P-value <0.05 was considered statistically significant.

Table 1. Primers used for PCR

Target	Gene	Product size (bp)	Primer sequence
Pan-fungal DNA	the small-subunit rRNA gene sequence of fungal organisms	580	Forward [5'-GATACCGTCGTAGTCTTA-3'] Reverse [5'-ATTCCTCGTTGAAGAGC-3']
T. rubrum DNA	internal transcribed spacer gene 2 (its2)	203	Forward 5'-TCTTTGAACGCACATTGCGCC-3' Reverse 5'- CGGTCCTGAGGGCGCTGAA-3'

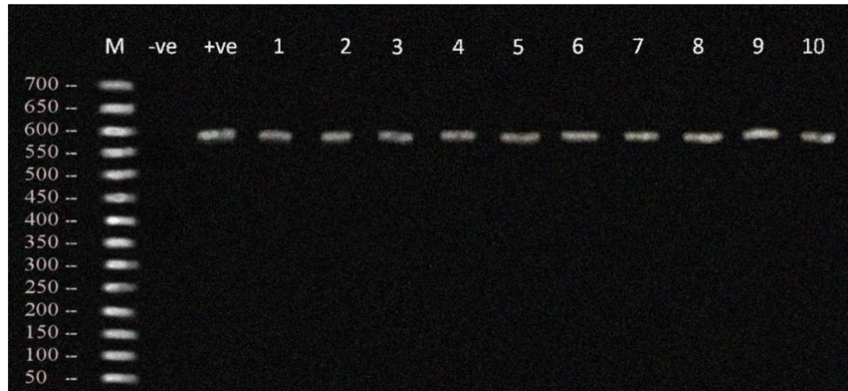

Fig. 1. Gel electrophoresis pattern of amplified Pan-fungal PCR products

M: molecular marker in bp; -ve: Negative control; +ve : Positive control; Lane1 -10: Positive samples (580 bp)

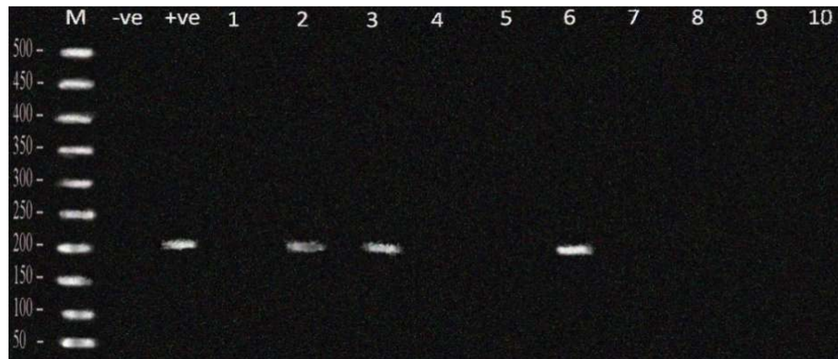

Fig. 2. Gel electrophoresis pattern of amplified *T. rubrum* specific PCR products

M: molecular marker in bp; -ve: Negative control; +ve: Positive control; Lane 2, 3 and 6: Positive samples (203 bp)

3. RESULTS

The age of the study population ranged between 18 and 60 years. The study population comprised 42 females and 8 males. Rural participants represented 66% of the study population while urban participants represented 34%. The majority of cases were housewives 35(70%) (Table 2).

Of the 50 patients with clinically suspected cases of onychomycosis, 54% (27/50) were positive for fungal elements by KOH microscopy. Positive fungal cultures were detected in 52% (26/50). The results of PCR showed that among 50 samples 37(74%) were positive by pan fungal PCR, while only 20(40%) samples were positive by *T. rubrum* specific PCR.

Dermatophytes were detected in 28% (14/50) and non-dermatophytic molds (NDMs) were isolated in 16% (8/50) of the studied cases while yeast represented 18% (9/50). Detailed results of fungal culture represented in (Table 3).

Table 2. Socio demographic data of the patients

Characteristic	Statistics
Age (years):	
Range	18-60
Mean±SD	31.1±10.8
Sex:	
Male	8(16%)
Female	42(84%)
Occupation:	
Housewives	35(70%)
Farmers	6(12%)
Manual workers	3(6%)
Students	6(12%)
Residence:	
Urban	17(34%)
Rural	33(66%)

The results of pan-fungal PCR were compared with the results of culture of all nail specimens and also results of *T. rubrum* specific PCR were compared with *T. rubrum* culture isolates (Table 4). The proportion of patient with positive fungal culture was lower than the proportion of patient with positive Pan-fungal PCR (52% vs. 74% respectively). This difference was of significance (P = 0.0001). Also the proportion of patients with positive *T. rubrum* culture was lower than the proportion of patients with positive *T. rubrum* specific PCR (22% vs. 40% respectively). This difference was of significance (P = 0.001).

Results of direct KOH examination and PCR were compared in the total studied samples and in the patients with *T. rubrum* (Table 5). The proportion of patient with positive fungal element on direct KOH microscopy was lower than the proportion of patient with positive pan-fungal PCR (54% vs. 74% respectively). This difference was of significance (P = 0.0009). Also the proportion of patients with positive *T. rubrum* on direct KOH microscopy was lower than the proportion of patients with positive *T. rubrum* specific PCR (6% vs. 40% respectively). This difference was of significance (P = 0.001).

4. DISCUSSION

Correct and rapid diagnosis of onychomycosis and identification of the causative agent are of a major importance as they allow appropriate treatment to be promptly started.

Diagnosis of onychomycosis is currently performed by direct mycological examination and culture on Sabouraud dextrose agar medium [20]. Microscopy is able to detect fungal hyphae in specimens but cannot identify the exact species, whereas culture will allow identification of the causative organisms but needs a long time (more than 2 weeks) for the fungus to grow, and has a high false-negative rate [21,22]. PCR assays have recently been developed to overcome these difficulties and is considered a more rapid and accurate method for fungal identification [23].

In this study, we aimed to compare PCR with the conventional diagnostic methods of direct microscopic examination and fungal culture for detecting fungal infection in nail specimens from patients with suspected onychomycosis.

In the current study occurrence of onychomycosis was detected between 18 and 60 year. This is in accordance with that reported by Bokhari et al. [24] and Garg et al. [25]. 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 growth [26].

In this study it has been found that onychomycosis is common in individual living in rural area 66% compared to those living in urban 34%, this was in accordance with a study that was carried out by Szepietowski et al. [27] who showed that 65% of the studied cases were living in rural area while 35% were living in urban areas and this observation could be related to the different types of jobs that rural resident perform compared to city residence. Lower education level and lower incomes compared to people living in the cities. All this may lead to delayed diagnosis of fungal infection, which could be responsible for the spread of disease to other body areas.

Our study included more female patients 84% affected by onychomycosis. This may be due to the fact that female commonly seek medical advice in condition associated with cosmotic aspect, also female are of high risk to develop onychomycosis due to frequent immersion of their hands in water, exposure to chemicals and other household activities. Low prevalence in men in our study may be due to lower presentation rate to the hospital. Tasic et al. [28] revealed that (67%) of onychomycosis patients were female. Also Brilhante et al. [29] and Bonifaze et al. [30] were in line with our result as they found that male- to –female ratio was 1:1.6 and 1:3 respectively.

Table 3. Distribution of dermatophytes and non dermatophytic fungi among subjects

	Organism	No of isolates		%
Dermatophytes	*T. rubrum*	11	14/50	
	T. mentagrophyte	2		28%
	T. schonelinii	1		
Non dermatophytic moulds	*Aspergillus niger*	2	3/50	
	Aspergillus fumigates	1		6%
Yeasts	*Candida* species.	9	9/50	18%
Total		26	26/50	52%

Table 4. Comparison between culture and PCR

Pan-fungal PCR	**Culture of nail specimens**		**Total**		**X^2**	**P**
	No	**%**	**No**	**%**	19	0.0001*
Positive	26	52%	37	74 %		
Negative	0	0 %	13	26 %		
Total	26	52 %	50	100 %		
***T. rubrum* specific PCR**	**Culture (*T. rubrum* isolates)**		**Total**		**X^2**	**P**
	No	**%**	**No**	**%**	10.3	0.001*
Positive	9	18%	20	40 %		
Negative	2	4 %	30	60 %		
Total	11	22 %	50	100 %		

Table 5. Comparison between PCR and KOH examination

Pan-fungal PCR	**KOH examination of all specimens**		**Total**		**X^2**	**P**
	No	**%**	**No**	**%**	6.8	0.009*
Positive	24	46%	37	74 %		
Negative	3	6 %	13	26 %		
Total	27	54 %	50	100 %		
***T. rubrum* specific PCR**	**KOH examination suspecting *T. rubrum***		**Total**		**X^2**	**P**
	No	**%**	**No**	**%**	4.8	0.02*
Positive	3	6%	20	40 %		
Negative	0	0%	30	60 %		
Total	3	6%	50	100 %		

On the other hand Ghannoum et al. [1] and Saunte et al. [4] detected that onychomycosi was twice or three times more in male patients than female patients. Veer et al. [31] 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 [32,33]. However, Roberts found that incidence was the same in both sexes [34].

In the current study housewives representing 70% of the total studied cases and this was supported by the results of Bokhari et al. [24] who reported that housewives represented 63%. Maceration from wet work, dishwashing, and contact with carbohydrates probably contribute to onychomycosis in housewives [24].

According to Mugge et al. [35] 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 Ricci [36] 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. [37] isolated high percentage of NDMs in onychomycosis in Egypt. Also El-Batawi et al. [38] showed

that most cases were caused by *Aspergillus* infection.

The difference in the results between the studies may be due to the geographic difference in mould distribution or difference in the criteria and mycological methods used for diagnosis of fungal infection. Epidemiological investigations should be performed in every country to determine the fungal species responsible for onychomycosis [39].

In the present study, positive samples for fungi represent (54%) by 20% KOH microscopic examination which is in accordance with Pontes et al. [40] and Brilhante et al. [29] who observed positive KOH microscopic examination in (68.4%) and (48%) of the examined samples respectively. On the other hand, Kam et al. [41] and El- Batawi et al. [38] found low percentage (14.3%) and (21.8%) respectively.

We also found that fungal culture on SDA was positive in 26 (52%) specimens which is in harmony with that detected by Chandran et al. [42] and Lopes et al. [43] who observed positive culture in (53%) and (56.6%) of the examined samples respectively. However, the percentage of positive samples for fungi by culture found by Pontes et al. [40] (66.5%), and El- Batawi et al. [38] (68.7%) were higher; this may be due to large number of examined cases included in their studies.

We found that KOH microscopy, culture and pan-fungal PCR yielded positive rates of (54%), (52%) and (74%) respectively. PCR picked up 11 specimens missed by culture. PCR had a higher positive rate than both KOH microscopy and culture (74% vs. 54% and 52% respectively). These results are consistent with that observed by Chandran et al. [42].

Our study results for *T. rubrum* showed that KOH microscopy, culture and *T. rubrum* PCR yielded positive rates of (6%), (22%) and (40%) respectively. PCR picked up 17 specimens missed by microscopy. *T. rubrum* specific PCR also had a higher positive rate than both KOH microscopy and culture (40% vs. 6% and 22% respectively). Our results are in agreement with that of Luk et al. [44].

5. CONCLUSION

This study demonstrates that both pan-fungal and *T. rubrum* specific PCR have higher positive rates for detection of fungal infection specifically *T. rubrum* compared with KOH microscopy or culture. We suggest that PCR should be used as a complementary method for confirmation of clinically suspected onychomycosis.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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