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Original article

Precision diagnosis: Onychomycosis detection with multiplex Real-Time PCR versus conventional methods

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ABSTRACT

Background: One of the most prevalent nail conditions is onychomycosis, that is mostly brought on by dermatophyte fungi, non-dermatophyte molds (NDMs) and yeast which may be confused with other nail lesions so, it is crucial to accurately identify the causative agent to initiate proper medications. Fungal culture and KOH microscopy have always been the gold standards for diagnosis. **Aim**: This study aimed to compare the results of a multiplex real-time PCR (RT-PCR) assay with those of conventional diagnostic methods (direct microscopy and culture) in detecting and diagnosing onychomycosis. **Methods**: A total of 100 nail samples from clinically suspected onychomycosis patients were divided into 3 pieces, one nail piece was set aside for microscopy, another for culture and the rest were kept at room temperature in sterile screwed vials in preparation for the multiplex RT-PCR test and DNA extraction. **Results**: of 100 nail samples, 30 had negative direct KOH and 70 had positive direct KOH. In the group of negative KOH samples, 10/30 had a positive culture. In the group of positive K OH samples, 70/100 had a positive culture. Multiplex RT-PCR demonstrated 92.8% sensitivity, 62.7% specificity, 61.9% VPP and 93.1% VPN. **Conclusion**: using multiplex RT-PCR can improve the detection of onychomycosis and decrease the turn round time which could improve the disease outcome.

Introduction

Onychomycosis is the most frequent nail infection linked mainly with dermatophyte fungi (*Trichophyton spp., Microsporum spp., and Epidermophyton spp.*) (60–70%), non-dermatophyte molds (NDMs) (20%) and yeast (10–20%). contact of surfaces contaminated with scales or keratin from affected patients can spread the infection from one person to another making it a serious public health concern [1].

The risk of developing onychomycosis rises with aging. Obesity, diabetes, history of tinea pedis, trauma and immunosuppression are additional risk factors. Onychomycosis negatively impact patients' life quality, as its distorted look can cause considerable psychological stress and the localized ache in highly dystrophic nails restrict daily life [2].

Onychomycosis often requires differential diagnosis due to its clinical presentation, as it may

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resemble non-infectious nail disorders such nail psoriasis, lichen planus, chronic micro trauma or subungual melanomahence, so confirmatory laboratory testing is recommended before starting treatment [3].

Onychomycosis is challenging to diagnose, and the conventional methods for detecting fungi are microscopy and culture techniques, which are easily accessible and reasonably priced for labs. However, the accuracy of both light microscopy (potassium hydroxide examination [KOH]) and culture for fungal detection varies. For genus/species identification, fungal growth may take weeks, and culture in particular exhibits high false negative rates. These factors make it challenging for KOH and culture to accurately and promptly diagnose patients who are awaiting therapy [4].

Several of molecular diagnostics have been designed for fungal etiology confirmation in clinical practice. The development of these molecular techniques, which enable detection and identification of fungi straight from the nail specimen, could be useful to affirm the diagnosis of onychomycosis, especially in patients who began empirical therapy without a mycological diagnosis or a noticeable amelioration in their condition. In these particular situations, a molecular approach would facilitate a quicker and more accurate diagnosis, promoting a more suitable use of the antifungal medications [5, 6].

This study aimed to compare the results of a multiplex real-time PCR (real-time PCR) assay with those of conventional diagnostic methods (direct microscopy and culture) in detecting and diagnosing onychomycosis.

Methods

A cross sectional study was conducted on 100 individuals who were suspected as onychomycosis. Onychomycosis was suspected based on the presence of one or more of the following symptoms: nail opaqueness, rising of the nail plate, deformation, nail thinness and inflammation of the adjacent tissues. Patients were brought in from the outpatient clinic of Dermatology, Venereology and Andrology Department of Benha University Hospitals between May 2024 and September 2024. The study was approved by the local Ethics committee of Benha Faculty of Medicine under number RC-8-6-2024. Prior to sample collection, each person gave their written permission.

Specimen collection

Nail scarification was performed on all enrolled patients, using a scalpel to cut tiny nail fragments from the area where an infection was suspected. Each specimen was separated into three parts: One part was kept for microscopy, one part was used for culture and the other part of the nail was kept at room temperature in sterile screwed vials for the PCR test and DNA extraction that followed.

- Direct microscopic examination

A piece of each nail was put on a slide and covered by two drops of 15% KOH solution, to dissolve larger keratinocyte material. The preparation was covered with a coverslip and left in a humidity chamber at room temperature for 30 min. After clarification, the coverslip was lifted and the slide was inspected for mycelial elements with light microscopy (yeasts, pseudohyphae, hyphae or arthrospores).

- Culture

For the culture, each sample was inoculated on both Sabouraud chloramphenicol and Sabouraud chloramphenicol plus cycloheximide dextrose agar plates (Biomérieux, France). After four weeks of incubation at 30 °C, agar plates were checked once weekly. Using conventional phenotypic techniques based on both macroscopic and microscopic morphological analyses, identification was carried out [7].

- Multiplex real time PCR

The DNA was extracted following the manufacturer's instructions. The multiplex PCR was performed according to manufacturer's instructions [8] using primers listed in table (1) [9], the nucleotide sequences of the different dermatophytes' primers were selected from the NCBI (National Center for Biotechnology Information) nucleotide database.

Two mixes were used. MIX 1 detects *C. albicans, T. tonsurans, T. mentagrophytes, T. rubrum/soudanensi* and *T. interdigitale* and MIX 2 detects *T. verrocusum, M. canis and Epidermophyton floccosum*. Every PCR protocol included a positive control and a negative template control (NTC). Positive molecular findings were verified after the inspection of melting curve analysis, which allowed differentiation of specific fungal species.

The reactions were set up with Universal PCR Master Mix (Real MODTM Green W2 2X q

PCR mix (iNTRON Biotechnology ®), the PCR reaction mix was prepared as follow (15 ml reaction) : 5 µl of DNA extract was mixed with 5 µl of QuaniTect SYBR Green master mix, 1.25 µl of each forward primer and 1.25 µl of each reverse primer. Amplification was performed on a Rotor-Gene Q real-time PCR machine (Qiagen; Germany) with the following PCR thermal cycling conditions: Initial hold at 95ºC for 10 min, followed by 40 cycles (including : denaturation for 20 sec at 95° C, annealing according to each primer set and elongation at 72° C for 30 sec).

Statistical analysis

Positive predictive value (PPV) and negative predictive value (NPV) of KOH and multiplex qPCR techniques were calculated and compared. Sensitivity and specificity were reported as percentages, categorical variables, such as the proportion of samples with positive and negative KOH and PCR results, were reported as percentages and compared using the two-tailed $\chi 2$ test or Fisher's exact test as appropriate. The distribution of positive and negative results across different culture types (negative culture, positive non-dermatophyte culture and positive dermatophyte culture) was also analyzed. For non-normally distributed continuous variables, medians with ranges were used, and comparisons were made using the Mann-Whitney U-test. The statistical significance level was set at < 0.05.

Results

The study included 100 nail samples. Of them, 70 were positive for hyphal or yeast forms on direct microscopy (figure 1) (70 %), 66 (66%) were positive in culture (figure 2), while 56 % were positive on both microscopy and culture, 30 had negative direct KOH and 70 had positive direct KOH. In the group of negative KOH samples, 10/30 had a positive culture. In the group of positive KOH samples, 70/100 had a positive culture (Table 2).

The most common isolated fungi were dermatophytes, according to species identification by RT-PCR, the most common isolated species were *Trichophyton rubrum* (Table 3).

When conventional culture was used as the gold standard, multiplex qPCR showed a sensitivity of 92.8%, specificity of 62.7%, VPP of 61.9% and VPN of 93.1% (table 3).

The mean time of response for positive culture was 13.5±5.01 days, while qPCR results were available in approximately 3h. No statistically significant difference, but qPCR is much faster than culture.

Table 1. Primers used for Multiplex real time PCR.

fungus name	Primer
trichophyton rubrum	F: CCC CCC ACG ATA GGG ACCG R: GAC TGA CAG CTC TTC AGA GAA TT
Tricophyton mentagrophytes	F: GCC CCC CAC GAT AGG GCC AA R: CTC GCC GAA CGG CTC TCC TG
Candida albicans	F: 5′-CGGAGATTTTCT CAATAAGGACCAC, R: 5′-AGTCAATCTCTGTCTCCCCTTGC
Microsporum canis	F: 5′ GTGTGATGGACGAC CGTCCCCCCT 3′ R:5′ATAATACATGGTGCGTTAGGCCAGCCTG 3′
Trichophyton tonsurans	F: (5'-TTCTAGGCTCCCAACCAC-3') R: (5'-ACAAGGGCGGAACTATCAGAC-3')
Trichophyton interdigitale	F: 5'-ATCATTAACGCGCAGGC-3', R: 5'-TGGCCACTGCTTTTCGG-3',
Epidermophyton floccusm	F: 5'-AAGTTGGTCAAACTCGGT-3', R: 5'-TGATCCTTCCGCAGGTT-3',
Trichophyton verrocusum	F: GAA GAA GAT TGT CGT TTG CAT CGT CTC -3′) R: 5′- CTC GAG GTC AAA AGC ACG CCA GAG

Table 2. PCR results according to KOH and culture results.

	PCR results		**Total**
	Negative	**Positive**	
Negative KOH	22	8	30
Negative culture	17	3	20
Positive non-dermatophyte culture	3	2	5
Positive dermatophyte culture	2	3	5
Positive KOH	6	64	70
Negative culture	2	12	14
Positive non-dermatophyte culture	1	10	11
Positive dermatophyte culture	3	42	45
Total	**28**	**72**	**100**

Table 3. Distribution of fungi causing onychomycosis detected by PCR.

Causative fungi	**NO of cases**	**% of cases**	**K OH positive (64)**	**Culture positive (57)**
Dermatophytes	60	83.3	54	45
Trichophyton rubrum	38	52.8	34	33
Trichophyton mentagrophytes	9	12.5	8	7
Trichophyton interdigitalis				
Trichophyton tonsurans	4	5.6	3	3
Trichophyton verrucosum	3	4.2	3	1
Epidermophyton floccucusum	2	2.7	2	0
Microsporum canis	4	5.6	4	1
	0	0	0	
Non dermatophytes candida, aspergillus and others	12	16.7	10	12
Total	72	100%	64	66

Table 4. Sensitivity and Specificity of K OH and multiplex RT-PCR in comparison to culture method.

Technique	**Sensitivity (%)**	**Specificity (%)**	**Positive predictive value (%))**	**Negative predictive value (%)**
KOH	90 %	62.1%	86.1%	85.7%
multiplex RT-PCR	92.8%	62.7%	61.9%	93.1%

Table 5. Comparison of mean Response Time between culture and qPCR methods.

Test method	**Mean response time**	**Statistical test (Mann-Whitney U)**	**p-value**
Culture	$13.5 \pm 5.01\ days$	U = 1.00	0.50
qPCR	~ 3 hours		

Figure 1. Direct microscopic examination of a nail specimen.

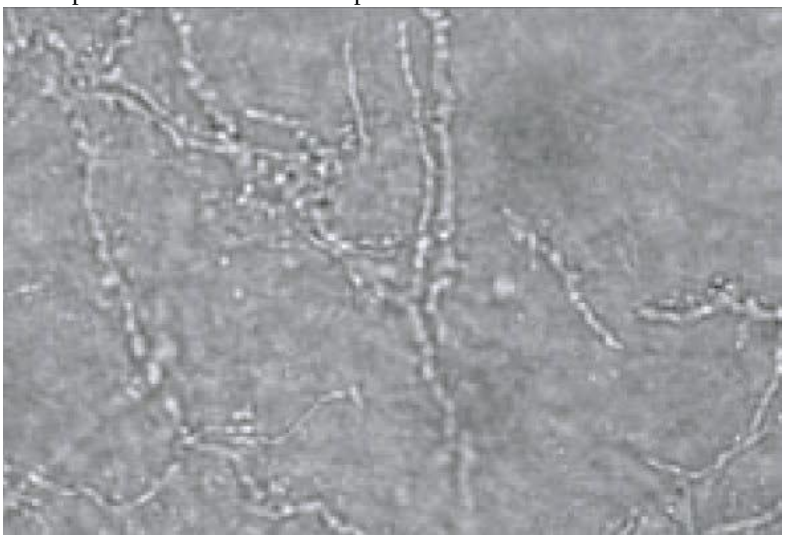


Figure 2. A culture plate showing *Trichophyton rubrum.*

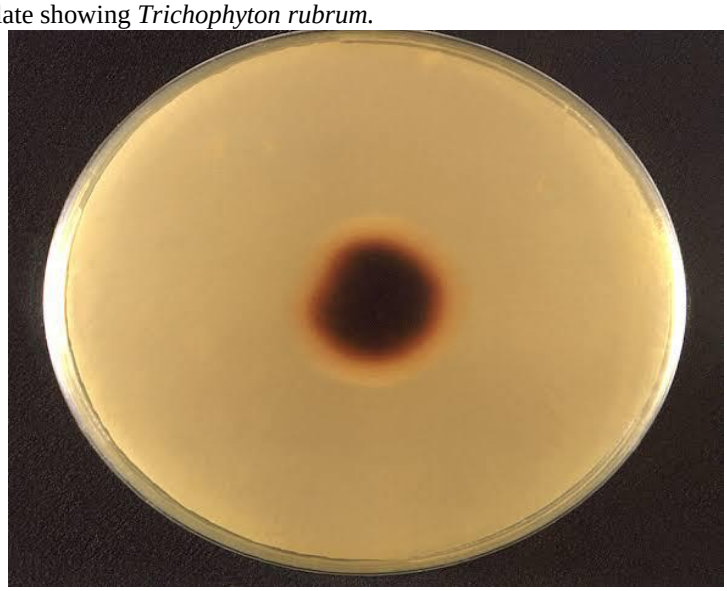


Figure 3. PCR results in positive KOH samples.

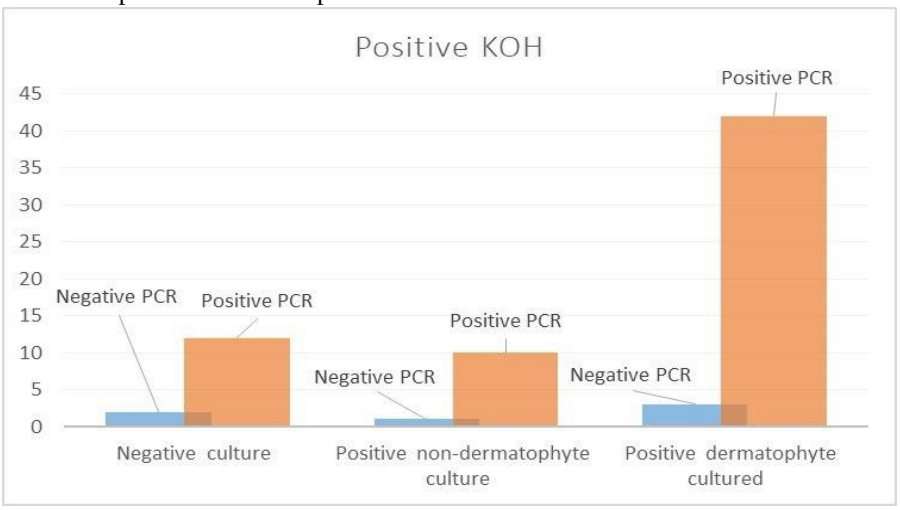
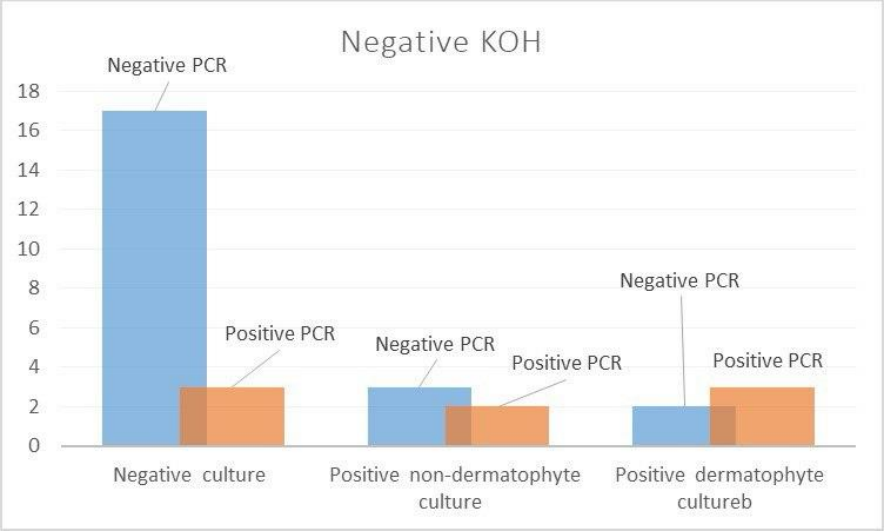


Figure 4. PCR results in negative KOH samples.



Discussion

Onychomycosis is the most prevalent nail infection observed in clinical settings [10]. The majority of onychomycosis cases are due to dermatophytes, clinical challenges arise from mixed infections because not all species respond to treatment in the same way. An ideal diagnosis should be both rapid and comprehensive, encompassing the identification of all active infecting species to facilitate the choice of the most effective antifungal therapy [11].

Our study included 100 nail specimens, of them 70 were positive for hyphal or yeast forms on direct microscopy (70 %), 66 (66%) were positive in culture, while 56 % were positive on both microscopy and culture.

By PCR, 72 specimens were tested positive, 9 (9%) specimens tested negative by PCR while yielded positive culture, which could be due to insufficient samples, heterogeneous distribution of fungal hyphae or conidia and absence of primer during PCR amplification as Aspergillus and other species of candida, Harel et al [12] also declared the rate of PCR failure at 5.6%, 10.3% and 11.9% for DermaGenius PCR assay and 3.5% and 9% for Conventional Diagnostics dermatophyte and fungi assay.

In addition the PCR detected 6 more specimens than culture alone

Regarding dermatophyte detection, it was detected in 50 % and 60 % by culture and RT-PCR respectively.

Our results agree with Ross et al [13] and Alexander et al [14] who reported that dermatophyte detection was 52.6 % and 72.2 and 51.6 % and 61 % by culture and RT-PCR respectively.

Also Lin et al [15] and Bergman et al [16] declared dermatophyte detection was 56% and 51% by RT-PCR

While Walser et al [17] reported that dermatophyte detection by culture was 19.6 % and by PCR 44.8 %, this is contrast to Gordon et al [18] who reported dermatophyte detection by culture 15.9% and by PCR 31.8 %, this difference could be due to presence of PCR inhibitors, varying microbial load and different techniques.

There was a 10% difference in dermatophyte detection between PCR and culture methods, which is agreed with Gordon et al [18] and Alexander et al [14] who reported 15.9% and 10.9% respectively, while In 9 nail samples, the cultured pathogens were not detected by RT-PCR

A dermatophyte was detected in 60 samples (60%) using the multiplex RT-PCR panel with *T. rubrum* the most prevalent pathogen, this agrees with Harel et al [12] who reported 99 (54.4%) and 186 (59.6%) samples tested positive for dermatophyte culture and dermatophyte PCR respectively,

Lin et al [15] reported A dermatophyte was detected in 92 samples (92/195, 47%) using the multiplex RT-PCR panel

The sensitivity of RT-PCR for the diagnosis of onychomycosis was 92.8% which agrees with Harel et al [12] who reported 94.3 %

Multiplex RT-PCR showed a sensitivity of 88.9% and specificity of 73.3%, using conventional culture as the gold standard.

In line with our results, Cuchí-Burgos et al [18] and Hayette et al [20] reported DermaGenius qPCR demonstrated 92.8% and 80% sensitivity, 62.7% and 74.4% specificity respectively.

This study might be limited in some aspects due to relative small sample size, limited population study to certain geographic distribution within Egypt and lack of PCR identification of some fungi.

Conclusion

Using multiplex RT-PCR can improve the detection of onychomycosis and decrease the turn round time which could improve the disease outcome.

Conflict of interest

The authors report no conflict of interest

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Data availability

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials

Authors' contribution

Amira E. Ramadan and ***Rasha A. Elsayed*** conceived the study, developed the theoretical framework and performed the practical tests. ***Marwa S.El-sayed*** aided in the analysis.***Miran K. Mohamed and Nora S. Nassar*** supervised the project. All authors discussed the results, revising it critically for important intellectual content, give final approval of the version to be submitted**)** and contributed to the final manuscript.

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