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Real-time PCR: A rapid and sensitive method for diagnosis of dermatophyte induced onychomycosis, a comparative study



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Abstract Onychomycosis is the most common nail disorder, accounting for up to 50% of all nail problems and about 30% of cutaneous fungal infections. Treatment of onychomycosis is expensive. It requires long-term therapy with an oral antifungal medication with potential side effects. Therefore, a proper diagnosis of infection is needed. *Aim of the study:* This study aimed to compare real time PCR using novel primers targeting the pan-dermatophyte-specific sequence of the chitin synthase 1 gene (CHS1) with nested PCR targeting the same gene, KOH microscopy, direct microscopy in relation to culture for diagnosis of clinically suspected onychomycosis.

Subjects and methods: This study was conducted during the period from April 2013 through May 2014. Eighty patients attending Outpatient Dermatology and Andrology clinic at Benha University Hospital were included in this study. They were 30 females and 50 males with suspected clinical diagnosis of onychomycosis. Their ages ranged from 22 to 77 years. Thirty eight of them were living in rural areas, while the other 42 came from urban areas. Nail scrapings were collected and examined by the following: direct KOH microscopic examination, culture, nested PCR using double sets of primers and finally real time PCR. *Results:* As regards direct microscopy by KOH examination, 66 (82.50%) cases were positive, while 14 (17.5%) were negative. Culture was positive only in 38 (47.5%) of nail samples revealing different fungi. Dermatophytes were isolated from 30 (37.5%) cases; most of them were *Trichophyton mentagrophytes*, and in 8 cases the only isolated

Abbreviations: (CHS1), Chitin synthase 1 gene; (SDA), Sabouraud Dextrose Agar.

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non-dermatophytic organism was *Aspergillus Niger* spp. (10.00%). Nested PCR was positive in 52 (65.00%) of nail samples while real time PCR was positive in 58 (72.5%) of nail samples. **Conclusion:** Real-time PCR followed by melting-point analysis, gives a diagnostic tool that has a higher sensitivity (93.3%) and is faster than nested PCR (73.3%) and other conventional methods.

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1. Introduction

Onychomycosis is a fungal infection of the toenails or fingernails. It is the most common nail disorder and is present in 2–13% of general population increasing up to 48% by 70 years of age. Onychomycosis can cause discomfort and disfigurement and may produce physical and occupational limitations, as well as reducing quality of life.¹ There are three groups of fungi associated with onychomycosis: dermatophytes as members of the genera *Trichophyton*, non-dermatophytes such as *Acremonium* spp., *Alternaria* spp. and *Aspergillus* spp. and yeasts as *Candida albicans* which is the principal pathogen responsible for candidal onychomycosis. *Candida* species other than *C. albicans* such as *C. krusei*, *C. parapsilosis*, *C. glabrata*, *C. guilliermondii*, *C. tropicalis* and *Blastoschizomyces capitatus* (*B. capitatus*) have also been found to cause onychomycosis.^{2,3}

Dermatophytes account for 90% of toenail and 50% of fingernail onychomycosis.⁴ The term “dermatophytosis” describes infections by members of the genera *Microsporum*, *Trichophyton*, and *Epidermophyton*. The species that most often cause onychomycosis are *Trichophyton rubrum* (*T. rubrum*), *Trichophyton mentagrophytes* (*T. mentagrophytes*), and *Epidermophyton floccosum* (*E. floccosum*): The first two species are much more often implicated than *E. floccosum*.⁵

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 factors.⁶

Treatment of onychomycosis is expensive. It requires long-term therapy with an oral antifungal medication with potential side effects. Therefore, a proper diagnosis of infection is needed.⁷

The laboratory diagnosis of onychomycosis routinely involves direct microscopic examination of clinical specimen followed by in vitro culture techniques. Microscopic identification of fungal elements directly from clinical specimen is a rapid diagnostic method but it lacks specificity and sensitivity, with false negative results in up to 15% cases, furthermore although rapid and economical, it does not provide genus or species identification and hence does not differentiate between dermatophytes and other molds. In vitro culture is a specific diagnostic test but it is a slow technique, and may take up to 8 weeks to give results.⁸

The advent of molecular technology has enabled the development of techniques like polymerase chain reaction (PCR), which is a highly sensitive and specific test and can be used for diagnosis of various microorganisms including fungal pathogens.⁹

PCR for detection of dermatophytes has been widely employed targeting the nontranscribed spacer (NTS) regions, metalloprotease gene,¹⁰ Chitin synthase (*CHS*) gene,¹¹ tubulin gene, promoter region within ribosomal intergenic spacer, transcription elongation factor 1, actin gene and calmodulin gene.¹²

2. Aim of the work

This study aimed to compare realtime PCR using novel primers targeting the pan-dermatophyte-specific sequence of the chitin synthase 1 gene (*CHS1*) with nested PCR targeting the same gene, KOH microscopy in relation to culture for diagnosis of clinically suspected onychomycosis.

3. Subjects and methods

This study was carried out at Microbiology and Immunology Department of Benha faculty of Medicine and Dermatology and Andrology Department, at Benha University Hospital, in the period from April 2013 through May 2014. A written informed consent (in Arabic language) was obtained from the patients before participation. Eighty patients attending Outpatient Dermatology and Andrology Clinic were enrolled in this study with clinically suggestive symptoms and signs of onychomycosis (discoloration, thickening, subungual keratosis, onycholysis, longitudinal and transverse grooves, and dystrophic nail). Each patient was subjected to the following sections:

3.1. Full medical history taking (personal, present and past history).

3.1.1. **Personal history:** Including sex, age, residence, and occupation.

3.1.1.2. **Complaint.**

3.1.1.3. **Present history:** Including the onset, course and duration of the lesion and associated nail changes.

3.1.1.4. **Past history:** History of fungal infection, previous trauma, previous treatment, diabetes mellitus, associated medical or immunological diseases and/or peripheral vascular diseases and peripheral neuropathy.

3.1.2. General examination (for associated conditions and lesions predisposing to and suspecting fungal infection as diabetic foot, peripheral vascular disease and concomitant fungal infections e.g. tinea pedis).

3.1.3. Local examination of the nail, mycological investigation and molecular detection of fungal DNA by PCR.

Forty six patients had one or more affected finger, and thirty four patients had one or more affected toes. Clinical types were distal, distolateral, proximal, proximal and distolateral and total dystrophic.

3.2. Mycological investigation

3.2.1. Samples

The suspected nails were cleaned with 70% alcohol to remove contaminants, Scrapings were taken with a sterile scalpel blade and collected in a clean paper, and the collected specimens were sent to the Mycology Unit, Medical Microbiology and Immunology Department at Faculty of Medicine, Benha University.

3.2.2. Methods

The collected specimens were divided into three portions. The first portion of the specimens was examined microscopically using 20% KOH. The second portion was cultured on two sets of media: SDA containing chloramphenicol (0.5%) with/without cycloheximide (0.5%). DNA extraction was performed on the third portion of specimen.

3.2.2.1. Direct microscopic examination¹³. The sample to be examined was placed on a clean glass slide. A drop of 20% KOH reagent was added and mixed with the sample. The softened nail material was examined under both low (10×) and high (40×) power fields of the microscope for the presence of fungal elements. The details regarding the hyphae, spores, budding cells and pseudo-hyphae were noted.

3.2.2.2. Culture¹³. All samples were cultured irrespective of the negative or positive direct microscopic examination results. Samples were inoculated on the two sets of media (SDA with/without actidione). Inoculated media incubated aerobically at room temperature for 4 weeks. Growth inspection was done daily for the first week and then twice weekly for the next 3 weeks. Negative cultures were discarded after 30 days. **Identification of the obtained growth was done by**

3.2.2.2.1. Macroscopic examination of the culture. The identification of isolates from SDA was done by observing the morphological characteristic of the colony including size, shape, consistency, margins, color 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.

3.2.2.2.2. Microscopic Lactophenol Cotton Blue (LPCB) stained preparation. It was used for microscopic examination of fungal structures. This was done by **tease mount** which is the most common technique used for rapid mounting of fungi for microscopic examination, and by **slide culture** which is the best method for preserving and observing the actual structure of a fungus. It is not a rapid technique, but it is useful for microscopic studying of fine fungal structures.

3.3. Molecular detection of fungal DNA by PCR

3.3.1. DNA extraction

DNA extraction was done using Gene JET Genomic (Thermo Scientific, Germany) DNA Purification Kit. Nail samples were digested with Proteinase K in lysis solution. RNA was removed by treating the samples with RNase A. The lysate was then mixed with ethanol and loaded on the purification column where the DNA bound to the silica membrane. Impurities were effectively removed by washing the column with the prepared wash buffers. Genomic DNA was then eluted under low ionic strength conditions with the elution buffer. Extracted DNA from nail samples was stored at -20°C till processing. For each of the samples, two sets of PCR were performed.

3.3.2. Nested conventional PCR¹⁴

It was performed by using primers targeting dermatophyte-specific chitin synthase 1 gene (CHS1), whose sequences were as follows 1S (5'-CAT CGA GTA CAT GTG CTC GC-3'; nucleotides [nt] 70 to 89) (Bioneer) and 1R (5'-CTC GAG GTC AAA AGC ACG CC-3'; nt 485 to 504) (Bioneer). Nested PCR was performed by reamplification of the first PCR product using a novel set of primers, whose sequences were as follows: JF2 (5'-GCA AAG AAG CCT GGA AGA AG-3'; nt 111 to 130) (Bioneer) and JR2 (5'-GGA GAC CAT CTG TGA GAG TTG-3'; nt 378 to 398) (Bioneer).

The PCR reaction was performed in a 50 μl reaction volume containing 25 μl Taq PCR Master Mix (Maxima Hot Start Green PCR Master Mix, Fermentas), 2.5 μl primer, 10 μl template DNA and 10 μl nuclease free water. The conditions of the reaction were as follows: One cycle of initial denaturation at 95°C for 5 min., 40 amplification cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 1 min., followed by one final extension cycle at 72°C for 15 min. Ten microliters of the PCR product were used as a template for the second (nested) PCR, using the same PCR conditions, except that the annealing temperature was 52°C . The specific band size of the PCR product was (288 bp).

3.3.3. Real time PCR¹⁵

The real time PCR was performed using the same primers targeting pan dermatophyte-specific chitin synthase 1 gene (CHS1). The PCR mixture contains 10 μl Super Real Pre Mix Plus (SYBR Green) TIANGEN Biotech (Beijing), 2 μl of Rox dye, 0.6 μl of each primer, and 2 μl of template DNA. To achieve a reaction volume of 20 μl , 4.8 μl of nuclease-free water was added. The real time PCR instrument used was ABI7900HT (Applied Biosystems, Foster City, CA, USA) with the following conditions: an initial denaturation at 95°C for 2 min., followed by 35 amplification cycles of 95°C for 3 s and 60°C for 30 s. Negative control (2 μl nuclease free water) was included in the run. Finally melting curve analysis was performed. The principle is when double-stranded DNA bound with SYBR Green I dye is heated, a sudden decrease in fluorescence is detected when the melting point (T_m) is reached, due to dissociation of the DNA strands and subsequent release of the dye. The fluorescence is plotted against temperature. The PCR product of pan dermatophyte gene melts at 81.9°C (Fig. 3).

4. Statistical analysis

Mean \pm SD and range were used to describe the quantitative data and proportion was used to describe the qualitative data of the studied population. The test of proportion (z test) was used to compare the results of the direct KOH microscopy, the nested PCR and RT-PCR with the results of the reference test (fungal culture) and a P -value <0.05 was considered statistically significant. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated for the different diagnostic tests against the reference test and Receiver Operating Characteristic (ROC) curve analysis was carried out. All statistical analyses were carried out in STATA/SE version 11.0 for Windows.

5. Results

The results of the study are represented in the following Tables 1–5 and Figs. 1–4. The age of the study population ranged between 22 and 77. The study population comprised 50 males and 30 females. Urban participants represented 52.5% of the study population while rural participants represented 47.5% (Table 1).

The most common associated clinical condition in suspected patients with onychomycosis was anemia which represented 35% of study population while the least common one was trauma which represented 12.5% of patients (Table 2).

The most common clinical types of onychomycosis in fingers & toes were Total dystrophic & proximal types respectively while the least common clinical type in fingers and toes was Proximal & distolateral for both (Table 3).

Of the 80 patients with clinically suspected cases of onychomycosis, 82.5% (66/80) were positive for fungal elements by KOH microscopy. Dermatophytes were detected in 65% and 72.5% of the cases by nested PCR and real time PCR respectively and isolated in 37.5% (30/80) of the cases by culture. Non-dermatophytic molds were isolated in 10% (8/80) (Table 4, Figs. 1 and 4).

The isolated dermatophytes were *T. mentagrophytes* detected in 14 cases, followed by *T. rubrum* detected in 8 cases and lastly *T. verrucosum* and *T. schoenleinii* detected in 4 cases for each. The only isolated non-dermatophyte was *Aspergillus Niger* in 8 cases.

The results of direct KOH microscopy, nested PCR and Real Time PCR (RT-PCR) were compared to these of fungal culture in Table 5 and Fig. 1 regarding sensitivity, specificity, PPV and NPV, and direct KOH microscopy shows the lowest sensitivity and specificity 80%, 16%, respectively, while RT-PCR shows the highest sensitivity and specificity 93.3%, 40%, respectively.

Real time PCR shows the highest sensitivity (93.3%) while nested PCR shows the least sensitivity (73.3%). Real time PCR

Table 1 Socio-demographic characteristics of the study population.

Characteristics		N = 80
Age	Mean ± SD; (range)	43.52 ± 15.04; (22–77)
Sex	Male/female; (% male)	50/30; (62.50)
Residence	Urban/rural; (% urban)	42/38; (52.50)

Table 2 Patients with suspected cases of onychomycosis.

Disease		N = 80
Trauma	Yes/no; (% yes)	10/70; (12.50)
DM	Yes/no; (% yes)	22/58; (27.50)
Anemia	Yes/no; (% yes)	28/52; (35.00)
PVD	Yes/no; (% yes)	16/64; (20.00)
Associated nail disease	No disease; (%)	42; (52.50)
	Paronychia; (%)	24; (30.00)
	Nail pitting; (%)	14; (17.50)
Treatment	Yes/no; (% yes)	30/50; (37.50)

DM: diabetes mellitus, PVD: peripheral vascular disease.

Table 3 Clinical features of patients with suspected cases of onychomycosis.

Fingers (46) = 57.5%	Toes (34) = 42.5%	Total (80) = 100%
Distal = 8	Distal = 4	12; (15.00)
Distolateral = 8	Distolateral = 10	18; (22.50)
Proximal = 7	Proximal = 11	18; (22.50)
Proximal & distolateral = 1	Proximal & distolateral = 1	2; (2.50)
Total dystrophic = 22	Total dystrophic = 8	30; (37.50)

Clinical features		N = 80
Nail shape	Distal; (%)	12; (15.00)
	Distolateral; (%)	18; (22.50)
	Proximal; (%)	18; (22.50)
	Proximal & distolateral; (%)	2; (2.50)
	Total dystrophic; (%)	30; (37.50)
Number of nails affected	One or more fingers; (%)	46; (57.50)
	One or more toes; (%)	34; (42.50)

Table 4 Comparison of different methods used for diagnosis of onychomycosis among the study group.

Test		N = 80
Direct KOH microscopy	Yes/no; (% yes)	66/14; (82.50)
Fungal culture	Negative; (%)	42; (52.50)
	Non-dermatophyte; (%)	8; (10.00)
	Dermatophyte; (%)	30; (37.50)
Nested PCR	Yes/no; (% yes)	52/28; (65.00)
RT-PCR	Yes/no; (% yes)	58/22; (72.50)

Table 5 Analysis for results of direct KOH microscopy, nested PCR and RT-PCR against the reference test (fungal culture).

	Direct KOH microscopy	Nested PCR	RT-PCR
Sensitivity	80.00%	73.3%	93.30%
Specificity	16.00%	40.00%	40.00%
Positive predictive value	36.40%	42.30%	48.30%
Negative predictive value	57.10%	71.40%	90.90%
Area under the curve (AUC)	0.48	0.58	0.67

is more accurate in diagnosis of onychomycosis as its area under the curve (AUC) was 0.67 compared with that of nested PCR and direct KOH microscopy they were 0.58, 0.48 respectively, and this is shown in Table 5 and Fig. 4.

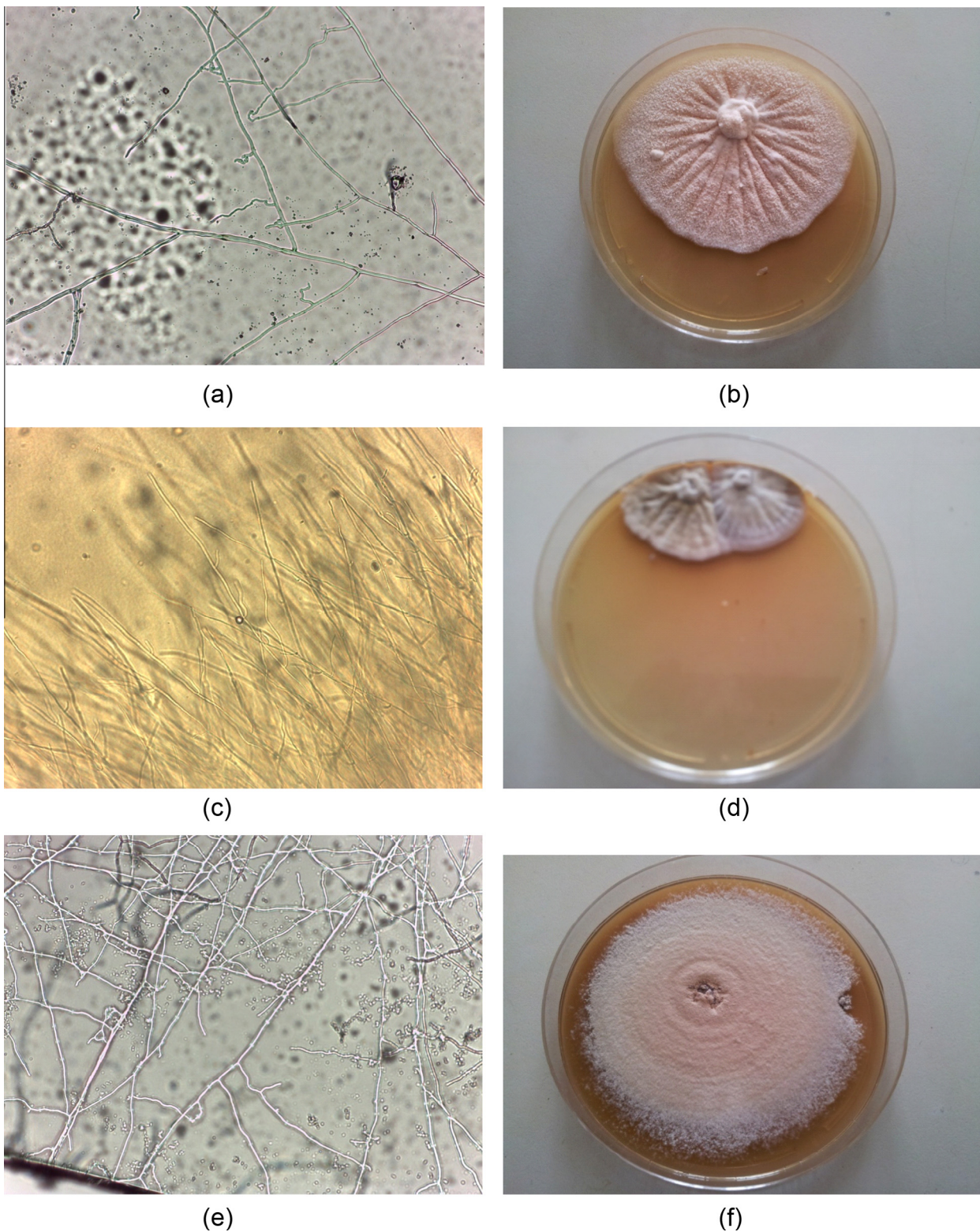


Figure 1 (a) Slide culture of *T. mentagrophytes*. (b) Culture of *T. mentagrophytes* on SDA (microscopic). (c) Slide culture of *T. verrucosum*. (d) Culture of *T. verrucosum* on SDA. (e) Slide culture of *T. rubrum*. (f) Culture of *T. rubrum* on SDA.

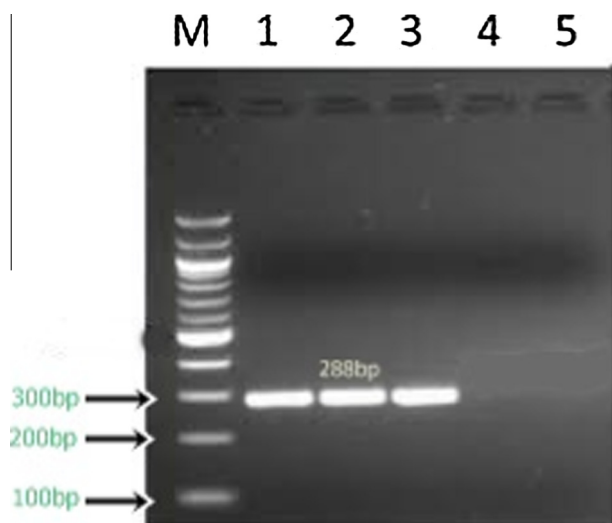


Figure 2 Results of nested PCR of clinical specimens from patients with onychomycosis. Lane M 100-bp DNA ladder (molecular mass markers); lane 1,2,3, nested-PCR-positive specimens (288 bp); lane 4,5 nested-PCR-negative specimens.

6. Discussion

Onychomycosis is a major problem in dermatology because it is so widespread. The prevalence has increased since World War I and today it is considered a disease of civilization, characterized by extreme chronicity and resistance to therapy.¹⁶

In our study, occurrence of onychomycosis was confined between 22 and 77 year. This is in accordance with that reported by Garg et al.¹⁷ and Bokhari et al.¹⁸ The increased prevalence of nail lesions by fungi in adults can be explained as a consequence of nail trauma and slow nail growth.¹⁹

Our study included more male patients (62.5%) affected by onychomycosis than females (37.5%). This is in line with Ghannoum et al.²⁰ and Heikkila and Stubb²¹ who detected that male subjects were found to have twice or three times as likely onychomycosis as female subjects respectively, which they attributed to the suggestion that "men exercise more" and so more liable to trauma. This higher incidence was observed in other studies conducted by Ching et al.²² and Neupane et al.²³

On the other hand Brillhante et al.²⁴ and Bonifaz et al.²⁵ founded that male-to-female ratio was 1:1.6 and 1:3

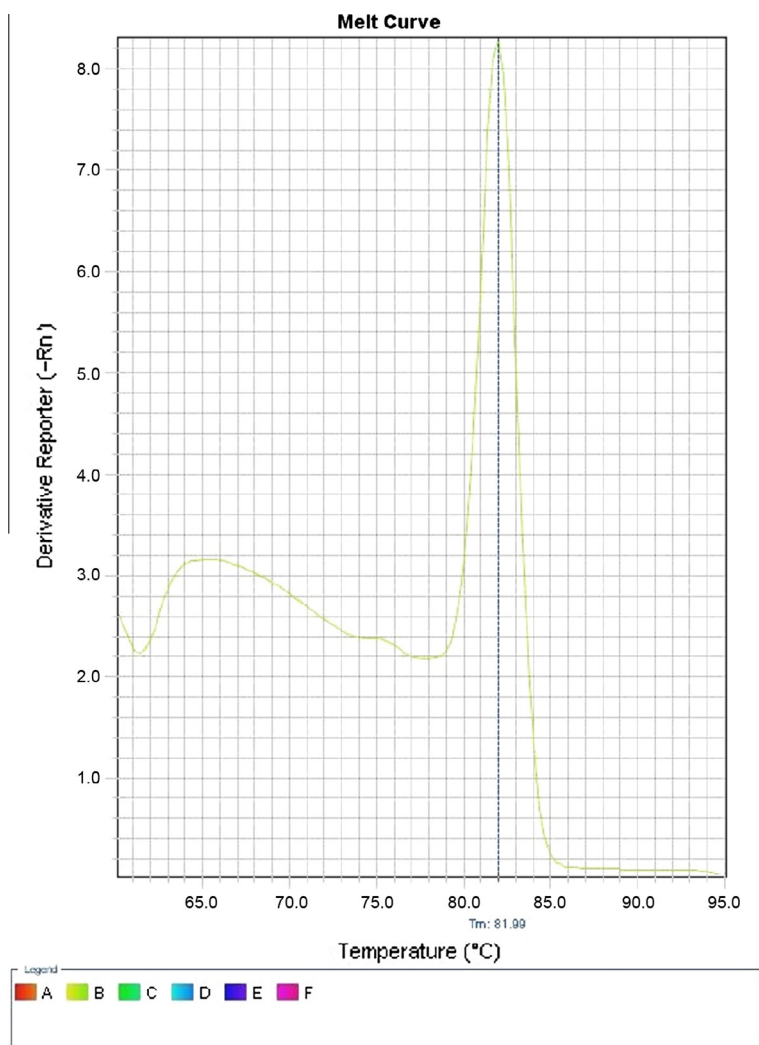


Figure 3 Melting curve analysis of PCR product of pan dermatophyte gene (melts at 81.9 °C).

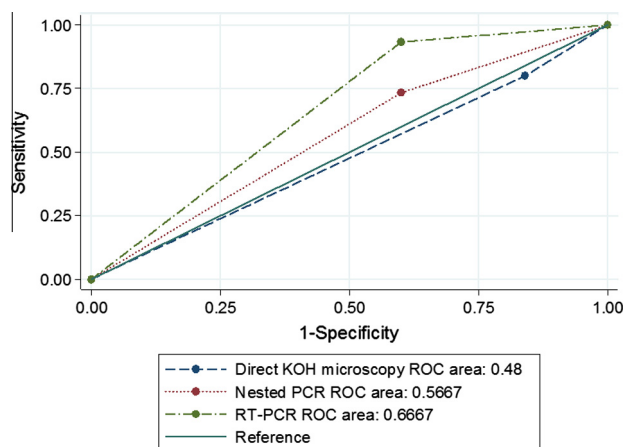


Figure 4 Receiver operating characteristic (ROC) curve for direct KOH microscopy, nested PCR and RT-PCR against the gold standard test (fungal culture) for the diagnosis of onychomycosis ($N = 80$). KOH microscopy: $n = 66$ positive and $n = 14$ negative; Area Under the Curve (AUC) = 0.48, $P < 0.001$ Nested PCR: $n = 52$ positive and $n = 28$ negative; AUC = 0.57, $P < 0.001$. RT-PCR: $n = 58$ positive and $n = 22$ negative; AUC = 0.67, $P < 0.001$.

respectively, probably because females see the doctor more often or for cosmetic reasons. Also Sais et al.²⁶ found that females were more affected, suggesting that trauma from wearing women's fashionable shoes might account for the difference or maceration from wet work and washing dishes. However Roberts, 1992²⁷ found the incidence to be the same between sexes.

Our study showed that patients with anemia represent 35% of study population while those with DM, peripheral vascular disease and trauma accounted for 27.5%, 20% and 12.5% respectively and patients with associated nail disease accounted for 47.5% of study population. This is in agreement with Dogra et al., 2002²⁸ who stated that the prevalence of onychomycosis has been shown to be significantly higher in diabetic patients than normal individuals. Increasing age, male gender, concurrent intake of immunosuppressive agents and peripheral vascular disease have been shown as independent risk factors for the development of onychomycosis.

Total dystrophic type of onychomycosis was the most common clinical type in our study followed by Proximal and Distal/lateral Subungual Onychomycosis (DLSO) types which is comparable to the findings of Agarwalla et al.²⁹; Wang and Ching.³⁰

It is essential that good laboratory methods are available for rapid and precise identification of the dermatophytes involved in onychomycosis, in order to apply appropriate treatment and preventive measures. The conventional methods of fungal detection have their own drawbacks; for example KOH microscopy has low specificity and fungal culture is associated with low sensitivity and takes long time. Thus newer fungal diagnostic methods are need of the hour for accurate diagnosis and identification of the etiological agent.³¹

In the present study, the number of positive samples for fungi was 66 (82.50%) samples by 20% KOH microscopic examination which was comparable with that of Pontes et al.³² (68.4%).

The number of positive samples for fungi by culture on SDA was 38 (47.50%) samples which is near to that detected by Brilhante et al.²⁴ (36%). However, the percentage of positive samples for fungi by culture found in Brazil by Lopes et al.³³ (56.6%) and Pontes et al.³² (66.5%), was higher; this may be due to selection and higher prevalence of mycotic infection from the start in such hot humid climates.

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

Dermatophytes were detected in 30 (37.50%) samples of fungal cultures and they were of *T. mentagrophytes*, *T. rubrum*, *T. verrucosum* and *T. schoenleinii* species. NDMs represent the second common isolated organisms constitute 10% (8 samples). *Aspergillus* species detected in all these 8 samples of positive cultures represent the only isolated NDMs. Similarly, dermatophytes were reported as principal pathogen followed by non-dermatophytic molds in studies from Mexico and Malaysia in patients of onychomycosis.^{34,35}

Dermatophytes were detected in 65% and 72.5% of the cases by nested PCR and real time PCR respectively and isolated in 37.5% (30/80) of the cases by culture. Possible explanation for culture negative/PCR positive results is that the nail sample may contain only dead nonviable organisms or maybe sometimes insufficient sample for culture was collected or lastly the most important reason which is the lack of sensitivity of the culture based detection of dermatophytes, confirming previously published results.

In our study, mycological culture was chosen as the reference method to assess the performance of each test. The sensitivity, specificity, accuracy, PPV and NPV were assessed. Real time PCR, nested PCR and direct microscopy have 93.3% 73.33% and 80% sensitivity respectively. The specificity was 40%, 40% and 16% respectively. This is in harmony with Winter et al.³⁶ who calculated the diagnostic sensitivity of the PCR assay as 79.0%. Also Bergman et al.¹⁵ and Garg et al.³⁷ reported that real time PCR results show higher sensitivity than nested PCR and direct microscopy respectively.

7. Conclusion

Real-time PCR followed by melting-point analysis, gives us a diagnostic tool that has a higher sensitivity and is significantly faster than nested PCR and other conventional methods for diagnosis of onychomycosis.

Conflict of interest

The authors declare that there are no conflicts of interest.

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