Translation elongation factor 1-alpha gene as a marker for diagnosing of candidal onychomycosis

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ABSTRACT

Background and Purpose: Culture-based identification methods have been the gold standard for the diagnosis of candidal onychomycosis. Molecular technologies, such as polymerase chain reaction (PCR) assays, can provide an alternative for the rapid detection of Candida species. The present study was conducted to investigate a pan-Candida PCR assay based on the translation elongation factor 1-alpha (TEF-1α) gene for the detection of the most prevalent pathogenic Candida species.

Materials and Methods: For the purpose of the study, an optimized pan-Candida PCR primer pair was designed, and the target was amplified and sequenced. The analytical and clinical diagnostic performance of the designed primers was tested using 17 reference strains, 137 nail scrapings suspected of onychomycosis, and 10 healthy nail specimens.

Results: The use of the universal pan-Candida primers designed on TEF-1α gene resulted in the successful amplification of a 270-base pair fragment in all Candida species tested, except for C. glabrata, and reacted neither with other fungi nor with E. coli. The sequence difference count matrix showed poor insertion/deletion differences (0-2 nt) among Candida species. Among 137 nail specimens, 35% (n=48), 30.7% (n=42), and 40.1% (n=55) of the samples were found to be positive by direct microscopy, culture, and pan-Candida PCR, respectively.

Conclusion: Based on the findings, the PCR-based detection targeting the DNA TEF-1α gene is a rapid and simple procedure for the diagnosis of candidal onychomycosis directly from nail sample.

Keywords: Candidal onychomycosis, Pan-Candida PCR assay, Translation elongation factor 1-alpha

Introduction

Candida species are common human commensal and important human yeast pathogens that cause a wide spectrum of diseases ranging from less severe superficial lesions to life-threatening systemic conditions in a vast spectrum of immunocompromised patients [1]. The incidence of infections due to Candida species has increased markedly as a result of a growing number of immunocompromised and critically ill patients [2]. Candida contains about 150 species. However, only a few of these species, including C. albicans, C. tropicalis, C. glabrata, C. parapsilosis, C. dubliniensis, and C. krusei, are implicated in human pathogenesis.

Superficial candidiasis may involve one or more mucocutaneous tissues, including the skin, mucous membrane, and nails [3]. Nail infections caused by Candida species known as candidal onychomycosis are normally associated with paronychia, onychia, or chronic mucocutaneous candidiasis. Further comprehensive studies have pointed out that Candida is the second most common etiology for onychomycosis after dermatophytes (tinea unguium) [4]. Conventional laboratory techniques for the diagnosis of candidal onychomycosis, such as direct microscopic investigation and mycological culture, are time consuming and laborious. Recent studies have shown that advances in molecular technologies are useful in detecting microbial infections without the need for microbial isolation [5, 6].

Nuclear ribosomal DNA (rDNA) regions, including ITS1 and ITS2, have been the most commonly used targets for the detection/identification of Candida.
species [7, 8]. Nevertheless, the description and characterization of new genetic markers for Candida species can clarify its taxonomy and might be helpful for detection/identification goals. The protein-coding genes (e.g., translation elongation factor 1-alpha [TEF-1α]) have been proven to be a powerful tool for the species delimitation of the taxonomically complex Fusarium species, considered an alternative to rDNA for species identification [9, 10]. Likewise, the gene is useful for developing robust phylogenetic inferences for other groups of pathogenic fungi, such as zygomycetes [11], dermatophytes [12], and Aspergillus species [13]. Since TEF-1α gene has not been used in Candida taxonomy and detection, our aim was to investigate TEF-1α as a new genetic marker to evaluate a pan-Candida PCR assay in the diagnosis of candidal onychomycosis.

Materials and Methods

Standard strains and clinical specimens

The specificity and sensitivity of the designed PCR system were determined against 17 standard strains of fungi. These strains included C. albicans (ATCC 9892, ATCC 2730, ATCC 562, ATCC 1912 and ATCC 64553), C. tropicalis (CBS 94 and ATCC 750), C. krusei (ATCC 6258), C. glabrata (ATCC 90030 and ATCC 863), C. dubliniensis (ATCC 8500 and ATCC 7987), C. parapsilosis (ATCC 4344 and ATCC 90018), Cryptococcus neoformans (ATCC 9011), A. flavus (ATCC 64025), A. fumigatus (ATCC 14110), and environmental isolates of penicillium chrysogenum and Fusarium solani. An standard isolate of Escherichia coli (ATCC 43894) was evaluated as a negative control.

A total of 137 nail scrapings collected from patients who referred to the Medical Mycology Laboratory of Razi Hospital in Tehran [14] were used as clinical samples. Nail specimens from 10 healthy volunteers were used as negative clinical controls to verify the diagnostic specificity of the tests. All specimens were examined by both direct microscopy and culture, and a portion of each sample was stored at -20°C for use in the PCR assay. This research was approved by the Ethics Committee of the Shiraz University of Medical Science (IRSUMSREC1398.020).

Mycological examination

A part of the samples was observed with 20% KOH, and the other part was inoculated onto three different points on Sabouraud dextrose agar (SDA) plate (Merck, Darmstadt, Germany) containing chloramphenicol (0.05 mg/l), with and without actidione (500 mg/l) (Merck, Darmstadt, Germany). The inoculated specimens were incubated at 28°C for 1-4 weeks. The culture plates were examined for fungal growth twice a week.

Primer design

The sequences of TEF-1α in Candida species and other causative agents of onychomycosis, such as dermatophytes and non-dermatophylic molds, were downloaded from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/pubmed/). The number of sequences for Candida species is limited in NCBI (a total of 13 TEF-1α sequences representing 5 species of Candida). Region of sequences that were conserved within Candida species but different from other fungi responsible for onychomycosis was chosen manually to design a pan-Candida primer pair (Table 1) considering the criteria specified by the Primer Biosoft [15] and optimized using the Multiple Primer Analyzer (https://www.thermofisher.com). The primers were also synthesized by the Bioneer Company (Bioneer, Korea).

Preparation of genomic DNA

DNA was extracted and purified from the fungal colonies using the boiling method [16]. Briefly, a small amount of fresh colony was suspended in 200 μL of distilled water, boiled at 95°C for 15 min, and centrifuged for 4 min at 7,000 rpm; subsequently, the supernatant was used for PCR. For clinical specimens, DNA was extracted by means of a commercial kit (Yekta Tajhiz Azma, Iran) according to the manufacturer’s protocol after being treated by 500 μL of a digestion buffer (containing 400 mM Tris-HCL [pH=8], 60 mM EDTA [pH=8], 150 mM NaCl, and 1% sodium dodecyl sulfate) for 30 min and crushed for 3 min by a mechanical grinder.

Polymerase chain reaction

The PCR reactions were performed using 2X PCR Master Mix (Amplicon, Denmark), 0.5 μM of each primer, 4 μL of DNA template, and sufficient distilled water to reach a final volume of 25 μL. The amplification was accomplished using one cycle at 95°C for 3 min, as well as 35 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, followed by a final extension step at 72°C for 3 min. Negative (i.e., sterile distilled water) and positive (i.e., C. parapsilosis [ATCC 4344]) controls were included in each extraction run. Furthermore, 5 μL of the aliquots of the amplicons was electrophoresed using a 1.1% agarose gel in Tris-Borate-EDTA buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA, and pH of 8.3) and visualized under ultraviolet irradiation after ethidium bromide staining. A 100-bp DNA ladder was used as a molecular size marker.

DNA Sequence Analysis

The PCR product of standard Candida species examined in this study was purified and sequenced unilaterally with the forward primer on an automated DNA sequencer (ABI PrismTM 3500 Genetic Analyzer, Genetic Group) and edited with the Geneious (http://www.geneious.com) software. The sequences were compared with those in the Genebank database using the web-based software BLAST, (http://www.ncbi.nlm.nih.gov/BLAST). The sequence...
Table 1. Standard and clinical strains of fungi sequences used in this survey for sequence analysis of translation elongation factor 1-alpha gene in silico studies

| Species                        | Strain               | EF-1α accession no. | Size of EF-1α partial sequence (bp) |
|--------------------------------|----------------------|---------------------|-------------------------------------|
| Candida albicans               | ATCC18804            | DQ4472251           | 742                                 |
|                                | ATCC 18804           | KC5074361           | 929                                 |
|                                | SC5114               | XM7050562           | 1377                                |
|                                | SC5114               | M299351             | 2369                                |
|                                | SC5114               | M299341             | 2411                                |
|                                | ATCC 13803           | DQ4472421           | 742                                 |
| Candida tropicalis             | MYA-3404             | XM0025474801        | 1377                                |
|                                | MYA-3404             | XM0025488371        | 1377                                |
| Candida parapsilosis           | ATCC90018            | DQ4472381           | 742                                 |
| Candida dublioniensis          | NCDF 3949            | DQ4472291           | 742                                 |
|                                | CD36-07890           | XM002417390         | 1377                                |
|                                | CD36-22520           | XM002418681         | 1377                                |
| Candida glabrata               | ATCC 66032           | DQ4472391           | 742                                 |
| Epidermophyton floccosum       | Clinical sample      | KM6781911           | 732                                 |
| Epidermophyton floccosum       | Clinical sample      | KM6781881           | 732                                 |
|                                | NBRC 9045            | KM6780601           | 732                                 |
| Microsporum canis              | CBS 130811           | KM6780521           | 719                                 |
|                                | CBS 130820           | KM6780521           | 719                                 |
| Trichophyton interdigital      | CBS 130927           | KM6780551           | 737                                 |
| Trichophyton rubrum            | DSM 107606           | MH8025051           | 644                                 |
|                                | DSM 107668           | MH8025061           | 624                                 |
| Trichophyton tonsurans         | CBS 130822           | KM6780541           | 756                                 |
|                                | nuns73               | MG3568841           | 667                                 |
|                                | nuns57               | MG3568801           | 669                                 |
| Aspergillus fumigatus          | AX 2102 I            | KJ4764101           | 873                                 |
|                                | JCM 10253            | KM9219611           | 674                                 |
|                                | CBS 101640           | KM9219681           | 612                                 |
| Aspergillus flavus             | JCM 2061             | KM9219751           | 684                                 |
|                                | JCM2061              | KM921975.1          | 684                                 |
| Aspergillus niger              | ITEM 7090            | FN6654031           | 758                                 |
|                                | ITEM 7496            | FN6656571           | 758                                 |
| Fusarium solani                | V04903               | FN6898261           | 691                                 |
|                                | dq240                | KR0255571           | 750                                 |
| Fusarium oxysporum             | NRRL 45915           | FJ9854261           | 630                                 |
|                                | NRRL 38544           | FJ9854081           | 629                                 |
|                                | NRRL 46859           | FJ9854381           | 631                                 |
| Acremonium recifei             | CBS 188.82           | KP0126471           | 763                                 |
|                                | CBS 541.89           | KP0126501           | 683                                 |
|                                | CBS 220.84           | KP0126481           | 554                                 |
| Scopulariopsis brevicaulis     | UTHSC 06-619         | HG3803651           | 957                                 |
|                                | UTHSC 06-1072        | LM6525861           | 957                                 |
|                                | UTHSC 09-1373        | LM6525871           | 967                                 |
| Penicillium chrysogenum        | AC4503 II            | KJ4764051           | 810                                 |
|                                | AL0202               | KJ4764061           | 921                                 |
|                                | MO5731               | KP0090001           | 990                                 |

of each sample was subjected to ClustalW pairwise and multiple alignments using the MEGA6 software, and a phylogenetic tree was built using the maximum-likelihood algorithm with the Tamura-Nei parameter.

**Analytical sensitivity of primers**

To determine the detection limit of this system, fungal inocula were prepared from culture for *C. albicans* (ATCC 5982). The inocula were adjusted to a turbidity of 0.5 on the McFarland scale, with approximately 1-5x10^6 CFU/ml, counted in a hemocytometer. The inoculum was diluted with sterile saline at a concentration of 10^1-10^6 CFU/ml. Subsequently, 200 µL of pool nail samples made from healthy nails added to each dilution. Commercial kit (Yekta Tajhiz Azma, Iran) was applied for DNA extraction from the nails treated with fungi. Eventually, PCR was performed on DNAs extracted from six serial dilutions and positive and negative controls.

**Statistical analysis**

The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated for the pan-*Candida* PCR against the culture (as the reference method). The method of chi square test was used for categorical data. Statistically, the
P-value < .05 was considered as significant. The data were analyzed using SPSS software Version 22.0.

Results

By aligning the TEF-1α gene sequences of most of the common Candida species, a highly conserved region was used for designing a new pan-Candida pair primer for PCR detection. The primers included CEFF: 5'-GGACAAAAACAGATTTGAA-3' as forward primer and CEFR: 5'-TTGCAATGGCAATCTCAAT-3' as reverse. The primers amplified an approximately 270-bp Candida-specific fragment in all tested Candida species, except for C. glabrata and reacted neither with other fungi nor with E. coli DNAs (Figure 1 left).

The sequence difference count matrix showed poor intraspecies differences among the species belonging to Candida (0-2 nt insertions/deletions). Pairwise and multiple alignment rates were also evaluated among the sequences. The mean similarity of two sequences for 9 Candida isolates was obtained as 94.6%, and the overall similarity of the sequences was estimated at 86.6%. Figure 2 depicts the multiple DNA sequence alignment of EF-1α sequences in the most common pathogenic Candida tested in this study. The consensus nucleotide sequence data determined in this study were deposited in the GenBank, under the accession numbers of MK568517-MK568521 and MN231082-MN231085.

Phylogenetic tree of TEF-1α sequences in Candida species indicated that the nine strains tested in this study (i.e., C. parapsilosis clinical isolate Ci 78, C. tropicalis CBS 94, C. dubliniensis Ci 390, C. albicans ATCC 5982, C. albicans ATCC 2730, C. albicans ATCC 562, C. albicans 1912, C. albicans ATCC 4344, and C. albicans ATCC 64553) were most closely related with strains derived from the gene bank and shared a common branch. In the phylogenetic tree, the studied species were divided into two clades. Clad one consisted of two clusters of C. albicans and C. dublinensis and clad two contained three species of C. parapsilosis, C. tropicalis, and C. glabrata (Figure 3).

For the determination of the analytical detection limit of the designed PCR reaction, serially diluted DNAs were assayed by pan-Candida PCR (Figure 1 right). According to the results, 10^1 cells/mL was the minimum number for reliable detection.

Figure 1. (A) Electrophoresis patterns of pan-Candida polymerase chain reaction using DNA template; lane 1) Candida albicans (ATCC 5982), lane 2) Candida tropicalis (ATCC 750), lane 3) Candida parapsilosis (ATCC 4344), lane 4) Candida albicans (ATCC 64553), lane 5) Candida tropicalis (CBS 94), lane 6) Candida krusei (ATCC 6258), lane 7) Aspergillus fumigatus (ATCC 14110), lane 8) Aspergillus flavus (ATCC 64025), lane 9) penicillium chrysogenum (clinical isolate), lane 10) Fusarium solani (clinical isolate), lane 11) Cryptococcus neoformans (ATCC 9011), lane 12) Escherichia coli (ATCC 43894), lane 13) negative control (sterile distilled water), and lane M) a 100-bp molecular size marker; (B) Determine the detection limit of this pan-Candida primers; lane 1) negative control (sterile distilled water), lane 2) 2-7 nails treated with serially diluted standard strain (C. albicans (ATCC 5982)) at a concentration of 10^2-10^6 CFU/ml, lane 8 positive control (C. parapsilosis (ATCC 4344)) and lane M) a 100-bp molecular size marker.

Figure 2. Multiple sequence alignments of translation elongation factor 1-alpha gene sequence from various Candida species sequenced in this study.
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**TEF-1α gene in the diagnosis of candidal onychomycosis**

Out of the 137 tested nail samples suspected of onychomycosis, 35% (n=48), 30.7% (n=42), and 40.1% (n=55) of the specimens were positive for yeast elements by direct microscopy examination, culture, and pan-Candida PCR, respectively (Table 2). The proportion of patients with positive pan-Candida PCR was higher than that of the patients with positive culture for Candida species (30.7% vs. 40.1%, respectively). This difference was statistically significant (\(P<0.001\)). Considering culture as the reference method to assess the performance of the molecular method used in this study, the PPV, NPV, specificity, and sensitivity were calculated as 63.3%, 91.4%, 78.9%, and 83.3% for pan-Candida PCR test, respectively.

**Discussion**

Rapid and accurate detection of Candida species in nail specimens is essential for the initiation of proper antifungal treatments. Among the routine methods, KOH preparation is easy to perform and economic; however, its sensitivity is inconsistent and influenced by such factors as inadequate sample volume, different sampling methods, and experience of the lab workers. Although fungal culture can identify specific pathogens, it takes a long incubation period and its sensitivity is much lower than that of KOH. In this regard, the false-negative rate of culture for the diagnosis of onychomycosis was approximately 30%, and the sensitivity was about 60% [17].

DNA-based diagnostic tests are increasingly being employed in the clinical microbiological laboratory for the detection of the etiological agent directly from the clinical specimens. The sensitivity of the detection molecular methods depends on various factors, one of the most important of which is the selection of the target DNA for amplification. Over the last two decades, the use of molecular diagnostic study for the detection of a specific sequence of Candida to investigate intra-species and inter-species differences has been reviewed [18, 19]. In such studies, when the main purpose was to design pan-Candida primers, the designed primer pair usually amplified common environmental contaminants, such as Aspergillus. The

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**Figure 3.** Phylogenetic analysis of elongation factor 1-alpha gene of 9 DNA isolates (standard and clinical isolates) of Candida species tested in this study and a number of sequences in this gene region related to Candida species in the gene bank (NCBI).

**Table 2.** Agreement of microscopy and Pan-Candida PCR methods with the gold standard method (culture).

| Culture          | True positive | True negative | Total |
|------------------|---------------|---------------|-------|
| Microscopy       |               |               |       |
| Positive         | n=42          | n=6           | n=48  |
| Negative         | n=0           | n=89          | n=89  |
| Pan-Candida PCR  |               |               |       |
| Positive         | n=35          | n=20          | n=55  |
| Negative         | n=7           | n=75          | n=82  |
| Total            | n=42          | n=95          | n=137 |

PCR: polymerase chain reaction
present study involved the design of a specific pan-
Candida primer set in the TEF-1α gene where the
primer sequences are conserved within Candida
species but distinct from a variety of fungi other than
Candida species and the bacterium Escherichia coli.
The nucleotide sequence of TEF-1α gene encoding a
part of the protein translation machinery appears to be
consistently single copy. The only study investigating
TEF-1α gene in Candida was conducted in 1990 by
Sundstrom et al. to determine whether any of the
characteristics of fungal TEF-1α proteins could be used
to delineate the phylogenetic relationship of C.
albicans with other fungi. Sequence analysis of the
study showed the presence of two genes, called TEF1
and TEF2, for TEF-1α in C. albicans. The coding
regions of TEF1 and TEF2 differed by only five
nucleotides and encoded identical TEF-1α proteins of
458 amino acids [20].

This study represents the first application of a PCR-
based diagnostic test for the most common Candida
species using primers amplifying 270-bp region of the
TEF-1α gene. Our results showed a great increase in
the detection rate of Candida species using pan-
Candida PCR (40.01%) over the direct microscopy
(35%) and culture (30.7%). In the same vein, Khan et
al. obtained more positive results by PCR than
conventional methods (52.2% % vs. 21.2 %) [21].

In our study, 7 out of 42 samples which were
positive in culture showed negative results in pan-
Candida PCR. The DNA from these samples was
diluted, and in another reaction, was exposed to pan-
fungal primers (ITS1 and ITS4); however, the results
stayed negative in the PCR. The positive and negative
controls in all experiment runs ruled out the possibility
of experimental error. The few negative results can be
due to the fact that positive material was not included
in the subsample set used for molecular testing.

The pan-Candida PCR detected Candida in 20
samples identified negative by culture. This
nonconformity that reduce the specificity (78.9%) and
PPV (63.3%) of pan-Candida PCR has been linked
rather to false-negative results of the culture method
because PCR is more sensitive than culture methods
and can detect all pathogenic fungi, both viable
nonviable types.

Our pan-Candida primers facilitated the detection of
DNA for all tested Candida species, except for C.
glabrata. This finding is in the same line with the
results obtained by Zhang et al. evaluating four pan-
Candida primer sets by conventional PCR assays [22].

The lower limit of detection in this assay was 10^1
cells/mL, which could be sufficient to find the low
number of fungal elements causing candidal onychomycosis in a small volume of nail samples
although this would require a highly efficient extracted
template DNA. Candida DNA was detected in a few
nail samples obtained from healthy volunteers,
suggesting that this represented commensal fungi
living on the nails. Accordingly, such methods as
quantitative real-time PCR can be important for the
appropriate evaluation of clinical samples containing
both pathogenic and commensal fungi.

Conclusion
As the results of the present study indicated, pan-
Candida PCR assay showed good performance
characteristics by increasing the detection rates of
Candida species and drastically reducing the clinical
turnaround time, compared to culture. However, it is
required to perform further research using more types
of standard isolates and larger clinical samples to
define the practical value of such an approach in the
mycology laboratory.

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Author’s contribution
M. M. and K. P. supervised all parts of the project
and wrote the paper. H. M. designed the study. F. F.,
F. G. H., R. A., and H. G. set up and performed the tests.
M. K. supported DNA sequencing. K. Z. edited the
final manuscript.

Conflicts of interest
No potential conflicts of interest have been
declared. The authors alone are responsible for the
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