Original Article

Molecular identification and *in-vitro* antifungal susceptibility testing of *Candida* species isolated from patients with onychomycosis

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Abstract

Background and Purpose: *Candida* species are the most opportunistic fungi affecting the nails and resulting in onychomycosis. In this study, we identified and evaluated *in-vitro* susceptibility of the recovered isolates against fluconazole (FLC), voriconazole (VRC), and clotrimazole (CLT) using the Clinical and Laboratory Standards Institute (CLSI) M27-A3 document.

Materials and Methods: From patients with either clinically or mycologically proven onychomycosis, 97 isolates comprising of seven *Candida* species were isolated, which were identified by both conventional and molecular techniques such as polymerase chain reaction-restriction fragment length polymorphism. In addition, *Candida dubliniensis* was confirmed by restriction endonuclease analysis. Antifungal susceptibility of each isolate against the three azoles applied in this study was determined using the CLSI microdilution reference method M27-A3.

Results: *Candida parapsilosis (C. parapsilosis)* was the most frequently isolated species (n=44), followed by *C. albicans* (n=23), *C. tropicalis* (n=13), *C. glabrata* (n=7), *C. krusei* (n=6), *C. guilliermondii* (n=3), and *C. dubliniensis* (n=1). All the isolates were susceptible to CLT. VRC had lower minimum inhibitory concentration (MIC) values for the isolates compared to FLC. Geometric mean MIC values of VRC, FLC, and CLT for *C. parapsilosis* isolates were 0.07 µg/ml, 0.8 µg/ml, and 0.35 µg/ml, respectively. Collectively, all species exhibited greater susceptibility to VRC in comparison to *C. albicans* ($P \leq 0.001$).

Conclusion: This study showed that non-*albicans Candida* species were the most common etiologic agents of non-dermatophyte onychomycosis. The major antifungal agents used in clinics to empirically treat yeast onychomycosis are FLC and CLT. Our data suggested that CLT is a better choice for the treatment of *Candida* onychomycosis, especially in drug resistant cases.

Keywords: *Candida albicans*, *Candidiasis*/microbiology, *Candidiasis*/pathogenicity, Clotrimazole, Fluconazole, Onychomycosis, Voriconazole

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Introduction

The genus *Candida* includes approximately 200 different species, of which only a few were adequately documented as human opportunistic pathogens. *Candida* infections, which may be superficial or invasive (or a combination of both), usually occur in immunocompromised patients [1]. Superficial infections that can be managed successfully with topical antifungal agents usually involve the skin, nails, or mucous membranes [2].

The azoles are the most effective antifungal agents for the management of yeast infections, which inhibit lanosterol biosynthesis from disrupting the function of the yeast cell membrane. They include imidazoles (e.g., miconazole, econazole, ketoconazole, and clotrimazole [CLT]) and triazoles (e.g., fluconazole [FLC], itraconazole, and voriconazole [VRC]), which were used successfully to treat yeast infections [2, 3].

Even though there may be some controversy regarding the correlation between *in-vitro* susceptibility testing data and clinical outcomes, *in-vitro* antifungal data can be beneficial for predicting potential outcomes and drawbacks such as resistance [3]. The

CLSI microdilution reference method is a testing platform used across the globe to obtain standardized results that can be compared with other studies.

Candida onychomycosis should to be distinguished from onychomycoses caused by either dermatophytes or a number of different filamentous fungi [4-9], as choosing the appropriate treatment without culture can result in non-responsive cases. Owing to the fact that onychomycoses may require months to resolve, providing the appropriate treatment during the early treatment phases seems to be imperative. Several groundbreaking studies, performed in Iran, identified the etiologic agents of onychomycosis, and then based upon *in-vitro* susceptibility studies evaluated the potential outcomes using different antifungal agents [9-13].

With the advent of molecular tools allowing for rapid genomic studies, determination of phylogenetic relationships as a way to identify yeast taxa was accentuated. This approach has resulted in several yeast taxa being redefined and reclassified. The separation of C. *dubliniensis* from *C. albicans* based on differences in their phylogeny is but an example.

Owing to the revision of many yeast species, especially in the *Candida* genus, the application of molecular methods has become mandatory for their accurate identification. Among the more novel molecular tools, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) is one of the most accurate methods for the identification of *Candida* species [14, 15].

Material and Methods

Isolates

Patients with clinically or mycologically confirmed onychomycosis were enrolled in this study. A total of 97 *Candida* isolates were isolated from fingernail specimens, collected using the standard aseptic techniques, and were placed on Sabouraud dextrose agar (Merck, Germany) and incubated at 30°C. The yeast colonies were purified using the standard methods to insure only one yeast, which is devoid of potential contaminating bacteria, was present.

The new subcultures were transferred to Sabouraud dextrose agar after which small portions of the purified colonies were added to microtubes containing distilled water and were maintained at -20°C. For the evaluation of conventional and molecular methods, both were applied for the identification of each isolate.

The conventional method

The isolates were initially identified according to the results of chlamydoconidia formation in Corn Meal Agar with Tween 80 (Merck, Germany) incubated at 30°C, germ tube formation in fresh serum incubated for up to three hours at 37°C, and colony color on the chromogenic medium of CHROMagar *Candida* (Biomeriux, France) incubated at 30°C. CHROMagar is used for presumptive identification of some yeasts and differentiation of *C. albicans, C. tropicalis,* and *C. krusei.*

The molecular method (PCR-RFLP)

We used a PCR-RFLP technique as described before [14, 15]. DNA extraction was performed using phenol-chloroform method [16]. A loop full of fresh yeast was harvested and suspended in 300 µl of lysis buffer (10 mM Tris, 1 mM ethylenediaminetetraacetic acid [EDTA] pH 8, 1% sodium dodecyl sulfate [SDS], 100 mM NaCl, and 2% Triton X-100) with phenol-chloroform and glass beads, and then was vortexed. Total DNA was precipitated with 2-propanol, washed with 70% ethanol, air-dried, and suspended in 50 µl of Tris–EDTA buffer (10 mM Tris, 1 mM EDTA), and was kept at -20°C before use.

Internal transcribe spacer (ITS) region of ITS1-5.8S-ITS2 segment of the ribosomal DNA gene was amplified [14]. A set of universal primers (ITS1, 5-TCCGTAGGTGAACCTGCGG and ITS4, 5-TCCTCCGCTTATTGATATGC) (Metabion International, Martinsried, Germany) were employed for amplification. PCR amplification was carried out in a final volume of 50 µl. Each reaction contained 1 µl of template DNA, 0.5 µM of each primer, and 0.20 mM of each deoxynucleoside triphosphate (dNTP), 5 µl of 10× PCR buffer, and 1.25 U of Taq polymerase (Roche Molecular Biochemicals, Mannheim,

Germany). An initial denaturation step at 94°C for five minutes was followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 45 seconds, and extended at 72°C for one minute, with a final extension step at 72°C for seven minutes. The PCR product was electrophoresed on 1.2% agarose gel and stained with ethidium bromide.

For differentiation between *Candida* species, amplified PCR products were digested with *Msp1* restriction endonuclease to achieve the best species-specific pattern, [14] and additional enzyme of *B1nI* (*AvrII*) was applied to differentiate *C. dubliniensis* from *C. albicans* [15]. Restriction fragments were separated by 1.8% agarose gel electrophoresis in Tris-Borate-EDTA (TBE) buffer for approximately one hour at 100 V and visualized by staining with ethidium bromide.

Antifungal susceptibility testing

Antifungal susceptibility testing of FLC, VRC, and CLT was performed according to the Clinical and Laboratory Standards Institute (CLSI) using micro-dilution method with minor modification [17, 18]. RPMI 1640 (with L-glutamine and phenol red, without bicarbonate; Sigma, USA) was prepared and buffered at pH 7.0 with 0.165 mol of 3-N-morpholino propanesulfonic acid (MOPS) (Sigma- Aldrich, Germany).

Serial dilutions of the drugs were prepared in 96-well microtiter trays using RPMI 1640 media buffered with MOPS (Sigma, St. Louis, USA). Stock inocula were prepared by suspending three colonies of each isolate in 5 ml sterile 0.85% NaCl and adjusting the turbidity to a 0.5 McFarland standard at 530 nm wavelength to achieve $1–5\times10^6$ cells/ml density.

Working suspensions were prepared by making a 1/1000 dilution with RPMI of the stock suspension for each *Candida* species. The trays were incubated at 35°C for 24-48 hours in humid atmosphere. The growth in each well was compared with control wells. Minimum inhibitory concentrations (MICs) were visually determined and defined as the lowest concentration of the drugs that produced no visible growth. Each experiment was performed in duplicate.

Candida albicans (ATCC10261) and *C. parapsilosis* (ATCC 4344) were used as controls. For FLC, MIC for susceptible, susceptible dose dependent, and resistant were ≤8 μg/ml, 16-32 μg/ml, and ≥64 μg/ml, respectively. For VRC, MIC for susceptible, susceptible dose dependent, and resistant were ≤1 μg/ml, 2 μg/ml, and ≥4 μg/ml, respectively [18].

Statistical analysis

To analyze the data, Chi-square test was performed, using SPSS version 15. Geometric mean of MICs was calculated, as well.

Results

The number of *Candida* species, as identified by the conventional and molecular methods, is summarized in Table 1. PCR-RFLP using MspI restriction endonuclease enzyme was able to distinguish seven *Candida* species as shown in Table 2 and Figure 1.

Table 1. Identification of *Candida* species by conventional and molecular methods

Candida species	Germ tube	Chlamidoconidia	Colony color on *CHROM*agar	Polymerase chain reaction- restriction fragment length polymorphism
C. parapsilosis (44)	1	0	White-pink(38)	44
C. ablicans (23)	21	22	Green (23)	23
C. tropicalis (13)	4	1	Blue (13)	13
C. glabrata (7)	0	0	Pink–purple (5)	7
C. krusei (6)	0	0	Pink–purple (6)	6
C. guilliermondii (3)	0	0	Blue-purple (3)	3
C. dubliniensis	1	1	Green (1)	1
Total				97

Table 2. Frequency of *Candida* species isolated from onychomycosis

Candida species	Polymerase chain reaction- restriction fragment length polymorphism	
	No.	Percent
C. parapsilosis	44	45.3
C. ablicans	23	23.7
C. tropicalis	13	13.4
C. glabrata	7	7.2
C. krusei	6	6.1
C. guilliermondii	3	3.09
C. dubliniensis	1	1.03
Total	97	100

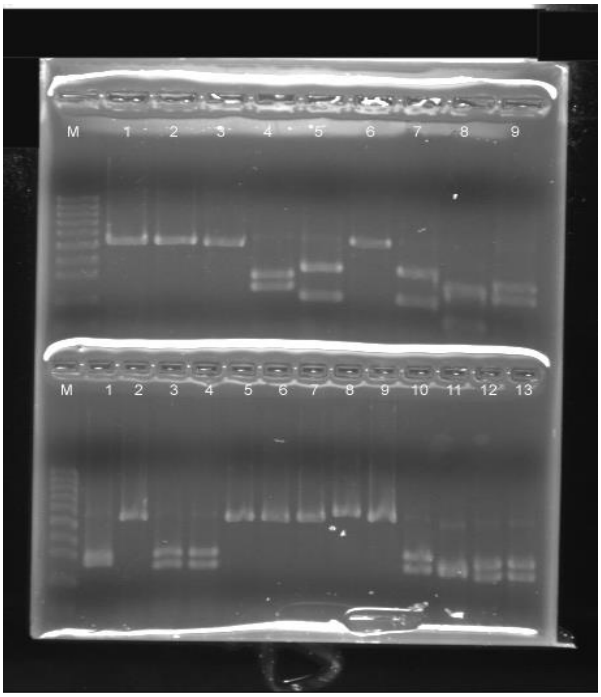

Figure 1. Polymerase chain reaction- restriction fragment length polymorphism profile of some isolates: *C. parapsilosis* (up 1-3, 6 and down 2, 5-9), *C. albicans* (up 4, 9 and down 3, 4, 10, 12, 13), *C.tropicalis* (up 5, 7) and *C. krusei* (up 8 and down 11)

C. parapsilosis was the most predominant species among the isolates (45.3%), whereas *C. albicans* was the second (23.7%) most common taxon. When *B1nI* (*AvrII*) restriction endonuclease enzyme was employed, one of the *C. albicans* isolates was re-identified as *C. dubliniensis*.

The results of antifungal activity of VRC, FLC, and CLT against the *Candida* species are presented in Table 3. CLT had potent activity against the isolates tested with MIC ranging from 0.03 µg/ml to 16 µg/ml. MIC_{50} and MIC_{90} of the isolates for CLT, VRC, and FLC were 0.5 mg/L and 0.06 mg/L, 0.5 mg/L and 5.6mg/L , as well as 8 mg/L and 16 mg/L, respectively.

Geometric mean MICs for CLT were 0.25 µg/ml for *C. albicans*, 0.35 µg/ml for *C. parapsilosis*, 1.8 µg/ml for *C. tropicalis*, 2 µg/ml for *C. glabrata*, 0.35 µg/ml for *C. krusei*, 0.14 µg/ml for *C. guilliermondii*, and 2 µg/ml for *C. dubliniensis*.

Furthermore, 19.5% and 7.2% of the isolates had low susceptibility to VRC and FLC, respectively. VRC was active against 79.3% of the isolates with geometric mean MIC values of 0.57 µg/ml for *C. albicans*, 0.07 µg/ml for *C. parapsilosis*, 1.06 µg/ml for *C. tropicalis*, 0.14 µg/ml for *C. glabrata*, 0.06 µg/ml for *C. krusei*, 0.5 µg/ml for *C. guilliermondii*, and 0.5 µg/ml for *C. dubliniensis*.

FLC geometric mean MIC values for *Candida* species are exhibited in Table 3. FLC was more effective compared to VRC ($P \leq 0.001$). There was a significant difference between *C. albicans* and non-*C. albicans* regarding sensitivity to VRC (P=0.001).

Table 3. Antifungal susceptibility of *Candida* species isolated from onychomycosis, S (Susceptible), SDD (Susceptible Dose Dependent), and R (resistant), GM (Geometric mean)

Species	Voriconazole				Fluconazole				Clotrimazole		
	S	SDD	R	GM (µg/ml)	S	SDD	R	GM (µg/ml)	S	R	GM (µg/ml)
C.parapsilosis	42	0	2	0.07	41	2	1	0.8	44	0	0.35
C. ablicans	13	0	10	0.57	22	0	1	0.9	23	0	0.25
C. tropicalis	6	0	7	1.06	8	1	4	8.9	13	0	1.8
C. glabrata	3	0	0	0.14	3	0	0	1.8	3	0	2
C. krusei	6	0	0	0.06	6	0	0	0.71	6	0	0.35
C.guilliermondii	7	0	0	0.5	5	2	0	1.41	7	0	0.14
C.dubliniensis	0	1	0	0.5	0	0	1	16	1	0	2
Total (%)	77 (79.3%)	1 (1%)	19 (19.5%)		85 (87.6%)	5 (5.1%)	7 (7.2%)		97 (100%)	0 (0%)	

Discussion

Candida species are the principal non-dermatophytic etiologic agents of onychomycosis in patients living in Shiraz, Iran. Identification of *Candida* spp. isolated from patients with onychomycosis using molecular tools was an important contribution to understanding their epidemiology in the city of Shiraz, southern Iran. PCR-RFLP is an ideal method for distinguishing *Candida* species from each other, especially *C. dubliniensis* [14, 15].

Using *B1nI* restriction enzymes, we were able to distinguish *C. dubliniensis* from among the *C. albicans* isolates. This is the first instance in which *C. dubliniensis* was demonstrated to be an etiologic agent of onychomycosis in Iran. Typical for this taxon and *C. albicans*, it produced germ tubes and chlamydoconidia, and developed green colored colonies on CHROMagar. *C. dubliniensis* was sensitive to CLT and VRC at 2 μg/ml and 0.5 μg/ml concentrations, respectively, but had a low MIC of 16 μg/ml to FLU.

Zomorodian et al. [19] recovered 16 isolates of *C. dubliniensis* from denture-related stomatitis from Iranian patients, whereas Ghahri et al. [20] used PCR-RFLP with MboI endonuclease restriction enzyme and could not distinguish *C. dubliniensis* from among 67 isolates of *C. albicans* recovered from onychomycosis in Tehran, Iran. This discrepancy in results might be pertinent to the type of restricted enzyme they used.

In the current study, *C. parapsilosis* was the most common isolate recovered from our patients. Our results are not congruent with the findings of other Iranian studies [21, 22]. Hashemi et al. identified *C. albicans* (41%) as the main etiologic agent of onychomycosis in Tehran, Iran [10]. Chadeganipour et al. reported *C. albicans* as the most prevalent yeast causing onychomycosis in Isfahan, Iran [11].

Moreover, Farasat et al., using an ITS sequencing method, identified *C. pulcherrima* as the causative agent of nail lesions [12]. Our findings are in line with those of the study by Segal et al. [23] and other studies performed in Spain and Hong Kong, which reported *C. parapsilosis* as the predominant species [24, 25]. The inconsistency among the results may be associated with the use of different identification tools and demographic groups as well as the fact that *C. parapsilosis* is a complex of closely related species, which require further studies. We purposefully chose our identification methods to avoid such problems

The CLSI broth microdilution method is recommended for evaluation of antifungal activity of *Candida* species [17, 18]. In our former study, we assessed antifungal activity among clinical *Candida* isolates and reported 96.6% susceptibility to FLC [19]. Khosravi et al. in a study conducted in Tehran, Iran, reported that 85.7% of onychomycosis isolates belonged to *Candida* species and that the isolates were susceptible to FLC [13].

In our study, MICs of non-*albicans* isolates were significantly lower than the *C. albicans* isolates (P=0.001). All the isolates had diverse geometric mean MICs to CLT; 19.5% of the isolates were resistant to VRC, and *C. albicans* were the most resistant isolates. VRC was employed for the treatment of disseminated candidiasis; however, it is not commonly used for the treatment of onychomycosis [26-34]. In Iran, VRC is used for highly resistant cases when the imidazoles are ineffective due to resistance or possible non-compliance. FLC is the most common drug of choice, used for the treatment of onychomycosis, primarily in combination with other antifungal agents [3, 26]. In our study, 87.6% of the isolates were sensitive to FLC; thus, it could be the best choice for combination therapy with CLT.

Conclusion

In summary, this study showed that PCR-RFLP is an efficient method for identification of *Candida* species and that FLC is more effective than the other agents tested against the isolates. We were unable to correlate in vitro antifungal data to in vivo response according to 90/60 rule [32] and it was not in the scope of this study to attempt to correlate *in-vitro* antifungal data to *in-vivo* response. The data can aid physicians to choose an effective potential drug for treating onychomycosis patients.

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Authors' Contributions

K. Pakshir contributed to study concept and design, drafted and revised the manuscript, and analyzed and interpreted the data. K. Zomorodian contributed to study concept and design, drafted and revised the manuscript, and interpreted the data. A. Zakaei contributed to sample collection and laboratory examination. M. Motamedi cooperated with sample collection and laboratory examination, interpreted the data, revised the manuscript, and performed statistical analysis. M. Rahimi Ghiasi and M. Karamitalab contributed to sample collection and laboratory examination. P. Jafari performed statistical analysis.

Conflicts of Interest

The authors declare no conflicts of interest.

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Authors have no financial interests related to the material in the manuscript.

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