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Original Article

A One-Enzyme PCR-RFLP Assay for Identification of Six Medically Important *Candida* Species

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Abstract

Early identification of *Candida* isolates to the species level is necessary for effective antifungal therapy, and can also facilitate control of hospital infections. Phenotype-based methods for identifying *Candida* species are often difficult and time-consuming. Molecular biological techniques provide a useful alternative approach. In the present study, the ITS1-5.8S-ITS2 regions of fungal rRNA genes were amplified with universal primers in 20 standard strains. Digestion of the PCR products with one restriction enzyme, *Msp*I, allowed discrimination of medically important *Candida* species, including *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, and *C. guilliermondii*. Using this method, we successfully identified 137 clinical isolates of *Candida*. Among them, *C. albicans* was identified as the most common species, followed by *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, *C. krusei*, and *C. guilliermondii*. This method is a simple, rapid, and cost-effective method for differentiation between species that is applicable in clinical laboratories.

Key words: *Candida*, PCR, RFLP, *Msp*I, identification

Introduction

Candida species are considered a major cause of opportunistic infections in humans. In recent years, despite advances in health care and therapeutic methods, the incidence of invasive systemic candidiasis has increased markedly. This is thought to be the result of the increase in size of populations at risk, such as transplant recipients, cancer patients, HIV-infected patients, and those receiving immunosuppressive and broad-spectrum antibiotic therapy[1−3]. Although the majority of *Candida* infections are caused by *C. albicans*, non-*albicans* species of *Candida*, such as *C. glabrata* and *C. krusei*, which are less susceptible to azoles derivatives have been reported with increasing frequency[4, 5]. Early diagnosis of invasive fungal infections is essential to reduce the mortality rates. In addition, identification of species is essential for effective antifungal therapy with regard to the emergence of resistance to antifungal drugs. Although *Candida* identification kits based on assimilation tests are commercially available, these kits require at least 1 to 5 days for identification of *Candida* at the species level. Recently, molecular techniques have provided alternative methods for diagnosis and identification of pathogenic fungi, including *Candida* species[6, 7]. Molecular methods with high discriminatory power are required for reliable identification of *Candida* at the species level, especially in epidemiological studies to assess the transmission routes as well as to determine appropriate antifungal drugs[8−10]. Although various methods have been reported for molecular identification of *Candida* spp.,

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such as RAPD (random amplified polymorphic DNA)-PCR, DNA sequence analysis, and mitochondrial large subunit ribosomal RNA gene sequencing[11, 12], these tests are still time-consuming and too expensive for routine use, especially in medical laboratories. Here, we report the application of a rapid PCR-based technique using a one-enzyme restriction fragment length polymorphism (RFLP), for discrimination of six clinically important *Candida* species.

Materials and Methods

Strains: Standard strains, which were provided by Teikyo University Institute of Medical Mycology (TIMM), Tokyo, Japan, are listed in Table 1. In addition, 137 clinical isolates from the Medical Mycology Laboratory, Tehran University of Medical Sciences, Iran, were also used. All yeasts were cultured on Sabauroud's dextrose agar and incubated at 32°C for two days. All clinical isolates were also cultured on CHROMagar *Candida* (Kanto Chemical Co., Ltd., Tokyo) and incubated at 35°C for 48 h for production of species-specific colors.

DNA extraction: Genomic DNA was extracted and purified using glass bead disruption[13]. Briefly, a loop full of fresh yeast was harvested and suspended in 300 μl of lysis buffer (10 mM Tris, 1 mM EDTA pH 8, 1% SDS, 100 mM NaCl, 2% Triton X-100). After adding 300 μl of phenol-chloroform (1 : 1) and 300 mg of glass beads (0.5 mm in diameter), samples were vortexed vigorously for 5 min to disrupt the cells completely. Cellular debris was separated by centrifugation at 10,000 rpm for 5 min and the aqueous layer was extracted once more with an equal volume of chloroform. Total DNA in the supernatant was precipitated with 2-propanol, washed with 70% ethanol, air-dried, resuspended in 100 μl of TE buffer (10 mM Tris, 1 mM EDTA), and preserved at −20°C until use.

PCR conditions: PCR amplification was carried out in a final volume of 100 μl. Each reaction contained 1 μl of template DNA, each forward (ITS1, 5'-TCC GTA GGT GAA CCT GCG G-3') and reverse (ITS4, 5'-TCC TCC GCT TAT TGA TAT GC-3') primer at 0.2 μM, each deoxynucleoside triphosphate (dNTP) at 0.1 mM, 10 μl of 10× PCR buffer, and 2.5 U of *Taq* DNA polymerase. An initial denaturation step at 94°C for 5 min was followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 45 s, and extension at 72°C for 1 min, with a final extension step of 72°C for 7 min. Amplified products were visualized by 1.5% (w/v) agarose gel electrophoresis in TBE buffer (0.09 M Tris, 0.09 M boric acid, and 20 mM EDTA, pH 8.3),

Table 1. Standard yeast strains used in the present study

Species	Strains
Candida albicans	ATCC 10261, ATCC 10231, ATCC 24432, TIMM 1768
C. glabrata	ATCC 90030, CBS 138
C. tropicalis	ATCC 0750, TIMM 0313
C. krusei	ATCC 6258, TIMM 3404
C. parapsilosis	ATCC 22019, ATCC 90018
C. guilliermondii	ATCC 9058, TIMM 0257
C. albicans var. *stellatoidea*	TIMM 1309
C. dubliniensis	CBS 7987
Cryptococcus neoformans	ATCC 90113
Saccharomyces cerevisiae	ATCC 9763, ATCC 2366
Trichosporon asahii	TIMM 3411

Table 2. Sizes of ITS1-ITS4 PCR products for *Candida* species before and after digestion with *Msp* I

Candida species	Size of ITS1-ITS4	Size(s) of restriction product(s)	*Accession number
C. albicans	535	297, 238	L47111
C. glabrata	871	557, 314	AF167993
C. tropicalis	524	340, 184	L47112
C. krusei	510	261, 249	L47113
C. guilliermondii	608	371, 155, 82	L47110
C. parapsilosis	520	520	L47109

*DDBG/EMBL/GenBank accession number

stained with ethidium bromide (0.5 μg ml^{-1}), and photographed.

RFLP analysis: The ITS1-ITS4 sequences of various *Candida* species obtained from DDBJ/EMBL/GenBank databases were aligned and restriction patterns of the PCR products of the species mentioned above were predicted for each of the known restriction enzymes using DNASIS software (Hitachi Software Engineering Co., Tokyo). Predicted restriction fragments were compared to choose the best discrimination. Finally, the enzyme *Msp* I was selected to achieve the best species-specific length patterns as shown in Table 2. Digestion was performed by incubating a 20-μl aliquot of PCR product with 10 U of *Msp* I (Roche Molecular, Mannhiem, Germany) in a final reaction volume of 25 μl at 37°C for 2 h. Restriction fragments were separated by 1.8% agarose gel electrophoresis in TBE buffer for approximately 45 min at 100 V and visualized by staining with ethidium bromide.

Results

Fungus-specific universal primer pairs (ITS1 and ITS4) were able to successfully amplify the ITS region of all yeasts tested, providing a single PCR product of approximately 510～870 bp (Fig. 1). After analysis of various restriction enzymes, *Msp* I was selected as the best enzyme

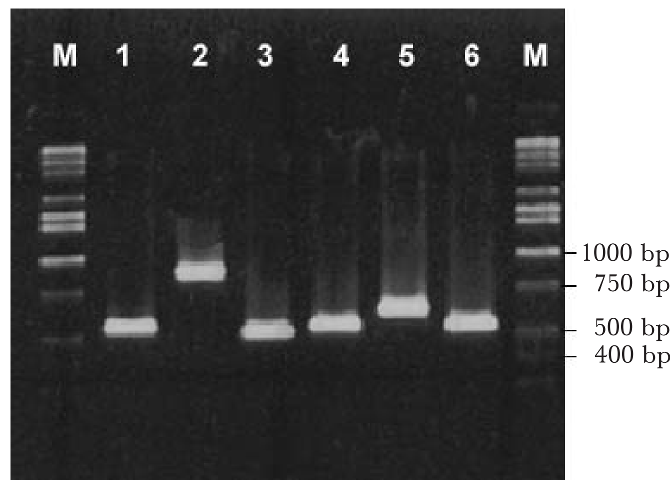

Fig. 1. PCR products from six *Candida* species. Lanes 1-6: *C. albicans* (ATCC 10261), *C. glabrata* (ATCC 90030), *C. tropicalis* (ATCC 0750), *C. krusei* (ATCC 6258), *C. guilliermondii* (ATCC 9058) and *C. parapsilosis* (ATCC 22019), respectively. Lanes M: Molecular size marker

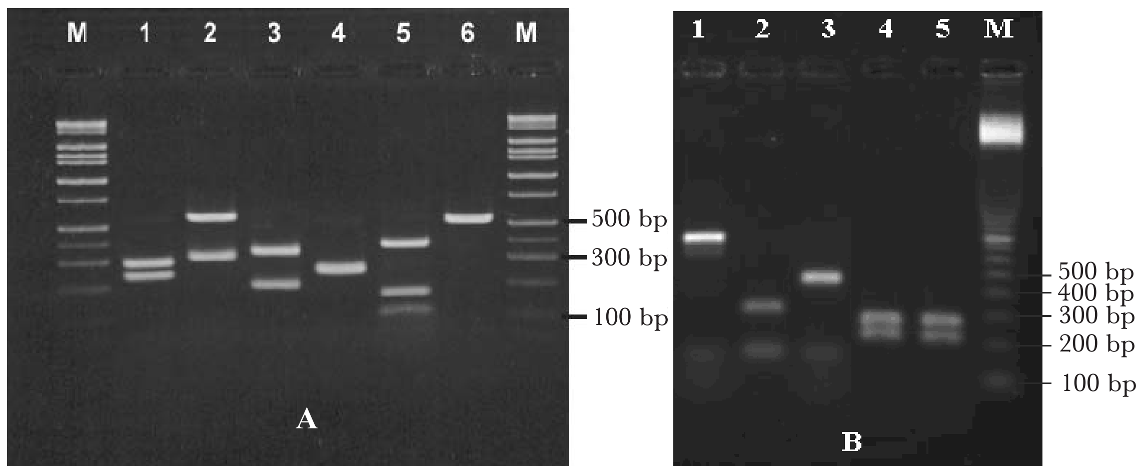

Fig. 2. A) Restriction digestion of PCR products of *Candida* strains with the enzyme *Msp* I. Lanes 1-6: *C. albicans* (ATCC 10261), *C. glabrata* (ATCC 90030), *C. tropicalis* (ATCC 0750), *C. krusei* (ATCC 6258), *C. guilliermondii* (ATCC 9058) and *C. parapsilosis* (ATCC 22019), respectively. Lane M: molecular size marker. B) Restriction digestion of PCR products of other strains with the enzyme *Msp* I. Lanes 1-5: *S. cerevisiae* (ATCC 9763), *T. asahii* (TIMM 3411), *C. neoformans* (ATCC 90113), *C. albicans* var. *stellatoidea* (TIMM1309) and *C. dubliniensis* (CBS 7987), respectively. Lane M: Molecular size marker

Table 3. Identification of clinical isolates of *Candida* species in Iran by PCR-RFLP amplification of the ITS1-ITS4 region and RFLP analysis with *Msp*I

Species	Number	%
C.albicans and/or *C. dubliniensis*	93	67.9
C. tropicalis	12	8.8
C. parapsilosis	12	8.8
C. glabrata	9	6.6
C. krusei	7	5.1
C. guilliermondii	4	2.9
Total	137	100

for differentiation between six medically important *Candida* species. PCR amplicons were digested with *Msp* I as described in Materials and Methods. The products of digestion are shown separately in Fig. 2, which shows that the bands generated corresponded to the predicted sizes. Digestion of the ITS region of *Candida* species by *Msp* I generated 2 bands for *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. krusei*, and 3 bands for *C. guilliermondii*. However, there were no recognition sites for this enzyme within the ITS region of *C. parapsilosis*, and its PCR and digestion products were the same size.

The RFLP pattern produced for each *Candida* species was completely specific so none of the species examined was mistaken for another (Fig 2, A). Identical patterns were seen for *C. albicans*, *C. albicans* var. *stellatoidea*, and *C. dubliniensis* (Fig2, B). None of the other yeasts tested, including *Cryptococcus neoformans*, *Saccharomyces cerevisiae*, and *Trichosporon asahii*, showed similar patterns (Fig2, B). The enzyme was also used for digestion of PCR products of 137 strains of *Candida* isolated from clinical specimens, including superficial, mucocutaneous, and deep seated specimens. Using this method, *C. albicans* was identified as the most common species (67.9%) followed by *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, and *C. krusei* (Table 3). In our study the results of PCR-RFLP tests for identification of the clinical isolates were completely identical to those obtained using CHROMagar *Candida*.

Discussion

Despite recent progress in the development of new molecular approaches for diagnosis of fungal infections, the goal of developing a simple, rapid, and cost effective test for diagnostic purposes remains elusive. In the present study, using the universal primers, ITS1 and ITS4, we amplified a fragment of 510－879 bp of the ITS1-5.8S-ITS2 rDNA region from genomic DNA of several strains of *Candida* species. Restriction fragment length polymorphism (RFLP) analysis of the PCR products with one enzyme allowed us to identify the 6 most medically important *Candida* species.

The ITS molecule contains several regions of highly conserved sequence useful for obtaining proper sequence alignments, but with sufficient sequence variability in other regions of the molecule that can serve as markers of species-specific restriction fragment length polymorphism (RFLP)[14]. This region has been used in part or completely by other investigators for species identification of some medically important fungi, particularly *Candida*[15, 16], *Aspergillus*[17], dermatophytes[18], *Trichosporon*[19], and *Malassezia*[20] species using various approaches, such as DNA probes, nested PCR, sequencing, and RFLP. Williams *et al.*[15] attempted to delineate medically important *Candida* species using restriction enzyme digestion (*Hae* III, *Dde* I, *Bfa* I) after amplification of the ITS1-ITS4 region, but their patterns were not suitable for identification, perhaps due to inaccessibility of related ITS1-ITS4 sequences. Maiwald *et al.*[21] introduced a method for presumptive differentiation of 12 clinically relevant yeasts to the species level by amplification of the small ribosomal subunit 18S-rRNA using six enzymes. Identification of *Candida* species by PCR-RFLP has also been applied recently by Deak and Pinto[22, 23]. While all of the mentioned studies discriminated *Candida* species using several restriction enzymes, we identified six species, which accounts for up to 95% of *Candida* infections, using only one enzyme.

In the present study, restriction digestion of the ITS amplification product with *Msp* I produced the predicted specific patterns for each species. Using this method, all standard stains were identified successfully. Moreover, the results of PCR-RFLP analysis of the clinical isolates examined were comparable with those obtained by CHROMagar *Candida* test. Unfortunately, digestion of *C. albicans* and *C. dubliniensis* with *Msp* I yielded similar patterns, and therefore additional enzymes for differentiation of these species are still required. Actually we have reported another PCR-restriction enzyme format for discrimination of these two *Candida* species[24].

We recommend this simple and easy-to-perform method for the identification of *Candida* isolates in the medical mycology laboratories.

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