

SIMPLE AND RAPID IDENTIFICATION OF MOST MEDICALLY IMPORTANT *CANDIDA* SPECIES BY A PCR-RESTRICTION ENZYME METHOD

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Abstract- Opportunistic fungal infections including candidiasis have increased dramatically in recent years. Most medically important fungi are *Candida* species. Rapid identification of *Candida* isolates to the species level in the clinical laboratory is necessary for more rapid and effective antifungal therapy and to facilitate hospital infection control measures. Conventional morphological methods for identification of *candida* species are often difficult and time consuming. Molecular DNA-based techniques provide useful alternative methods. In this study using universal primers, ITS1-ITS4 region of the fungal rRNA genes were amplified. Digestion by the restriction enzyme *MspI* allowed us to identify of *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicali*, and *C. guilliermondii*. *C. guilliermondii* produces 3 bands whereas the others gave two distinctive bands after digestion. This panel of PCR- restriction enzyme could be rapid, simple and useful in diagnostic studies of candida and candidiasis.

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Key Words: *Candida*, PCR, restriction enzyme

INTRODUCTION

Opportunistic fungal infections have increased dramatically in recent years. This had often been as a result of advanced medical treatments, such as more intensive regimens of cancer therapy, complications of abdominal or cardiothoracic surgery, organ transplantation, prolonged broad spectrum antibiotic therapy, invasive devices such as indwelling catheters and prolonged hospital stays (1,2). Under these conditions an antibiotic-resistant replacement flora, including *Candida* species, can proliferate in the gut and invade deep tissues from mucosal foci. This is especially the case when mucosal integrity has been disrupted due to chemotherapy or surgery. At least 150 fungal species have been identified as human pathogens but most opportunistic fungal infections are caused by yeasts, and the most important yeasts are *Candida* species (3). Rapid identification of *Candida* isolates to the species level in the clinical laboratory has become more important because the incidence of candidiasis continues to rise in proportion to an increasing number of patients at risk for infection with

Candida albicans and recently, with innately azole-resistant non-*albicans Candida* species. Some *Candida* species including *C. glabrata* and *C. krusei* are emerging possibly because they are innately less susceptible to azole drugs (4). Consequently, rapid identification to the species level is necessary for more rapid and effective antifungal therapy, to facilitate hospital infection control measures and generally because epidemiological studies. Identification of this increasing diversity of pathogen by conventional methods is often difficult and sometimes impossible (5). Morphological features and reproductive structures useful for identifying isolated fungi may take days to weeks to develop in culture, and evaluation of these characteristics requires expertise in mycology. Commercial methods used to identify yeast including API system require 2 to 3 days before biochemical reactions can be interpreted (5). In addition, their databases sometimes are ambiguous. Molecular techniques utilizing amplification of target DNA, provide alternative methods for diagnosis and identification of some organisms (6). PCR - based detection of fungal DNA sequences can be rapid, sensitive and specific (7). In this study using universal primers complementary to the coding regions of the fungal rRNA genes, we amplified a 510 to 871 base pair segment of ITS1, 5.8s rRNA, and ITS2 region from genomic DNAs of numerous isolates of the *Candida* species. Restriction enzyme analysis (REA) of the PCR products allowed us to identify most

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medically important *Candida* species including *C. albicans*, *C. tropicalis*, *C. krusei*, *C. glabrata* and *C. guilliermondii*. This panel of PCR-restriction enzyme can be useful in diagnostic studies of *Candida* and candidiasis.

MATERIALS AND METHODS

Yeasts isolates: Yeasts were isolated from clinical specimens submitted to Medical Mycology Laboratory, Faculty of Public Health, Tehran University of Medical Sciences, Tehran, Iran, for suspected fungal infection. Patients' samples were cultured on glucose 4%, pepton 1%, agar 1.5% and were incubated for 3 days in 30°C.

DNA extraction: Yeast cells were subcultured on YEPD broth (1% yeast extract, 2% pepton, 2% dextrose) and were incubated for 2 days at 30°C under shaking conditions (150 rpm). The yeast cells were collected by centrifugation (5 ml) of the broth culture at 3000 rpm for 5 minutes, suspended in 1 ml sterile saline, centrifuged again and resuspended in 0.5 ml sterile saline and freezed in -25°C until use. For DNA extraction, 200 microliter of lyses buffer (10 mM tris, 1 mM EDTA (pH 8), 1 % SDS , 100 mM NaCl, 2% triton X-100), 200 microliter of phenol-chlorophorm (1: 1) solution, and 200 microliter of 0.5 mm diameter glass beads, were added to yeast pellet. After 5 minutes vigorous shaking, folowed by 5 minutes centrifugation in 10000 rpm, the supernatants were isolated and transmitted to a new tube and equal volume of chlorophorm was added, mixed gently, centrifuged and its supernatant was transferred to a new tube. For alcohol precipitation, 0.1-volume sodium acetate (pH 5.2) and 25-volume cold absolute ethanol were added and the mixture was gently shaken and centrifuged in 10000 rpm for 10 minute in 4°C. After once washing with 70% ethanol, the pellet resuspended in 100 microliter TE buffer (10 mM Tris, 1 mM EDTA) until it was used for PCR amplification.

PCR amplification: The PCR assay was performed with 1 microlitre of test sample (about 1 ng) in a total reaction volume of 100 microliter, consisting of 10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl, 0.2 mM each dATP, dGTP, dCTP, and dTTP, 0.2 mM each primers and 5U of Taq DNA polymerase. Thirty-five cycles of amplification were performed in a model Techne-Progene thermal cycler. After initial

denaturation of DNA at 95°C for 5 minutes, each cycle consisted of a denaturation step at 94°C for 30s, an annealing step at 56°C for 30s an extension step at 72°C for 1 minute and a final extension step at 72°C for 7 minute following the last cycle. After amplification, the samples were stored at -20°C until used. Appropriate negative controls were included in each test running.

Restriction enzyme analyses: ITS1-ITS4 sequences of various *Candida* species were derived from Gene-Bank (Table 1). On the basis of that sequences the restriction sites of various restriction enzymes were determined by DNAsis software and the best enzyme was selected. For restriction digestion, 21.5 microlitre of PCR products were digested directly and individually by 10 U (1 microlitre) of the restriction enzyme *MspI* and 2.5 microlitre related buffer (total reaction= 25microlitre) by 90 minute incubation at 37°C.

Agarose gel electrophoresis: The resulting restriction fragments were analyzed by 2.5% agarose gel. Electrophoresis gel was conducted in TBE buffer (0.1 M tris, 0.09 M boric acid, 20 mM EDTA (pH= 80) at 100 V for 45 minutes. The gel was stained with 0.5 microgram per milliliter of ethidium bromide and photographed by IVP.

All materials were prepared from Roche Molecular Biochemicals.

RESULTS

The intergenic spacer regions of all isolates tested were successfully amplified (Fig. 1). The fungus-specific universal primer pairs, generated PCR products of approximately 510 bp to 871 bp related to different *Candida* species (Fig. 2).

Table 1

1	2	3	4	5	6
<i>C. albicans</i>	535	1	297	238	
<i>C. glabrata</i>	871	1	557	314	
<i>C. guilliermondii</i>	608	2	371	155	82
<i>C. tropicalis</i>	524	1	340	184	
<i>C. krusei</i>	510	1	261	249	
1. Organism		2. Size of ITS region			
3. Number of restriction site	4,5,	and	6.	size of restriction product	

On the basis of results of the application of some enzymes on its sequences of various *candida* derived

from Gene-Bank (Table 1), it was found that *MspI* was a suitable and useful restriction enzyme for delimitation of most important *Candida* species. This enzyme gives two bands for each *C. albicans*, *C. tropicalis*, *C. krusei* and *C. glabrata* and three bands for *C. guilliermondii* (Fig. 1 and 2). The produced bands were well distinguishable so that none of mentioned *Candida* spp. are mistaken with each other. Furthermore it gives good pattern for identification of another medically important yeast *Cryptococcus neoformans*, another agent of systemic fungal infection. It does not have any site for *C. parapsilosis*, thus does not cleave it and there is only one undigested band after electrophoresis.

DISCUSSION

Rapid identification of *Candida* species in clinical laboratory is becoming increasingly important as the incidence of candidosis continues to rise in the growing at-risk patient population. During the period 1986-90 *Candida* species emerged as the sixth most common nosocomial pathogens in the hospital survey. Among the nosocomial bloodstream infections, *Candida* species ranked fourth hospital-wide. *Candida* species accounted for 10.2% of all cases of septicemia and for 25% of all urinary tract infections in intensive care units (8).

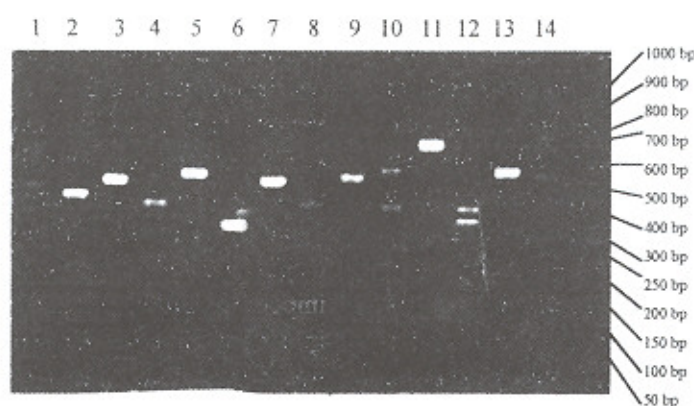


Fig. 1. ITS1-ITS2 PCR products from six *Candida* species: Lanes 1-6: *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. guilliermondii*, and *C. parapsilosis* respectively, Lane M: 100 bp ladder molecular size marker

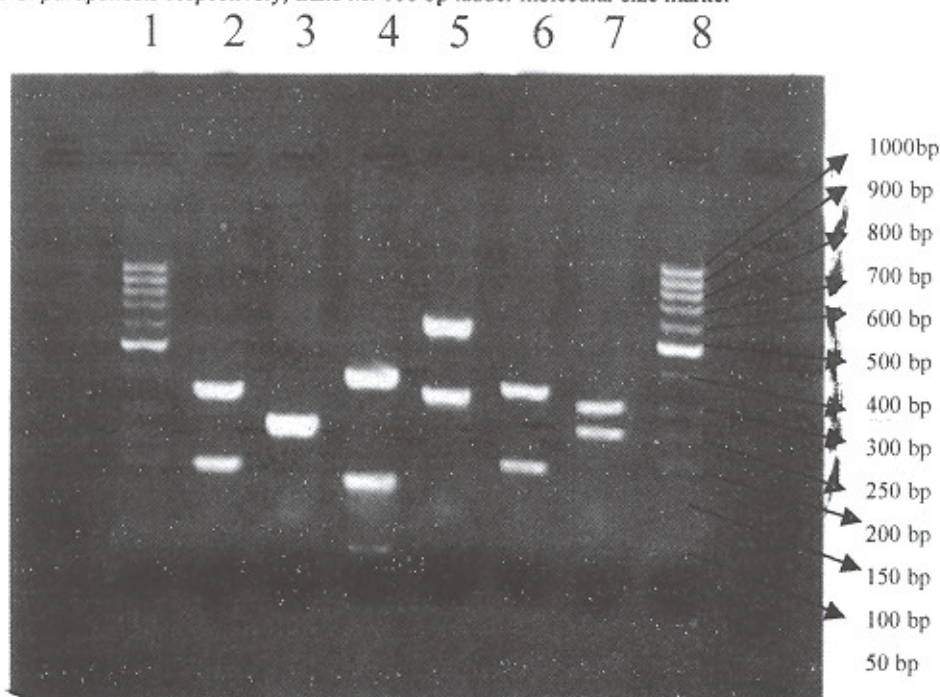


Fig. 2. Patterns of PCR products of *Candida* species after digestion by the restriction enzyme *MspI*, Lanes 1-6: *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. guilliermondii*, and *C. parapsilosis*. Lane M: 100 bp ladder molecular size marker

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On the other hand the recent increase in infections caused by non-*C. albicans* *Candida* species to certain antifungal drugs (8), make test for rapidly differentiating *Candida* species, valuable for targeted drug therapy. The traditional methods of identifying *Candida* species are often based on examination of phenotypic characteristics. This approach can be time-consuming and the reliance on the variable expression of phenotypic characteristics can lead to inconsistent results. For example the phenotypic switching of *Candida* species has been well-documented (9). Consequently genotype based approaches may be preferable. Several DNA-based methods such as karyotyping (8,9), DNA probing, DNA fingerprinting, restriction fragment length polymorphism analysis (10) and specific amplification of certain genes by the polymerase chain reaction (PCR) (7,11-16) have been used in the past to recognize species of the genus *Candida* in clinical specimens. However most of these techniques are laborious, cumbersome and time-consuming and only a few of the techniques mentioned above are actually able to differentiate between *Candida* species other than *C. albicans* (14). Coding regions of the 18s, 5.8s and 28s nuclear rRNA genes evolve slowly, are relatively conserved among fungi, and provide a molecular basis of establishing phylogenetic relationship (14). Between coding regions are the internal transcribed spacer 1 and 2 regions (ITS1 and ITS2 respectively) which evolve more rapidly and may therefore vary among different species within a genus. Thus PCR amplification may facilitate the identification of its region DNA sequences with sufficient polymorphism to be used for identifying fungal species especially *Candida* species. In addition, this region offers distinct advantages over other molecular targets including increased sensitivity due to the existence of approximately 100 copies per genome. This advantage is important in detecting fungi DNA in clinical samples. This region has been used in identification of some medically important fungi, particularly *Candida* and *Aspergillus* species (14,17) by the various approaches such as DNA prob and RFLP. For these reasons, in this study ITS sequence polymorphism is evaluated by their PCR amplification and consequent restriction enzyme analysis. Restriction enzymes are endonucleases that cleave DNA in response to recognition site on the DNA. The recognition site (restriction site) consists of a specific sequence of nucleotides in the DNA duplex, typically 4-8 base pairs. Experiment with restriction enzymes is simple, relatively inexpensive and their result is reproducible. These enzymes have vast application in the molecular biology especially the diagnostic

purposes. The pattern in restriction enzyme profile is important. When the restriction enzyme is applied for differentiation between some related organisms, it must be distinguishable according to the size and number of bands and in general produced patterns must be so clear that can identify the given organism. Williams and coworkers (18) tried to delineate medically important *Candida* spp. by some restriction enzymes (*HaeIII*, *DdeI*, *BfaI*) after amplification of ITS-ITS4 region, but their bands were not suitably suitable for identification perhaps due to unavailability to related ITS1-ITS4 sequences. Also, Morace (15) targeted the amplification of a 300-350 bp segment in P-450 LI AI gene and subsequent digestion by 3 enzymes (*HincII*, *NsiI* and *Sau 3A*) for identifying 8 *Candida* species. In the present study only one enzyme (*MspI*) has been used. *MspI*, has a 4 base pair restriction site (CCGG). It cleaves ITS region of the various species of the genus *Candida* and produce suitably different segments that is clearly distinguishable after the agarose gel electrophoresis (Table 1 and Fig. 1 and 2). We think that this PCR-RE is a suitable and useful method in identification of most medically important *Candida* species and another medically important fungus *Cryptococcus neoformans*. This method can determine the *Candida* isolate during one or even half of a workday.

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