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Rapid Molecular Diagnosis for *Candida* species Using PCR-RFLP

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Abstract: Rapid identification of *Candida* species in clinical laboratory is becoming increasingly important since the incidence of Candidiasis continues to rise as the hospital surveys show. Molecular techniques utilizing amplification of target DNA provide quick and precise methods for diagnosis and identification of *Candida* species. In this study, using universal primers, the ITS1-ITS4 region was amplified. The restriction enzyme *Msp*I digests this region and was used to identify of *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis* and *C. guilliermondii*. Electrophoretically, ribosomal DNA of *C. guilliermondii* produced three bands whereas the other species gave two bands upon digestion. Accordingly these enzymes behave as valuable application tools in molecular diagnosis of *Candida* species in Candidiasis maladies and can be substituted with the classical diagnosis of the pathogen.

Key words: *Candida*, PCR, restriction enzyme, molecular diagnosis

INTRODUCTION

Candida albicans is an important opportunistic fungal pathogen in human which can cause mucosal and systemic infections (Bautiste-Muñoz *et al*., 2003; Dendis *et al*., 2003; Karababa *et al*., 2004). When immune system is weak (e.g., in immunocompromised patients as the result of cancer chemotherapy or HIV infection) or when the competing flora are eliminated (after antibiotic treatment), *C. albicans* colonizes and invades host tissues (Warren and Hazen, 1995). The pathogen is the fourth most common hospital-acquired infection in the United States, the treatment of which is estimated to cost more than 1 billion $US annually. The genus *Candida* includes around 154 species that show different levels of resistance to antifungal agents. Consequently, it is important to identify the causative organism to the species level correctly. Identification of *C. dubliniensis* in particular, remains problematic because of the high degree of phenotypic similarity between this species and *C. albicans* (Neppelenbroek *et al*., 2005). Morphological features and reproductive structures useful for identifying isolated yeasts may take days to weeks to develop in culture and evaluation of these characteristics requires expertise in mycology (Mirhendi *et al*., 2006). Molecular techniques utilizing amplification of target DNA provide alternative methods for diagnosis and identification of some organisms. Identification of *Candida* species has been achieved by Restriction Fragment Length Polymorphism (RFLP) analysis of the ribosomal DNA (rDNA) (Cirak *et al*., 2003). At one study in China, a sensitive and speedy technique was established to identify three medically important fungal species, *Candida*, *Aspergillus* and *Cryptococcus* by using PCR-RFLP *in vitro*. The result showed that PCR-RFLP is sensitive, specific and fast. Internal transcribed spacer1 (ITS1) and ITS2 and 5.8S rDNA were amplified by PCR and semi-nested PCR to detect fungal DNA (Consuelo *et al*., 2001). Various techniques have been reported to separate different fungi detected by universal primers, including restriction fragment length polymorphism (Mirhendi *et al*., 2001). Amplification Product Length Polymorphism (APLP) in PCR-APLP-RFLP assays can be useful in the diagnosis of fungal infections in immunocompromised patients It is documented that PCR-RFLP is sensitive, specific and fast method for detection of the medically important fungi (Morace *et al*., 1997; Zhenyu *et al*., 2000; Xu *et al*., 2002; Mirhendi *et al*., 2006). 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

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genomic DNAs of numerous isolates of the *Candida* species. Restriction Enzyme Analysis (REA) of the PCR products allowed us to identify most medically important *Candida* species including *C. albicans*, *C. tropicalis*, *C. glabrata* and *C. gilliermondii* in 27 collected samples from hospital referred vaginitis patients. This panel of PCR-restriction enzyme analysis can be useful in diagnostic studies of *Candida* species and Candidiasis and hence leading to proper treatment. This research was performed in 2006 at the Department of Medical Parasitology and Mycology, College of Medicine, Medical University of Kerman, Iran, Department of Plant Pathology and Biotechnology, College of Agriculture, Bahonar University of Kerman and International Center for Science and High Technology and Environmental Sciences, Mahan, Iran.

MATERIALS AND METHODS

Fungal isolates: *Candida* species were isolated from clinical specimens of vaginitis patients of Kashani and Afzalipoor hospitals who were submitted to the medical mycology laboratory. These samples were cultured on glucose 4%, peptone 1% and agar 1.5% and were incubated at 37°C for 2 days to develop proper growth for further studies.

DNA extraction: For DNA extraction, 300 μL of lysis buffer (10 mM Tris, 1 mM EDTA (pH 8), 1% SDS, 100 mM NaCl, 2% triton X-100), 300 μL of phenol-chloroform (1:1) solution and equal to 300 μL of 0.5 mm diameter glass beads, were added to yeast (Mirhendi *et al.*, 2005). After 5 min of vigorous shaking which followed by 5 min centrifugation at 10000 rpm, the supernatant was isolated and transferred to a new tube and equal volume of chloroform was added, mixed gently, centrifuged and its supernatant was transferred to a new tube. For alcohol precipitation, 0.1 mL volume sodium acetate (pH 5.2) and 2.5 mL volume of cold absolute ethanol were added and the mixture was gently shaken and centrifuged at 10000 rpm for 10 min at 4°C. After washing with 70% ethanol, the pellet resuspended in 100 μL TE buffer (10 mM Tris, 1 mM EDTA) until it was used for PCR amplification.

PCR amplification: The PCR assay was performed with 1 μL of test sample (about 1 ng) in a total reaction volume of 50 μL, consisting of 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each dATP, dCTP, dGTP and dTTP, 0.2 mM of each primers and 5U of Taq DNA polymerase. Thirty-five cycles of amplification were performed in a thermal cycler model Techne-progene. After initial denaturation of DNA at 95°C for 5 min, each cycle consisted of a denaturation step at 94°C for 30s an extension step at 72°C for 1 min and a final extension step at 72°C for 7 min following the last cycle. After amplification, the samples were stored at -20°C before use. Appropriate negative controls were included in each test.

Restriction enzyme analyses: ITS1-ITS4 sequences of various *Candida* species were derived received from Mirhendi *et al.* (2006). On the basis of the sequences, the restriction sites of various restriction enzymes were determined by DNAsis software and the best enzymes were selected. For restriction digestion, 21.5 μL of PCR products were digested directly and individually by 10 U (1 μL) of the restriction enzyme *Msp*I and 2.5 μL related buffer (total reaction of 25 μL) by 90 min 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 = 8, at 100 V for 45 min. The gel was stained with 0.5 μg mL^{-1} of ethidium bromide and photographed by Ultra Violet Photography (Integrated Vision Products®).

RESULTS

Using PCR-RFLP method, from the 27 tested samples, 22 identified as *C. albicans*, 2 as *C. glabrata*, 2 as *C. guilliermondii* and 1 as *C. tropicalis*. The intergenic spacer regions of all tested isolates were successfully amplified. The yeast-specific universal primer pairs generated PCR products of approximately 510 to 871 bp related to different *Candida* species. According to the results of the application of some enzymes on the sequences of various *Candida* species (Mirhendi *et al.*, 2006). It was well conclusive that *Msp*I is a suitable and useful restriction enzyme for delineation of most important species of the yeast. The enzyme gave two bands for each of *C. albicans*, *C. tropicalis*, *C. krusei* and *C. glabrata* and three bands for *C. guilliermondii* (Mirhendi *et al.*, 2006). The produced bands were so well distinguishable that none of mentioned *Candida* spp. were mistaken with each other. As shown in Fig. 1 and 2, it is noticeable that yeast isolates No. 19, 11, 24, 22, 29, 23, 25, 15 and 28 (Fig. 1) and No. 27, 14, 13, 30, 21 and 18 (Fig. 2) have 2 bands around 338 bp and 297 bp which are indicative of *C. albicans* and the isolates No. of 20 (Fig. 1) which is representative of *C. glabrata* and 15 (Fig. 2) has 2 bands around 557 bp and 314 bp and isolate No. of 20 and 15 (Fig. 1 and 2) are *C. glabrata*, 29 (Fig. 1) is *C. tropicalis* and 17 (Fig. 2) is *C. guilliermondii*.

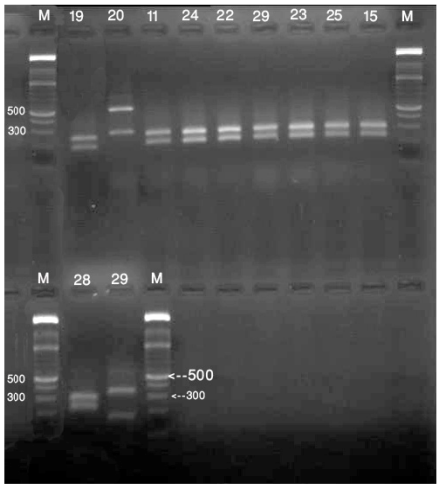

Fig. 1: Patterns of PCR products of *Candida* isolates after digestion by the restriction enzyme *MspI*. Lanes of 19, 11, 24, 22, 29, 23, 25, 28 and 15 represent *C. albicans*; 20, *C. glabrata* and 29, *C. tropicalis*. Lane M is 100 bp ladder molecular size marker

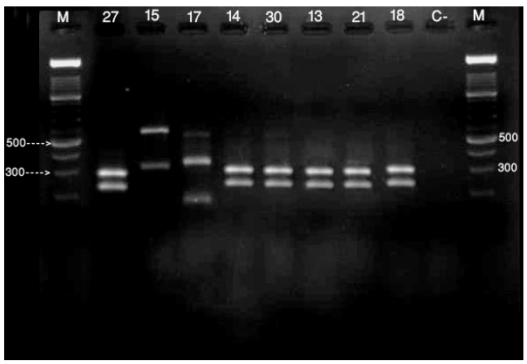

Fig. 2: Patterns of PCR products of *Candida* isolates after digestion by the restriction enzyme *MspI*. Lanes of 27, 14, 30, 13, 21 and 18 represent *C. albicans*; 15, *C. glabrata* and 17, *C. guilliermondii*. Lane M is 100 bp ladder molecular size marker and C is negative control

DISCUSSION

Rapid identification of *Candida* species in clinical laboratory is becoming increasingly important as the incidence of Candidiasis continues to rise as the hospital surveys indicate. Among the nosocomial bloodstream infections, *Candida* species were ranked fourth hospital-wide. *Candida* species were responsible for 10.2% of all cases of septicemia and 25% of all urinary tract infections in intensive care units. On the other hand, the recent increase in infections caused by non *C. albicans* species resistant to certain antifungal drugs, *C. dubliniensis* has resulted in problems in the identification of clinical samples (Dooley *et al*., 1994; Chavasco *et al*., 2006). In a retrospective study carried out on a collection of yeasts, it was demonstrated that 2% of the isolates originally identified as *C. albicans* were actually *C. dubliniensis* (Soh, 2000). Making tests for rapid differentiation of *Candida* species is valuable for targeted drug therapy (Birincia *et al*., 2004; Mirhendi *et al*., 2005). The traditional methods of identification of *Candida* species include laborious and subjective morphological and assimilation tests that can take several days for identification (Consuelo *et al*., 2001; Watton *et al*., 2004). Furthermore, microscopical tests have been reported to misidentify clinical isolates (Dooley *et al*., 1994). Restriction enzymes are endonucleases that cleave DNA in response to recognition site on the DNA. The recognition site consists of a specific sequence of nucleotides in the DNA duplex, typically 4-8 bp. Experiment with restriction enzyme is simple, relatively inexpensive and their result is reproducible. These enzymes have vast application in the molecular biology especially for the diagnostic purposes (Mirhendi *et al*., 2005, 2006). Several DNA-based methods such as karyotyping, DNA probing, DNA fingerprinting, restriction fragment length polymorphism analysis and specific amplification of certain genes by the polymerase chain reaction (PCR) have been used in the past to recognize species of the genus *Candida* in clinical specimens (White *et al*., 2003; Mirhendi *et al*., 2006). However, RAPD analysis is difficult to interpret and lacks reproducibility. Furthermore, RFLP without a hybridization probe generally produces patterns that are too dense and unresolved for computer analysis and hence it does not lend itself to studies in which cluster analyses of moderately related isolates are necessary (Soh, 2000; Bautiste-Muñoz *et al*., 2003). However, most of these techniques mentioned above are actually able to differentiate between *Candida* species other than *C. albicans*. Coding regions of the 18s, 5.8s and 28s nuclear rRNA genes evolve slowly and so are good candidates, since they are present in high copy number and the sensitivity of their detection may be dramatically increased by the use of nested PCR (Cirak *et al*., 2003) and relatively conserved among fungi providing a molecular basis of establishing phylogenetic relationship. Identification of rDNA genes has been used to identify pathogenic fungi (Baere *et al*., 2002). Sequence variability of the internal transcribed spacer 2 (ITS2) region of fungi is potentially useful in rapid and accurate diagnosis of

clinical fungal isolates. PCR with fungus-specific primers targeted toward conserved sequences of the 5.8 and 28S ribosomal DNA (rDNA) results in amplification of the species-specific ITS2 regions, which are variable in amplicon length (Turenne *et al*., 1999). 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 (Berman and Sudbery, 2002). We like to express that these enzymes and technique has been used previously by Mirhendi *et al*. (2006) which their results comprehend with ours coherently. The overall conclusion of our PCR-restriction enzyme analysis is that this procedure can be used as diagnostic and differentiating tool about the *Candida* species and Candidiasis which would lead to more accurate medical treatment of the patient malady.

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