IDENTIFICATION OF *CANDIDA* SPECIES BY RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) ANALYSIS OF COLONY LYSATES

KOLONİ LİZATLARIN RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) ANALİZİ YOLU İLE CANDIDA TÜRLERİNİN TANIMLANMASI VE CANDIDA ALBICANS SUŞLARINDA FLUKONAZOL DİRENCİNİN ARBITRARILY PRIMED POLYMERASE CHAIN REACTION (AP-PCR) İLE SAPTANMASI

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ABSTRACT

Purpose: *The aim of this study was to identify Candida species by using genotypic methods such as PCR (polymerase chain reaction) and RFLP (restriction fragment length polymorphism) analysis. The identification of Candida species by PCR and RFLP analysis was based on the size and primary structural variation of rDNA intergenic spacer regions (ITS).* ***Methods:*** *Forty-four clinical Candida isolates comprising 5 species were included to the study. The amplification products were digested individually with 3 different restriction enzymes HaeIII, DdeI and BfaI.* ***Results:*** *All the isolates tested yielded the expected band patterns by PCR and RFLP analysis. The results obtained from this study demonstrate that Candida species can be differentiated as Candida albicans and non-C. albicans strains only by using HaeIII restriction enzyme and that BfaI confirms the differentiation of these non-C. albicans species. This differentiation seems to be highly efficient especially in cases of fungemia.* ***Conclusion:*** *The identification of Candida species with the amplification of the ITS region and RFLP analysis is a practical, rapid and reliable method when compared with conventional, time-consuming Candida species identification methods*

Key Words: *Candida albicans, Identification, RFLP Analysis.*

ÖZET

Amaç: *Bu çalışmada Candida türlerini PCR (Polymerase chain reaction) ve RFLP (restriction fragment lenght polymorphism) analizi ile getotipik yöntemlerle tanımlamaktır. Candida türlerinin PCR ve RFLP analizi ile tanımlanması, rDNA intergenic spacer (ITS) bölgelerinin büyüklük ve primer yapısal varyasyonlarına dayanır.* ***Yöntem:*** *Beş tür halindeki 44 klinik Candida izolatı çalışmaya dahil edilmiştir. Amplifikasyon ürünlerinin herbiri 3 farklı restriksiyon enzimi Hae III, Dde I ve Bfa I tarafından parçalanmıştır.* ***Sonuçlar:*** *Test edilen bütün izolatlar PCR ve RFLP analizi ile beklenen bant örneklerini vermiştir. Bu çalışmadam elde edilen sonuçlar Candida türlerinin sadece Hae III restriksiyon enzimi kullanılarak Candida albicans ve non-C. albicans suşları olarak ayırılabileceğini ve Bfa I'in ayırt edilen non- C. albicans türlerinin doğruladığını ortaya koymaktadır. Bu tanımlama özellikle fungemi vakalarında oldukça önemlidir.* ***Sonuç:*** *Candida türlerinin ITS bölgesinin amplifikasyonu ve RFLP analizi ile tanımlanması Candida türlerini tanımlamak için kullanılan zaman alıcı konvansiyonel yöntemlerle karşılaştırıldığında pratik, kısa ve güvenilir bir yöntemdir.*

Anahtar Kelimeler: *Candida, Restriksiyon Parça Uzunluk Polimorfizm Analizi.*

INTRODUCTION

Candida species are becoming increasingly important nosocomial pathogens especially in immunocompromised, intensive care and postoperative patients (1). *Candida albicans* ranks first among the identified species, but the proportion of non-*C. albicans* species seems to be increasing (2,3). Hence the accurate identification of Candida at a species level is becoming of increasing importance. The treatment of Candida infections is generally effective and usually involves the use of topical or systemic antifungal therapy with drugs such as the polyenes and the azoles (4). Azole antifungal agents have therapeutic activity against different

Candida species. Among the azole drugs, fluconazole shows satisfactory tolerance and efficiency. However, in recent years increasing resistance to fluconazole has been reported and antifungal drug resistance is becoming a major problem, especially in immunocompromised patients (5,6). This resistance also favors the emergence of *C. krusei* and *C. glabrata* (7,8).

Conventional methods for the identification of Candida species are based on assimilation, fermentation reactions and morphology (9,10). Given the many limitations of fractional typing techniques, techniques from the field of molecular biology have been adapted for use as molecular identification methods. Recent advances in the use of molecular DNA analysis have facilitated the development of identification systems at a species level (1,9-13). The identification of Candida species has been achieved by restriction fragment length polymorphism (RFLP) analysis of the ribosomal DNA (rDNA) (genes encoding rRNA) repeat of Candida species in previous studies (1-3,9,12,13).

The aim of this study is to identify Candida species by using genotyping methods like PCR and RFLP analysis.

MATERIALS AND METHODS

Isolates

A total of 44 Candida strains isolated from clinical samples, comprising 5 species were included in the study. Thirty-three of these isolates were from blood, 5 of from urine, 4 from the vagina, 1 from an abscess drainage and 1 from aspiration material. *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258, *C. albicans* ATCC 64551, *C. albicans* ATCC 64569, and *C. glabrata* ATCC 90030 reference strains were also included in the study. Clinical isolates were identified by using an ID32 C (API system; bioMérieux, Marcy l'Etoile, France) kit, according to their biochemical features. The identified species were *C. albicans*, *C. parapsilosis*, *C. krusei*, *C. glabrata*, *C. tropicalis* and *C. rugosa*.

DNA extraction

Fungi were subcultured on Sabouraud dextrose agar for 48 hours at 30 °C and then suspended in sterile 0.9% NaCl solution at a concentration of 10^6 colony-forming units (CFU)/mL (McFarland 0.5 corresponds to 10^6 cells). DNA was extracted as described previously with limited modifications (14). For the extraction of whole-cell DNA, yeast suspensions were incubated with lysis buffer containing 0.1 mg/mL proteinase K, 150 mM NaCl, 25 mM EDTA, 10 mM Tris-HCL (pH 8.0), and 0.5% SDS for 3 hours at 55 °C. After performing the phenol-chloroform extraction, the pellet was kept overnight at -20 °C in 95% ethanol with sodium acetate. The supernatant was discarded, and the pellet was allowed to dry. The DNA was then resuspended in 50 μλ of sterile water and 5 μλ of DNA was used for the amplification procedure.

PCR

DNA was amplified using the primers ITS1 (5'-TCC GTA GGT GAA CGT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3'), which are designed for the amplification of the intergenic spacer region (ITS) of ribosomal DNA (rDNA) as described previously (9).

Typing by RFLP

PCR products were digested individually with 10 U of restriction enzymes HaeIII, BfaI and DdeI (New England Biolabs, Hitchin, United Kingdom) by overnight incubation at 37 °C. The resulting restriction fragments were analyzed by agarose gel electrophoresis using 3% 3:1 NuSieve agarose (FMC BioProducts, Kent, UK) gels in 1x Tris-phosphate-EDTA buffer (7,9).

RESULTS

Thirty-four *C. albicans*, 3 *C. parapsilosis*, 2 *C. krusei*, 4 *C. glabrata*, 2 *C. tropicalis* and 1 *C. rugosa* strains were identified by the ID32 C kit. The intergenic spacer region was successfully amplified from all of the tested isolates, and a distinct product size was obtained for all isolates of a given species. All isolates yielding a product size of approximately 800 bp were identified as *C. glabrata* and an isolate with a product size of approximately 500 bp was identified as *C. rugosa*. These two Candida species were the only species that could be identified by the size of the PCR products. A product of approximately 520 bp was obtained from the remaining isolates.

These isolates were studied further by RFLP analysis following digestion of the PCR products

by the restriction enzymes HaeIII, BfaI and DdeI. The size of the fragments obtained from the products of the restriction enzymes are shown in Table 1. Fig. 1a shows a gel electrophoresis of the PCR products obtained from the Candida ATCC strains digested by the HaeIII restriction enzyme. Fig. 1b and 1c show PCR products obtained from Candida clinical isolates digested by the HaeIII restriction enzyme. Fig. 2a and 2b demonstrate the restriction digestion of PCR products with the enzyme DdeI, while Fig. 3 demonstrates products related to the digestion with the enzyme BfaI.

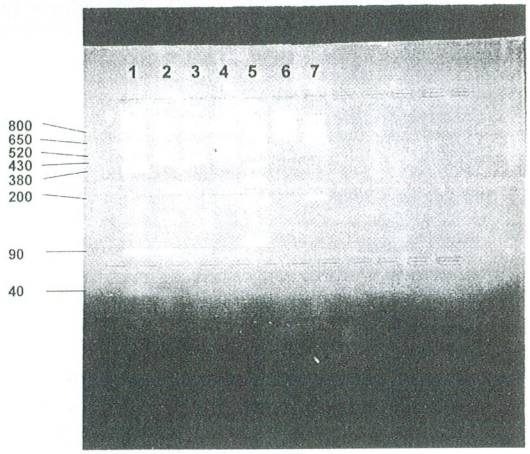

Fig. 1a: Restriction digestion of PCR products of Candida ATCC strains with the enzyme HaeIII.
1. *Candida krusei* ATCC 6258
2. *Candida albicans* ATCC 64551
3. *Candida albicans* ATCC 64569
4. *Candida krusei* ATCC 6258 (uncut)
5. Molecular weight marker (100-1000 bp)
6. *Candida glabrata* ATCC 90030 (uncut)
7. *Candida glabrata* ATCC 90030

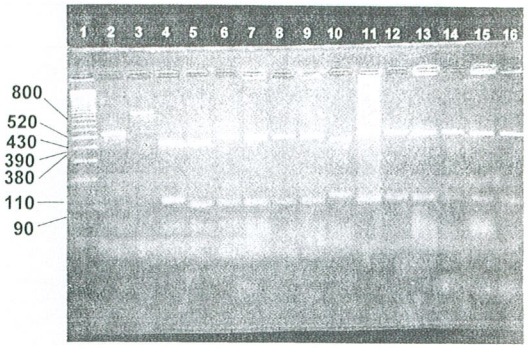

Fig. 1b: Restriction digestion of PCR products of Candida clinical isolates with the enzyme HaeIII.
1. Molecular weight marker (100-1000 bp)
2. *Candida albicans* (uncut)
3. *Candida glabrata* (uncut)
4. *Candida parapsilosis*
5. *Candida krusei*
6-9. *Candida albicans*
10. *Candida parapsilosis*
11-16. *Candida albicans*

differentiating enzyme. As *C. parapsilosis* and *C. krusei* had similar restriction products, they could not be differentiated by HaeIII alone. *C. krusei* could be differentiated by BfaI digestion. DdeI was used to confirm the *C. albicans* species. All of the *C. albicans* species yielded 100 and approximately 400 bp products with DdeI restriction.

DISCUSSION

The identification of pathogenic fungi has changed dramatically over the past decade through direct examination of the tremendous variation present in DNA (19). Typing methods based on phenotypic characteristics are usually cheap and easy to perform but they have shown a lack of reproducibility. However, genotypic

Table 1: Size of fragments obtained from the products of restriction enzymes.

	BfaI	**HaeIII**	**DdeI**
Candida albicans	-	90, 430	100, 420
Candida parapsilosis	-	40, 110, 390	-
Candida tropicalis	80, 360	80, 440	110, 410
Candida krusei	120, 200	40, 90, 380	-
Candida curvata	-	60, 150, 200, 450	90, 630
Candida glabrata	-	200, 650	-

All of the studied isolates were found to yield the expected band patterns excluding *C. tropicalis* and one strain of *C. krusei*. RFLP analysis of the PCR products of the isolates demonstrated that HaeIII was the most

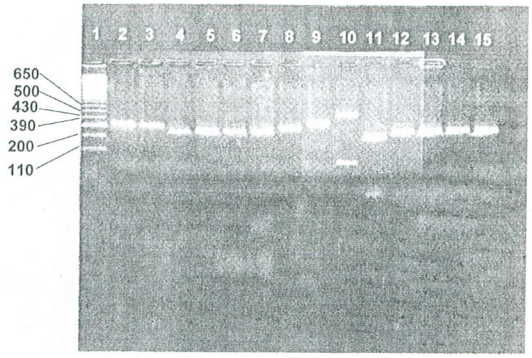

Fig. 1c: Restriction digestion of PCR products of Candida clinical isolates with the enzyme HaeIII.
1. Molecular weight marker (100-1000 bp)
2. *Candida albicans* (uncut)
3. *Candida krusei*
4-8. *Candida albicans*
9. *Candida rugosa* (uncut)
10. *Candida glabrata*
11. *Candida parapsilosis*
12-16. *Candida albicans*

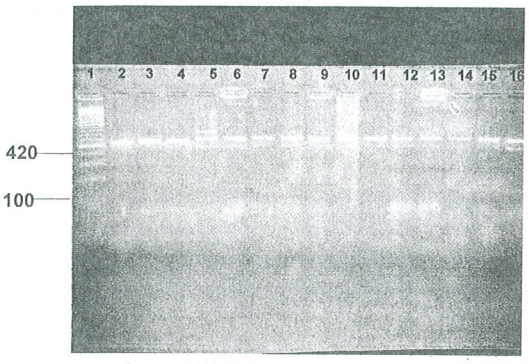

Fig. 2: Restriction digestion of PCR products of Candida clinical isolates with the enzyme HaeIII.
1. Molecular weight marker (100-1000 bp)
2. *Candida albicans*
3. *Candida parapsilosis*
4. *Candida krusei*
5-8. *Candida albicans*
9. *Candida glabrata*
10-16. *Candida albicans*

methods are expensive and require sophisticated technology, but they usually have good reproducibility (10).

Restriction enzyme analysis, called restriction fragment length polymorphism (RFLP), is based on the digestion of chromosomal DNA. Every organism possesses a

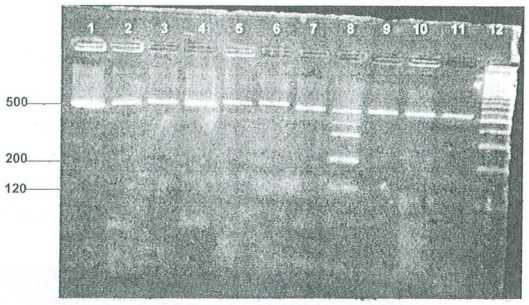

Fig. 3: Restriction digestion of PCR products of Candida clinical isolates with the enzyme BfaI.
1. *Candida parapsilosis*
2. *Candida glabrata*
3. 3-4. *Candida parapsilosis*, *Candida parapsilosis*
5-6. *Candida albicans*
7. *Candida rugosa*
8. *Candida krusei*
9-11. *Candida albicans*
12. Molecular weight marker (100-1000 bp)

unique nucleotide sequence that distinguishes it from every other organism on the basis of the number and size of the fragments. DNA is extracted from isolates and cleaved into fragments by restriction endonucleases; the fragments are then separated by gel electrophoresis (1,13). RFLP requires only a moderate amount of time and work, and the method has been used for genotyping a variety of pathogens within the last few years (13).

The RFLP method used in this study has been successfully applied for the exact identification of a variety of species. All the species examined by the RFLP method have proven to be reasonably homogeneous internally and quite distinct from each other. The differences in the restriction patterns for the rDNA regions of the various Candida species serve as a rapid means of differentiation among these organisms. A HaeIII digestion was definitive for distinguishing *C. albicans* species from other non-Candida albicans species. BfaI was found to be useful in the differentiation of *C. parapsilosis* and *C. krusei*. DdeI digestion also seemed to be an efficient way to identify *C. albicans* species.

Our identification of the samples, based on RFLP with HaeIII, BfaI and DdeI, was similar to

that of previous investigators (9). Williams et al. demonstrated that *C. glabrata, C. guilliermondii* and *C. pseudotropicalis* could be discriminated on the basis of PCR product size alone. The results of BfaI digestion were found to be very similar for the differentiation of *C. parapsilosis* and *C. krusei.* The DdeI restriction enzyme does not allow the identification of *C. albicans* species if necessary from *C. tropicalis* species, as mentioned before (9).

In this present study, the genotyping of Candida species has been performed by using RFLP analysis following the amplification of rDNA intergenic spacer region or Candida species. The results obtained from this study demonstrated that by using merely the restriction enzyme HaeIII the differentiation of *C. albicans* and non-*C. albicans* strains could be performed easily and further analysis by Bfa I restriction enzyme allowed the identification of *C. parapsilosis* and *C. krusei.* The analysis of RFLPs derived from the DNA of Candida species has the advantage of being easy, rapid and reliable when compared with phenotypic methods, which are insensitive, exhibit a lack of reproducibility and are hard to standardize.

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