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Use of PCR-RFLP and PCR-HWP1 for Identification of *Candia* Species Isolated from Cystic Fibrosis Patients

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Abstract

Background: Due to the predisposing conditions in patients with cystic fibrosis (CF) caused by defective mucociliary clearance facilitating colonization and invasion with *Candida* species has dramatically increased. Traditional methods for identifying problems are imminent and time-consuming. Therefore, molecular techniques utilizing amplification of target DNA provide quick and precise methods for the diagnosis and identification of *Candida* species. Therefore, the aim of the current study was to identify the most medically common isolated *Candida* species from the air way of CF patients by PCR-RFLP and amplification of *HWP1* gene.

Materials and Methods: A total of 42 CF patients presenting symptoms who referred to pediatric respiratory diseases research center were screened for the presence of *Candida* spp. The isolates initially were phenotypically identified and confirmed by molecular approaches based on restriction fragment length polymorphism (PCR-RFLP) for the discrimination of *C. albicans* of non-albicans and the amplification of *HWP1* gene for the discrimination of *C. albicans* from *C. dubliniensis* and *C. africana* was conducted.

Results: The results show that *C. albicans* was the most frequently isolated species (83.8%) followed by non-albicans included *C. parapsilosis* (7.1%), *C. glabrata* (3.2%), and *C. tropicalis* (3.2%). The restriction patterns of each *Candida* species were perfectly specific. Since *MspI* could not discriminate between the three morphological related species, *C. albicans, C. dubliniensis and C. africana*, we used PCR amplification of *HWP1* gene, which (7.1%) species from *C. albicans* identified as *C. dubliniensis*, however *C. africana* strains were not found.

Discussion: The present study found that *C. albicans* as predominant species wereisolated from the CF patients. It could be concluded that molecular diagnostic methods are reliable and would be useful for the identification of medically important *Candida* species in clinical samples. Therefore, considerable attention has been paid to the prevention and treatment of microbial growth, which has resulted in the improvement of patient management.

Keywords: *Candida* species, PCR-RFLP, *HWP1* gene, Cystic fibrosis

Introduction

Cystic fibrosis (CF) is an autosomal recessive disorder that causes abnormalities of ion transport of epithelial cells and presents as a multisystem disease (1, 2). The airways of CF patients are colonized by pathogenic micro-organisms and most patients experience recurrent acute respiratory episodes (3). Understanding the microbial flora of the CF respiratory tract is of considerable importance, as patient morbidity and death are primarily caused by chronic respiratory infections (4). Most studies of CF pathogens have focused on four major bacterial species. Also, both yeasts and filamentous fungi have been identified as microbial pathogens in CF (5, 6, 7). One particular fungal genus isolated at high frequencies from sputum culture is *Candida* and some studies have shown that airway colonization with *Candida* may cause symptoms in CF patients (8). The frequent use of broad-spectrum antibiotics, impairs salivary secretion, the use of corticosteroid treatments, and CF-related diabetes predisposes CF patients to colonization of the upper and lower airways with *Candida* spp. (9-11). It is still controversial whether *Candida* species are transient or persistent colonizers of the airways or more simply an oral carriage (12). Due to the predisposing conditions of *Candida* colonization in these patients, identification and discrimination of ethological agents for early treatment, and preventing the invasion is highly recommended. Therefore, due to the high degree of phenotypic similarity between *Candida* species, identification of the problems are imminent. Conventional approaches for identification down to the species level are based on morphological and physiological criteria, need several days or weeks to be concluded, and are frequently unspecific. However, today molecular tools are well established. Sequencing of the partial ribosomal operon is relatively expensive. Therefore, alternative molecular assay with high specificity, reproducibility and sensitivity are necessary. Thus, the objective of this study was to identify the most common *Candida* species isolated from CF patients using both the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay and amplification of *HWP1* gene.

Materials and Methods

Yeast isolates

From February 2012 through April, Forty-two CF patients were surveyed for colonization of their air way by *Candida* species. Sputum samples were collected from these patients during routine clinical visits or during an admission to the respiratory disease research center in Masih Daneshvari Hospital (Tehran, Iran). The specimens were inoculated onto Sabouraud dextrose agar containing antibiotics, and incubated at 37°C for 2 days. Yeast growth was semi quantitatively noted as none, light (<10 colonies), moderate (10–50 colonies), or heavy (>50 colonies). Primarily, these strains were identified by phenotypic methods such as colony color on CHROMagar *Candida* medium (CHROMagar Company, Paris, France), germ-tube tests in serum at 37 ºC for 2–3 h, microscopic morphology on corn-meal agar (DIFCO laboratories, Detroit, Mich., USA) with 1% tween 80. One colony on each identification strain was sub-cultured for molecular identification.

DNA Extraction

Genomic DNA was extracted, using the method of glass bead disruption (13). Briefly, 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. 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 pellets resuspended in 100 µL TE buffer (10 mM Tris, 1 mM EDTA) and were stored at -20°C prior to use.

RCR- RFLP analysis

The PCR-RFLP method was performed as previously described. Briefly, PCR amplification of ITS1-5.8S-ITS2 rDNA regions was achieved using the universal primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′ and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (MWG-Biotech AG, Germany). To amplify ITS domains, PCR amplification was performed in a final volume of 50 µl. Each reaction consists of 2 µl template DNA, 0.5 µl of each primer at 25 pmol, 1.25 µl of dNTP (BIORON GmbH, Germany) at 5 mM, 0.5U Taq DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany) and 5 µl 10× PCR buffer. The amplification parameters consist of 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min. In the first cycle, the denaturation step was 94°C for 5 min and in the final cycle the final extension step was 72°C for 7 min. Subsequently, PCR products were digested in a final reaction volume of 15µl containing 3 µl water, 1.5µl buffer, 1U of restriction enzyme *MspI* and 10 µl PCR product at 37˚C for 2h.

Amplified and digested products were visualized by 1.5% and 2% agarose gel electrophoresis in TBE buffer (0.09 M Tris, 0.09 M boric acid and 2 mM EDTA, pH 8.3), respectively, and stained with ethidium bromide (0.5 µg/ml) and photographed. The size of DNA fragments was determined directly with comparison of molecular size marker and distinct banding patterns which was demonstrated in similar studies.

PCR-HWP1 for Discrimination of C. albicans complex

Definitive species identification and discrimination of all members of the *C. albicans* species complex (*C. albicans, C. dubliniensis, C. africana, and C. stellatoidea*) was performed *via* the amplification of the hyphal wall protein 1 (*HWP1*) gene as described by Romeo and Criseo (14). PCR amplification of *HWP1* gene was achieved using the forward, 5′-GCTACCACTTCAGAATCATCATC-3′and reverse, 5′-GCACCTTCAGTCGTAGAGACG-3′ primer pairs. The method is also identified because it produces 3 different DNA fragments: approximately 700 bp for *C. africana*, 941 bp for *C. albicans*, and 569 for *C. dubliniensis.*

Sequence analysis of ITSr DNA region

Randomly for confirmation, the identity of the species sequenced. Sequence analysis of ITS1 and ITS2 regions of the rDNA was performed according to Lott *et al.* [17] through company MWG (Eurofins MWG Operon, Ebersberg, Germany) with a Big Dye Terminator Cycle Sequencing Kit on an ABI 3730 Genetic Analyzer (Pleasanton, CA, USA). The sequences generated were compared to the available data in the NCBI database using the Basic Local Alignment Search Tool (BLASTn) (http://www.ncbi.nlm.nih.gov/).

Results

After the digestion with *Msp*I enzyme, the results were evaluated based on the sizes of PCR products for *Candida* species, i.e. 297, 238 bp for *C. albicans*; 557, 314 bp for *C. glabrata*; 340, 184 bp for *C. tropicalis*; 261, 249 for *C. krusei*; 520 bp for *C. parapsilosis.* The ITS regions of all tested isolates were successfully amplified. The digestion of ITS region of *Candida* species by *Msp*I enzyme created bands for *C. albicans*, *C. glabrata*, and *C. tropicalis*. For *C. parapsilosis*, the size of the PCR and digestion product was similar (Figure 1).

Figure 1 demonstrates the patterns of ITS-RFLP for *Candida* strains after digestion with *Msp*I. As shown, the fragment lengths were exactly the same as the estimated sizes in the computational sequence analysis. Results show that *C. albicans* was the most frequently isolated species (83.8%) followed by non-*albicans* including *C. parapsilosis* (7.1%), *C. glabrata* (3.2%), and *C. tropicalis* (3.2%).

The restriction patterns of each *Candida* species were perfectly specific. Since *Msp*I is not able to discriminate between three morphological similar species, *C. albicans*, *C. dubliniensis and C. africana*, we used PCR amplification of *HWP1* gene, which (7.1%) species from *C. albicans* identified as *C. dubliniensis;* however *C. africana* strains were not found (Figure 2).

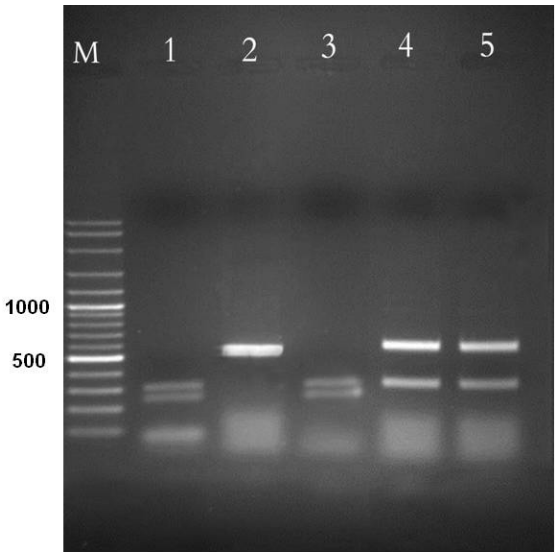

Fig1. Patterns of PCR products of *Candida* isolates after digestion by the restriction enzyme *Msp*I. Lanes of 1, 3 represent *C. albicans*; 2, *C. parapsilosis*; and 4, *C. glabrata*. Lane M is 100 bp ladder molecular size marker.

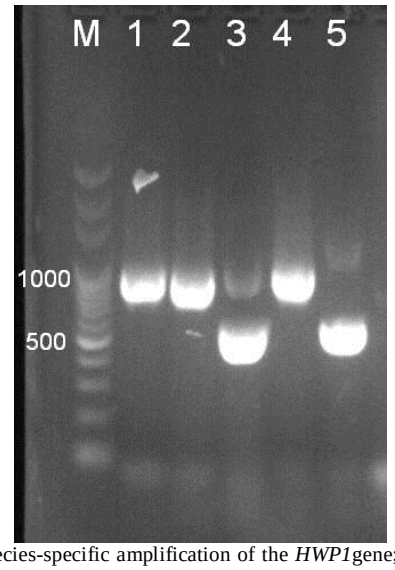

Fig 2. Species-specific amplification of the *HWP1*gene; Lane 1, 2 and 4 for *C. albicans*; Lane 3 and 5, for *C. dubliniensis*

Discussion

Although recently molecular approaches have been developed for the identification and discrimination of *Candida* species, novel techniques for the diagnosis based on species level have been established in routine laboratories. These tools are crucially required for the identification of *Candida* species to reduce morbidity and mortality, and for the treatment of patients suffering from candidiasis. Given the recent evidence that *Candida* species would be of clinical impact on respiratory function in patient with CF (15-17), we investigated to know the prevalence of *Candida* species in sputum samples from CF patients using the PCR-RFLP method.

Consistent with the results of previous studies, the overall *Candida* colonization rate was high in our study group (8, 11, 12). A striking result of this study is that *C. albicans* as predominant species were isolated from CF patients. Prior studies showed that *C. albicans* was the most frequent species in the CF patients (18, 19), although, it remains controversial as to whether *Candida* species are transient or persistent colonizers of the respiratory tract in CF (8). However, the regular assessment of airway colonization is one of the basic guidelines in clinical follow-up of these patients. Newer airway *Candida* species for example *C. dubliniensis* also emerged over the last decade (20). This new organism was subsequently described in the non-HIV population particularly in individuals receiving high antibiotic burdens such as CF patients (21-23). The present study found that 7.1% of *Candida* species were isolated from CF patients identified as *C. dubliniensis*. In this study, molecular analysis performed by amplification of the *HWP1* gene showed that 7.1% of *Candida* species isolated from CF patients identified as *C. dubliniensis*. Molecular diagnostic provide a rapid and frequently highly discriminatory means of identifying infectious organisms (24). The use of a PCR-RFLP method to identifying infectious organisms is a rapid, almost inexpensive and completely valid method for the identification of *Candida* spp. This method is useful for clinical and epidemiological investigation both mucocutaneous and systemic forms (25). In this study, we apply a PCR-RFLP method to identify the medically important *Candida* species and similar to other studies, the same results were obtained *via* PCR-RFLP methods in detecting different *Candida* spp. (24, 26, 27). In conclusion, in this study we found that CF patients are often colonized with *Candida* species and *C. albicans* as predominant species isolated from this patients. It can be concluded that molecular diagnostic methods are reliable and would be useful for the identification of medically important *Candida* species in clinical samples.

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Conflict of Interest

The authors declare that they have no conflict of interest in this article.

Authors' Contributions

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