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A PCR-RFLP Method to Identification of the Important Opportunistic Fungi: *Candida* Species, *Cryptococcus neoformans, Aspergillus famigatus* and *Fusarium solani*

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ABSTRACT

Deep-seated fungal infection present with non specific symptoms and involove a large number of different organisms. DNA-based technology offers for eariler detection of fungal pathogens and then earlier initiation of antifungal therapy. In this study universal primers common to almost all fungi were used to amplification of internal transcribe spacer 1 and 2 region. Subsequent restriction enzyme analysis of PCR products, using *HpaII* allows us to identify the most medically opportunistic important fungi: *Candida albicans*, *C. glabrata*, *C. tropicalis*, *C. kruzei, C. guilliermondi, Cryptococcus neoformans*, *Aspergillus fumigatus* and *Fusarium solani*, according to sizely different bands in polyacrilamid gel electrophoresis. It seems that this panel of PCR-RFLP could be a rapid and useful molecular approach in diagnostic studies of invasive opportunistic fungal infections.

INTRODUCTION

Invasive fungal diseases are increasing and significant cause of mortality and morbidity in the immunocompromised patients. Many immune system defects may cause increased risk for these opportunistic fungal infections, but the major predisposing factors are neutropenia and AIDS (9). Most important fungal pathogens in these settings are *Candida albicans* and some other candida species, *Cryptococcus neoformans, Aspergillus fumigatus, Rhizopus arhizus, Fusarium solani* and some other rare agents (1,2).

During the period 1980-90 Candida species emerged as the sixth most common nosocomial pathogens in a hospital-wide survey. Among the nosocomial blood stream infections, *Candida* species ranked fourth hospital-wide.*Candida spp.* accounted for 10.2% of all cases of septicemia and for 25% of all urinary tract infections in intensive care unit. The risk of Candidemia is especially high in patients with acute leukemia. In one tertiary care cancer hospital, the incidence was 2.9% with an associated mortality rate of 50% (20). Some *Candida* species including *C.glagrata* and *C.kusei* are emerging as pathogens because they are innately resistant to therapy with azole antifungal compounds.Thus rapid species-specific identification is necessary for time targeted therapy and to facilitate hospital infection control measures.

Invasive aspergillosis (IA) is second nosocomial fungal infection in immunocompromised patients, specially in those with severe granulocytopenia as a result of receiving a bone marrow transplant for the treatment of leukemia or those receiving therapy for the management of solid organ transplants or systemic corticosteroid therapy. Aspergillus species were isolated from 36% of 55 patients with nosocomial pneumonia in one bone marrow transplant unit (20).

Cyptococcus neoformans is the most frequent lifetreating fungal infection in patients with HIV infection that result in 10% mortality (19).

A number of other fungi including *Rhizopus orizus, Fusarium solani, Sacharomyces cerevisia, Trichosporon beigelii* and *Malassezia furfur* have also been recognized as pathogens in the immunocompromised host.

Earlier detection of infection permit prompt initiation of antifungal therapy with a greater likelihood for improved survival and reduced morbidity. Because of its ability to detect extremely small quantities of DNA, PCR technology offer potentialy earlier detection of fungal pathogens, allowing earlier initiation of antifungal therapy and perhaps improved chances of survival. PCR technology can directly detect the presence of fungi with high level of sensitivity and specificity (4,8,11,13).

Universal primers common to all fungi have been used as a promising approach for clinical microbiological diagnosis (5, 8, 10, 17, 21, 22). Various techniques have been reported to separate different fungi detected by universal primers, including restriction fragment length polymorphism (12, 15, 16, 18, 22), hybridization of the ampelicon with a specific probe (5, 7, 8, 14, 17), single-strand conformational polymorphism (SSCP) (21) and others.

In this study we present a PCR-restriction enzyme for recognition of the medically important opportunistic fungi including *C. albicans, C. tropicalis, C. glabrata, C. krusei, C. guilliermondii, Cryptococcus neoformans and Fusarium solani*

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using the universal primers: ITS1 and ITS4 to amplification the ITS1 and ITS2 region in the rDNA gene.
This panel can be an exact and simple method for identification of related infections directly from the clinical samples and indirectly after isolation of fungi from clinical specimens.

MATERIALS AND METHODS

Fungi Isolates

Candida species were isolated from patients with superficial and deep candidiasis. *Cryptococcus neoformans* was isolated from patients with cryptococcal meningitis. *Aspergillus fumigatus* was isolated from a patient with invasive pulmonary aspergillosis and *Fusarium solani* was prepared from a collection in Canada. All of the fungi studied except *Fusarium*were isolated from clinical specimens submitted to medical mycology laboratory, Faculty of public Health, Tehran University of Medical Sciences, Tehran, Iran,for suspected fungal infections. Yeasts were subcultured on YEPD broth (1% yeast extract, 2% peptone, 2% dextrose) and were incubated for 2 days at 30°c under shaking conditions (150 rpm). Moulds were sub-cultured on Sabouraud broth (1% peptone and 2% dextrose) and were incubated 3 days at 25°C under shaking conditions (150 rpm). All fungi were harvested by centrifuging in 5000 rpm and washed by sterile saline and freezed in -25°C until use.

DNA Extraction

200 microlitre of lyses buffer [10 mM EDTA (pH 8), 1% SDS, 100 mM NaCl, 2% Triton X-100], 200 microlitre of phenole-chlorophorm (1:1) solution and 200 microlitre of 0.5 mm diameter glass beads, were added to fungi pellet. After 5 minutes vigorous shaking and 5 minutes centrifugation in 10000 rpm, the supernatants were isolated and its DNA were precipitated by 0.1 volume sodium acetate (pH 5.2) and 2.5 volume cold absolute ethanol. After centrifugation for 10 minutes at 4°C and washing by 70% ethanol, the pellet resuspended in 100 microlitre TE buffer(10 mM tris, 1 mM EDTA pH 8) and stored at -20°C until using 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 microlitre, consisting of 10 mM Tris-HCI, 1.5 mM MgCl2, 50 mM KCI, 0.2 mM each dATP, dGTP, dCTP, and dTTP, 0.2 mM each primers (ITS1: 5´- TTC GTA GGT GAA CCT GCG G-3´ITS4: 5´-TCC TCC GCT TAT TGA TAT GC-3´and 5U of Taq DNA polymerase. Thirty cycles of amplification were preformed in thermal cicler (model Techne-Progene England). After initial denaturation of DNA at 95°c for 5 minute, 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 minutes following the last cycle. After amplification, the sample were stored at -20°C until used. Appropriate negative and positive controls were included in each test running.

Restriction Enzyme Analysis

ITS1-ITS4 sequences of various tested fungal species were derived from Gene Bank. On the basis of that sequences the restriction site of various restriction enzymes were determined by DNAsis software and the best enzyme was sellected. For restriction digestion, 25 microlitre of PCR products were digested directly and individually by 10U of the restriction enzyme *Hpa*II by 90 minutes incubation at 37°c.

Polyacrylamid Gel Electrophoresis

5 microlitre of restriction digestion product was electrophoresed for 120 min at 120 V in a vertical 10% polyacrylamid gel in TBE buffer (0.09 M tris-HCL, 0.09 M boric acid, 0.01 M EDTA (pH 8.4).

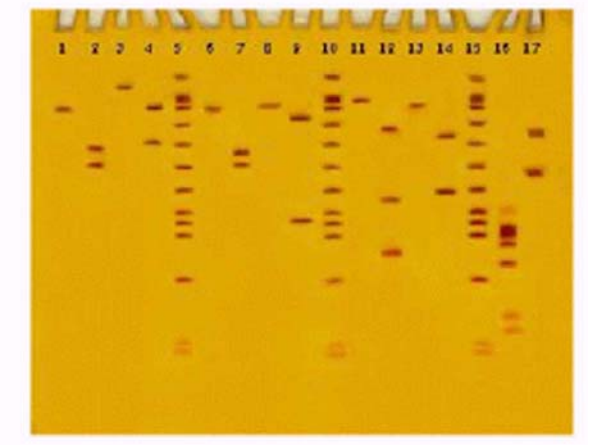
Silver Staining

The gel was rinsed in ethanol (10%) acetic acid (0.04%) solution for 6 min. After repeating this stage and twice washing in water, gel rinsed in silver nitrate (0.1%) for15 min. Then rinsed in 1 M NaOH, formalin (0.4%) solution until appearing the bands.

RESULTS

The intergenic spacer regions of all isolates tested were successfully amplified. The fungus-specific universal primer pairs, generate PCR products of 535, 871, 524, 510, 608, 535, 556, and 589, for *C. albicans, C. Glabrata, C. tropicalis, C. Krusei, Aspergillus fumigates, Cryptococcus neoformans* and *Fusarium solani,* respectively. On the basis of results of the application of some enzymes on ITS sequences of various fungi derived from the Gene Bank (Table 1), it was found that *Hpa*II is a suitable restriction enzyme for delineation of fungi under study. This enzyme produced 2 segments for each of *C. albicans, C. glabrata, C. tropicalis, C. Krusei, Cryptococcus neoformans* and *Fusarium solani,* 3 segments for *C. guilliermondii* and 6 segments for *Aspergillus fumigatus.*The enzyme had not any digestion site for *C. parapsilosis,* another important Candida species and so produced only one segment equal to undigested segment. The size of different bands of digested PCR product have been listed in Table 1.The polyacrylamid gel electrophoretic pattern of bands has been showed in Fig. 1 .

Fig.1. **Polyacrylamide gel electrophoresis of ITS PCR product before and after digestion by *Hpa*II. Bands are stained by silver nitrate**



5,10 and 15. marker number 8, 1 and 2. *C. albicans,*3 and 4, *C. glabrata,* 6 and 7. *C. Krusei,*8 and 9. *Cryptococcus neoformans*, 11 and 12. *C. guilliermondii,* 13 and 14. *C. tropicalis before and after digestion, respectively,*16. *Aspergillus fumigatus* after digestion, 17. *Fosarium solsni* after digestion.

DISCUSSION

Deep-seated mycoses often present with non-specific symptoms and involve a large number of different organisms. Therefore, an appropriate diagnostic procedure will require a universal primary step to fungus detection to differentiation of a fungus infection from other infectious diseases. This may be accomplished by PCR with universal fungal primers. A second general method should allow the identification of a causative agent, preferably to the specific level. This may be accomplished by some complementary method such as hybridization with probes specific for common fungi species such as *Candida* or *Aspergillus* ,or restriction digestion polymorphism. This proporsed molecular approach would overcome several limitations of classical identification techniques: 1) delayed production or lack of characteristic fruiting bodies or macroconidia; 2) lacking of the sexual reproduction cycle in many imperfect fungi; 3) special nutritional requirements of certain fungi; 4) similarity of micromorphology or macromorphology or both at the genus level; 5) Antigenic cross-reactivity between important species and genera and; 6) possibly hazarodous cultures for the health of laboratory personal (20). In addition problems originating in the availability of clinical samples and poor viability of fungal elements in smears or tissues may be overcome. As mentioned in the introduction, universal primers common to all fungi are suitable for detecting fungi in clinical samples. Coding regions of the 18s, 5.8s and 28s in fungal nuclear rRNA genes evolve slowly and are relatively conserved among different fungi(5). Between coding regions are the internal transcribed spacer 1 and 2 regions (ITS1 and ITS2) which evolve more rapidly and may therefore vary among different species within different genus or even within different species of a genus. On the other hand this gene is high-copy-number (40 to 80 repeat copies per haploid genome) so can be more sensitive than other single copy. Thus PCR amplification and consequential distinguishing may facilitate the identification of the fungi in the species level according to sequential polymorphism present in this region (1). For these reasons this gene was selected in this study as a universal region for amplification and restriction enzyme analysis for identifing of different medically important fungi. Many invsetigators have also used this region for identifying fungi. For example Henry and coworkers compared the ITS1 and ITS2 nucleotide sequences of clinicaly important Aspergillus species to identify them (10). ITS2 has been applied for identification of yeast specially *Candida* species in various methods such as, DNA sequence polymorphisms (1), Microtitiration plate enzyme immunoassay (8) and nested PCR (3,6). In another study, species identification of eight species of *Candida* was carried out on the basis of size and enzyme variation of rDNA intergenic spacer regions (22).

As it clear in the Fig. 1, and Table 1 the bands are sizely enough different to be distinguishable from each other and this help us for rapid and simple identification of fungi after amplifying their DNA and digesting the PCR products by restriction enzyme. Althogh we used polyacrylamide gel electrophoresis for showing small size bands in this study, but agaros gel electrophoresis also is applicable for separating the bands.

We concluded that although molecular approach for identification of medically important fungi is a little problematic here in Iran, but regarding to its sensitivity amd specificity, this method can be an suitable alternative method for identifying and diagnosing some important mycotic infections agents. We have plane to apply this method in a nested-PCR-RFLP system to detection and identification of major opportunistic fungal infections in animal model as well asreal human clinical samples.

Table 1. **Sizes of ITS1-ITS4 PCR products for 6 *Candida* species, *Aspergillus fumigatus*,*C.neoformans* and *F.solani* before and after digestion by the restriction enzyme *Hpa*I**

***Candida* species**	**Size of ITS1-ITS4**	**Size of restriction products**
C. albicans	535	297, 338
C. glabrata	871	557, 314
C. tropicalis	524	340, 184
C. krusei	510	261, 249
C. guilliermondii	608	371, 155, 82
C. parapsilosis	520	520
Cr. neoformans	556	428, 128
A. fumigatus	597	4, 19, 38, 43, 57, 94, 101, 108, 115
F. solani	526	

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