Invasive Fungal Infection in Renal Transplant Recipients Demonstrated by Panfungal Polymerase Chain Reaction

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Objectives: **Invasive fungal infections following renal transplant are associated with high morbidity and mortality rates. This study reports our experience using molecular assay to diagnose invasive fungal infections in renal graft recipients.**

Patients and Methods: **One hundred twenty patients who had undergone renal transplant at the Organ Transplant Unit of Nemazi Hospital in Shiraz, Iran, between September 2004 and January 2006 were followed up for fungal infections for 6 months following transplant. Blood samples were cultured by bedside inoculation to BACTEC fungal medium. Whole blood specimens were collected prospectively once per week and were evaluated for any invasive fungal infections using panfungal polymerase chain reaction and polymerase chain reaction–enzyme-linked immunosorbent assay. The female-to-male ratio was 44.2% to 55.8%, the mean age of the recipients was 34.7 years, and the mean length of hospitalization was 10.92 days.**

Results: **The sensitivity and specificity for proven and probable infections were 80% and 95.6%, respectively. Using panfungal polymerase chain reaction–enzyme-linked immunosorbent assay, 4 recipients were diagnosed as having invasive fungal infections. The etiologic agents were C. albicans in 3 patients, and C. albicans and A. fumigatus in 1 patient. The mean interval of polymerase chain reaction–enzyme-linked immunosorbent assay positivity in blood samples before clinical signs was 27 days (range, 7-60 days).**

Conclusions: **Polymerase chain reaction–enzyme-linked immunosorbent assay may improve early diagnosis of invasive fungal infections; however, correlating the results of polymerase chain reaction–enzyme-linked immunosorbent assay with clinical outcomes in renal transplant recipients will require further evaluation.**

Key words: *Opportunistic fungus disease, Early diagnosis, Mycoses, PCR*

Along with increased survival for patients undergoing renal transplant has come an increase in opportunistic fungal infections owing to the immunocompromised state of this population. Compared with viral and bacterial infections, these infections are associated with high morbidity and mortality rates despite their lower incidence. Delayed diagnosis of invasive fungal infections (IFIs) or delayed treatment in renal transplant recipients (RTRs) results in high mortality [1].

A high index of suspicion and close attention to clinical signs are required to diagnose fungal infections. Unfortunately, reliable diagnostic techniques are not available, and patient presentation is nonspecific. Successful treatment depends on 3 factors: early diagnosis; aggressive antifungal therapy; and a decrease in, or removal of, immunosuppressive drug therapies. The clinical microbiology laboratory plays a vital role in assisting the physician with the medical management of transplant patients with suspected or confirmed mycotic disease. Laboratory diagnosis of fungal infections in solid organ transplant recipients includes 3 approaches for isolating the organism: serodetection of the antibody or antigen and histopathologic evidence of invasion [2].

Fungi traditionally identified by morphologic and

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metabolic characteristics may require days or weeks to be isolated in cultures. Histopathologic diagnosis can be more rapid, but it is an invasive method and is rarely performed in a transplant unit. Serologic tests on a single serum sample to detect circulating antifungal antibodies also may be inconclusive [2]. The management of IFIs has been hampered by an inability to diagnose the infection during the early stages of the disease. To alleviate this, several groups have developed methods based on polymerase chain reaction (PCR) [3-6].

In this study, we used a PCR–enzyme-linked immunosorbent assay (PCR-ELISA) method to amplify and differentiate major fungal pathogens that cause infections in RTRs. This method allows amplification and specific identification of DNA from all fungi. Fungal DNA amplified with universal fungal primers (ITS^1, ITS^4) and a universal probe (ITS^3) is directed toward the rRNA gene [7]. This study reports our experience using panfungal PCR to diagnose IFIs in kidney graft recipients who were followed up at our transplant unit.

Patients and Methods

In this cross-sectional study, 139 patients who had undergone renal transplant at the Organ Transplant Unit of Nemazi Hospital in Shiraz, Iran, between September 2004 and January 2006 were followed up weekly for 6 months for any fungal infections. If a patient did not attend follow-up for more than 2 weeks, he or she was excluded from the study.

On admission to the hospital, all patients were evaluated for fungal colonization by providing swabs from the mouth, vagina (women), and rectum. Midstream urine samples were centrifuged for 10 minutes at 2500 rpm and then cultured on Sabouraud's dextrose agar (Merck, Darmstadt, Germany). Colonization was defined as the presence of fungus in 1 or more surveillance cultures in the absence of local or systemic symptoms or signs of infection [8]. Recipients received no antifungal prophylaxis. They received triple therapy consisting of prednisolone, cyclosporine A, and azathioprine. None of the patients received antilymphocytic therapy.

During follow-up, whenever the clinician suspected a fungal infection, laboratory tests were performed using the clinical samples that had been obtained (ie, urine, cerebrospinal fluid, pleural and abdominal fluid from tap, bronchoalveolar fluid from lavage, and sputum). All clinical samples (skin, nail, wound, urine, blood, sputum, biopsy) were examined for fungal infections by routine methods. Histologic analyses were performed when a biopsy specimen was provided. Blood samples were cultured by bedside inoculation to BACTEC fungal medium (Becton-Dickinson, Sparks, MD, USA). EDTA-anticoagulant whole blood specimens were collected prospectively once per week and stored at –20°C until the examination. Erythrocytes, leucocytes, and fungal cell walls were lysed according to Van Burik and associates [9]. For spheroplast lysis, protein precipitation, and elution of DNA, a QIAamp DNA Minikit (Qiagen, Hilden, Germany) was used in accordance with the manufacturer's recommendations. The optical densities of the DNA solutions were measured at 260 nm for DNA and 280 nm for proteins. Amplification and agarose gel electrophoresis were performed as previously described [9]. All primers and probes were synthesized by TIB MOLBIOL (Berlin, Germany) and are listed in Table 1.

To determine the sensitivity, and to limit the assay, for fungal pathogens in blood, suspensions of EDTA-anticoagulated blood samples with *Candida* (*C. albicans* and *C. tropicalis*) and *Aspergillus* (*A. flavus* and *A. fumigatus*) conidia (1-10^5 conidia/mL) were diluted and removed for DNA extraction and amplification. As human DNA can obscure detection of weak fungal

Table 1. Demographic characteristics of the subjects

Primer or probe	Nucleotide sequence (5' to 3')	Reference	Chemistry and location
Primers			
ITS1	TCC GTA GGT GAA CCT GC G G	10	18S rDNA universal fungal 5' primer
ITS4	TCC TCC GCT TAT TGA TAT GC	10	28S rDNA universal fungal 3' primer
Probes			
ITS3	GCA TCG ATG AAG AAC GCA GC	10	5'-end-labeled biotin probe universal probe
AFLA	GAA CGC AAA TCA ATC TTT	11	5'-end-labeled digoxigenin probe; ITS2 region of *A. flavus*
AFUM	CCG ACA CCC ATC TTT ATT	11	5'-end-labeled digoxigenin probe; ITS2 region of *A. fumigatus*
ANIG	GAC GTT ATC CAA CCA TTT	11	5'-end-labeled digoxigenin probe; ITS2 region of *A. niger*
ATER	GCA TTT ATT TGC AAC TTG	11	5'-end-labeled digoxigenin probe; ITS2 region of *A. terreus*
ASPEN-G	CCT CGA GCG TAT GGG GCT	11	5'-end-labeled digoxigenin probe; ITS2 region of *Aspergillus* and *Penicillium spp.*
CA	AT TGC TTG CGG CGG TAA CGT CC	12	5'-end-labeled digoxigenin probe; ITS region of *C. albicans*
CT	AA CGC TTA TTT TGC TAG TGG CC	12	5'-end-labeled digoxigenin probe; ITS region of *C. tropicalis*
CP	AC AAA CTC CAA AAC TTC TTC CA	12	5'-end-labeled digoxigenin probe; ITS region of *C. parapsilosis*
CK	GG CCC GAG CGA ACT AGA CTT TT	12	5'-end-labeled digoxigenin probe; ITS region of *C. krusei*

ITS, internal transcribed spacer, AFLA, *Aspergillus flavus*, AFUM, *Aspergillus fumigatus*, ANIG, *Aspergillus niger*, ATER, *Aspergillus terreus*, ASPEN-G, *Aspergillus universal probe*, CA, *Candida albicans*, CT, *Candida tropicalis*, CP, *Candida parapsilosis*, CK, *Candida krusei*.

DNA bands on agarose gels [9], and the sensitivity of PCR-ELISA is 10 to 100 times that of ethidium bromide for monitoring DNA in blood samples, PCR-ELISA was used to monitor the DNA in samples that had weak fungal DNA and to identify species that are pathogens in human beings. A commercial kit was used for PCR-ELISA (PCR ELISA [DIG Detection] Roche, Mannheim, Germany).

To identify the species of fungi, another oligonucleotide (an ITS[3] universal probe) was used as an internal capture probe in the PCR-ELISA. This probe was used in conjunction with the specific probes. This oligonucleotide was biotin labeled at the 5' end. PCR-ELISA was performed according to the manufacturer's instructions, and color changes were measured in an ultraviolet Max microplate reader (Multiskan Ascent ELISA, Thermo Labsystems, Vantaa, Finland) at 405 and 492 nm. All collected blood samples before and after the patients had been treated for fungal infections were assayed for presence of bands on ethidium bromide-stained gels, and hybridized with DNA probes specific for *C. albicans* and *Aspergillus spp.* Hybridization for non-*C. albicans* was performed only when bands were present on gel electrophoresis but hybridization was negative for *C. albicans* and *Aspergillus spp.* If hybridization with *Aspergillus spp.* was positive, hybridization with *A. flavus*, *A. fumigatus*, *A. terreus*, and *A. niger* was performed. IFIs were defined as "proven" or "probable" according to the criteria proposed by the European Organization on Research and Treatment in Cancer and the Mycoses Study Group, and Pappas and associates [13, 14]. Briefly, a proven IFI was defined as one of the following: 1 positive blood culture for *Candida* species or other pathogenic fungi, or a positive culture for pathogenic fungus from a specimen collected from a normally sterile site or biopsy. A probable IFI was defined as a clinical illness consistent with an IFI in the absence of other causes of sepsis, together with positive fungal cultures from 1 or more nonsterile sites , and supporting radiographic or other diagnostic methodologies but without histopathologic confirmation of disease [14]. Data were analyzed with the chi-square and Fisher exact tests to determine the prevalence, time of onset, infection rate, and mortality of IFIs in RTRs.

Results

Among 139 renal recipients transplanted between September 2004 and January 2006, 120 recipients (of 51 deceased- and 69 living-donor grafts) were followed up for 6 months; the remaining recipients (n=19) were lost to follow-up. The female-to-male ratio was 44.2% to 55.8%; the mean age of the recipients was 34.7 years (range, 4-73 years); and the mean length of hospitalization was 10.92 days (range, 5-37 days). Fifty-seven recipients had *Candida* colonization in different sites of their bodies before renal transplant. The lower limit of detection of this PCR assay was 2 colony-forming units/mL of whole blood. Blood samples from recipients were analyzed simultaneously by PCR-ELISA to assess the clinical application of the assay, and 9 recipients had positive results. Five recipients (11 samples) who neither developed clinical signs of IFIs nor had received empirical antifungal treatments had positive PCR-ELISA results for *C. albicans* (false positive). The sensitivity and specificity of panfungal PCR-ELISA in proven and probable recipients were 80% and 95.6%, respectively. Twenty-one samples from 4 recipients (3.3%) with positive PCR-ELISA results had IFIs according to the definitions described. Two patients with IFIs were probable; 2 patients with severe esophagitis were proven. The etiologic agent was *C. albicans* in 3 recipients and *C. albicans* and *A. fumigatus* in 1 recipient. Two recipients had signs of pneumonia (cough, dyspnea, abnormal results on chest radiograph, positive culture for fungi from sputum or bronchoalveolar lavage fluid) [15, 16, 17], and in 2 recipients, IFIs developed with esophagitis. Four recipients with IFIs had been colonized with *Candida spp.* before renal transplant. Antifungal treatment was begun when the cultures were determined to be positive by a clinician; however, 2 patients died despite antifungal therapy (Table 2). In patients

Table 2. Features of recipients with IFIs

Patient No.	Age (y)/sex	Time to PCR positivity (d)*	Time to clinical sign (d)*	Localization of infection	Diagnosis of IFI	Site of colonization	Causative agent	Outcome
1	58/F	7	20	Lung	Sputum/radiology[1]	Mouth/vagina	*C. albicans*	Remission
2	46/F	17	45	GI	Biopsy/endoscopy[2]	Mouth/vagina	*C. albicans*	Died
3	16/F	30	37	Lung	Sputum/radiology[1]	Mouth/vagina	*C. albicans*	Remission
4	62/F	15	75	GI	Biopsy/endoscopy[2]	Mouth/vagina	*C. albicans*	*A. fumigatus*

*After transplant
[1]Probable [2]Proven
GI, gastrointestinal; IFI, invasive fungal infection

treated with amphotericin B, if the treatment was successful, the results of a fungal PCR assay became negative after 14 days; if the treatment failed, the results of a PCR-ELISA were positive until the patient died.

Clinical signs in all patients developed within 2 months of the kidney transplant (mean, 48 days after transplant). The mean time to a positive result on PCR assay in blood before clinical signs were apparent was 27 days (range, 7-60 days). None of the patients with IFIs had a positive blood culture result. The mean age of patients with IFIs was 45.5 years, and the mean length of hospitalization was 27.5 days (range, 7-46 days). After surgery, 9 patients had cutaneous and mucocutaneous infections including tinea versicolor (4 patients), thrush (4 patients), and otomycosis (1 patient). The etiologic agents for cutaneous infections were *C. albicans*, *Malassezia furfur*, and *A. niger.* None of recipients with cutaneous and mucocutaneous infections had a positive PCR blood result.

Discussion

Despite ongoing refinements in immunosuppressive agents, graft preservation, and surgical techniques, fungal infections remain a significant cause of morbidity and mortality in organ transplant recipients [15, 18]. During the first 6 months following transplant, as the anatomic barrier of the mucosa changes and the immunosuppressive regimen is at its strongest, fungal infections caused by opportunistic fungi such as *Candida spp.* and *Aspergillus spp.* occur. We therefore followed-up recipients for 6 months, and if the recipients had signs of rejection or infection, they were followed-up until they were well. *Candida spp*. is a normal flora in the oropharynx and gastrointestinal tract in 30% to 60% of individuals [16, 19]. According to our study, 47.5% of RTRs and the 4 recipients with IFIs had been colonized with *Candida spp.*

Opportunistic fungi can cause infectious complications in 2% to 14% of kidney transplant recipients [1, 20]. At autopsy, fungal infections have been found in 26.1% of kidney transplant recipients [21]. The diagnosis of an IFI is often difficult because of the low sensitivity of a fungal culture from infected tissues. Diagnosis is often made only when the infection is widely disseminated and the chance of cure is slim, or the infection is unsuspected during life but found at the time of autopsy. An assay with high sensitivity is essential for detecting fungal pathogens in blood, particularly in recipients with hepatosplenic candidiasis and invasive aspergillosis, as these blood samples will rarely culture positively [22].

This study assessed the ability of panfungal PCR and PCR-ELISA targeted toward the DNA region encoding the RNA genes of the amplification products to detect and identify fungi from whole blood samples. As the sensitivity of plasma PCR is lower than that of PCR performed on a whole blood sample [23], whole blood from the recipients was used to extract the DNA.

PCR for early diagnosis of fungal infections in RTRs has not been assessed. Based on an analysis of 1205 serum samples from 167 patients, the sensitivity and specificity of the assay for proven and probable invasive aspergillosis were 63.6% and 89.7% respectively, when 2 consecutive positive results were considered significant [24]. Other studies have recognized PCR-ELISA as a useful diagnostic tool for detecting and identifying fungal infections [25, 26].

The prevalence rate for IFIs in kidney recipients is 1.4% to 14% according to Gallis and associates (1975), 3.5% according to Nampoory and associates (1996) and Martinez and associates (1994), 3.5% according to Dictar and associates (2000), and 4% according to Altiparmak and associates (2002) [15, 27, 28, 29, 30]. In the current study, 3.3% of our RTRs had positive results on PCR-ELISA testing, which is similar to earlier reports from other countries [15, 27, 28, 29, 30], with the difference being that the recipients in our study were checked by molecular assay. We found no other study that used molecular assay to diagnose IFIs in RTRs.

Altiparmak and associates (2002) reported that the lungs and central nervous system were the most frequently affected sites in renal transplant recipients, and the most common etiologic agent was *A. fumigatus* [15]. *Candida spp.* is the most common fungal pathogen among RTRs, and its manifestations of infection include pneumonitis, esophagitis, urinary tract infection, and line-related infections [31]. Among transplant recipients, infections due to *Aspergillus spp.* are less common than are candidal infections or infections from bacterial or viral pathogens, which typically occur in less than 6% of patients [32]. *C. albicans* was the most frequent etiologic agent in our study, and manifestations of its infection included pneumonitis and severe esophagitis.

It has been reported that intensive immunosuppression, long-term hospitalization, antibiotic consumption, and older age may be significant factors affecting the high incidence of fungal infections. Recipients with coexisting diabetes mellitus have been reported as being prone to fungal infections [1, 9, 22, 33]. In this study, similarly, we found that colonization of *Candida* in mucosal tissues of the body (mouth and vagina), length of hospita-

lization, and older age are influential in fungal infections. However, as the number of transplant recipients with IFIs was low, we did not find a significant correlation.

A priority of PCR to clinical and radiologic signs for fungal infections has been reported in other studies [24, 34]. Hibberd and associates (2000) have reported that invasive fungal infection precedes clinical evidence by a mean of 5.75 days (range, 0-14 days) in febrile neutropenic patients without a history of IFIs [34]. In our study, infections occurred at an average of 27 days (range, 7-60 days) before the onset of clinical signs. In transplant recipients, this interval may be correlated with a decrease in white blood cell count, human cytomegalovirus infection, and rejection. Recipients with any of these conditions exhibited clinical manifestations earlier than did other recipients. None of the recipients in our study with cutaneous and mucocutaneous infections had positive results of PCR testing in whole blood. The results of our study demonstrate the specificity of PCR-ELISA for detecting IFIs.

In conclusion, PCR-ELISA may improve the early diagnosis of IFIs. Correlating the results of this test with clinical outcomes in RTRs requires further evaluation.

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