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Study on invasive fungal infections in immunocompromised patients to present a suitable early diagnostic procedure

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Summary
Objectives: The incidence of invasive fungal infections has increased considerably in recent years. The aim of this study was to present a suitable early diagnostic procedure in immunocompromised patients, using a molecular assay.
Methods: From September 2005 to January 2007, 310 immunosuppressed patients were followed for fungal infections for a 6-month period. EDTA-anticoagulant whole blood specimens were collected prospectively once per week and stored at −20 °C until use in molecular assays.
Results: Molecular assays were positive in 55 (17.7%) patients. The etiologic agents were *Candida albicans* (67.3%), *Aspergillus flavus* (20.0%), *Aspergillus fumigatus* (7.3%), *Candida tropicalis* (3.6%), and *Candida krusei* (1.8%). The sensitivity, specificity, and positive and negative predictive values of PCR—ELISA with proven and probable invasive fungal infections were 84.6%, 92.7%, 75.3%, and 95.8%, respectively. The results showed that the mean clinical manifestation time was 38.96 days and the mean time of positivity of the molecular test (time of infection) was 17.69 days. A linear model for predicted infection and clinical manifestation time was found to be as follows: $Y = 11.64 + 1.147X$, $r^2 = 0.812$, where Y is the time at presentation of clinical signs and X is the time of infection (positive PCR—ELISA result).

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Conclusion: It may be concluded that the molecular assay would help in the diagnosis of invasive fungal infections at the early stage of infection, before clinical manifestations.

Introduction

The incidence of invasive fungal infections (IFIs) has increased considerably in recent years because of the increasing population with HIV infection, transplant recipients, cancer patients, and other individuals receiving immunosuppressive treatments. Among patients undergoing transplants or treatment for malignancies, more intensive regimens have resulted in more profound levels of immunosuppression for longer periods, and IFIs are more common in these patients. The prevalence of IFIs ranges from 5% to 50% in kidney and liver transplants,[1] and in neutropenic patients these infections are the major causes of morbidity and mortality.[2] *Candida spp* and *Aspergillus spp* are responsible for more than 80% of all fungal infections in solid organ transplant recipients.[1] Survival of patients from such life-threatening infections depends on early diagnosis, but clinical manifestations of IFIs are nonspecific, and laboratory methods are often unable to diagnose the infection in its early stages.

The laboratory diagnosis of invasive mycoses involves one or more of three methods, including isolation of the fungus by culture, serological detection of antibody, antigen, or metabolites from the fungus, and histopathological evidence of invasion.[3] Unfortunately, the diagnosis of IFIs remains difficult, and is sometimes only confirmed late, by biopsy; this late on, most patients are too ill to undergo a biopsy. Pathogenic fungi may require 2—3 weeks or longer in terms of growing times. The detection of galactomannan antigen in serum by enzyme immunoassay has been an important adjunct in the diagnosis of invasive aspergillosis. Although this test is promising as a screening tool, the effect on specificity of a newly introduced lower positive cut-off value is still under investigation.[4]

Various researchers have reported methods for the molecular identification of fungal infections.[5—9] A PCR assay for detection of fungal nucleic acids may be the optimal diagnostic approach because it offers the potential of: (1) being more sensitive than current culture-based methods, (2) encompassing multiple fungal genera, and (3) being applied to a variety of specimen types.[10]

The aim of this study was to present a suitable early diagnostic procedure in immunocompromised patients by molecular assay.

Materials and methods

This study was a cross-sectional investigation carried out with immunocompromised patients. Previous studies[1,11,12] have revealed the incidence of IFIs among solid organ recipients and neutropenic cases to be 5—50% (mean 25%). We conducted the study on 310 cases to raise the confidence interval to 95%.

From September 2005 to January 2007, recipients who underwent transplantation at the Organ Transplant Unit (kidney and renal transplants) and leukemia patients with neutropenia receiving chemotherapy at the Oncology Unit of Nemazi Hospital affiliated to Shiraz University of Medical Sciences in Southern Iran, were followed for fungal infections for at least a 6-month period.

Upon admission to the hospital, all patients were evaluated for any fungal colonization by providing swabs from the mouth and vaginal and rectal tissues. Midstream urine samples were centrifuged for 10 min at 2500 rpm and were then cultured on Sabouraud—dextrose agar (Merck, Darmstadt, Germany). Colonization was defined as the presence of fungus in one or more surveillance cultures in the absence of local or systemic symptoms or signs of infection.[13] Once the patient had received graft or chemotherapy, they were then entered into our study.

Liver transplant recipients received fluconazole (400 mg/day) for 21 days after transplantation, and other patients did not have any antifungal prophylaxis. We defined 'time of infection' as the time at which there was presence of fungal nucleic acid in the blood with no clinical signs. At this stage, the number of fungal organisms is very low and no spread, replacement, or affecting of other organs has occurred. We defined the 'clinical manifestation time' as the time when the fungal infection appears in the organs and damages them. Unfortunately, the existing anti-fungal drugs are not effective enough against the infection at this stage of disease. The infection time and time of clinical manifestation are measured in terms of days following the immunodeficiency condition, that is, when the patients underwent organ transplantation or chemotherapy.

During the follow-up, whenever the patients developed fever after 96 hours of antibiotic therapy, or presented radiological or mycological evidence of IFIs, the clinician suspected a fungal infection. Clinical samples (consisting of biopsy, urine, cerebrospinal fluid, pleural and abdominal tap, bronchoalveolar lavage, and sputum) from these patients were examined for fungal infection. All samples were cultured on Sabouraud—dextrose agar, and direct microscopic examination was also performed. Histological study was performed for patients when a biopsy specimen was taken by the clinician. Blood samples were cultured by bedside inoculation onto BACTEC medium (Becton—Dickinson, Sparks, MD, USA).

Four milliliters of ethylenediaminetetraacetic acid (EDTA)-anticoagulant whole blood specimens were collected prospectively once per week and stored at −20 °C for PCR—ELISA analysis. Erythrocytes, leukocytes, and fungal cell walls were lysed according to van Burik et al.[10] For spheroblast lyses, protein precipitation, and elution of DNA, QIAmp DNA minikits (Qiagen, Hilden, Germany) were used in accordance with the manufacturer's recommendations. The optical densities of DNA solutions were measured at 260 nm for DNA and 280 nm for proteins (Pharmacia Biotech, Ultroscopec 3000, UV/visible spectrophotometer, Cambridge, UK).

Amplification and agarose gel electrophoresis were performed as previously described.[10] All primers and probes

Table 1 Primer and probes used for identifying fungi by PCR—ELISA

Primer or probe	Nucleotide sequence (5′ to 3′)	Ref.	Chemistry and location
Primers			
ITS1	TCC GTA GGT GAA CCT GC G G	14	18S rDNA universal fungal 5′ primer
ITS4	TCC TCC GCT TAT TGA TAT GC	14	28S rDNA universal fungal 3′ primer
Probes			
ITS3	GCA TCG ATG AAG AAC GCA GC	14	5′-end-labeled biotin probe universal probe
AFLA	GAA CGC AAA TCA ATC TTT	15	5′-end-labeled digoxigenin probe; ITS2 region of *A. flavus*
AFUM	CCG ACA CCC ATC TTT ATT	15	5′-end-labeled digoxigenin probe; ITS2 region of *A. fumigatus*
ANIG	GAC GTT ATC CAA CCA TTT	15	5′-end-labeled digoxigenin probe; ITS2 region of *A. niger*
ATER	GCA TTT ATT TGC AAC TTG	15	5′-end-labeled digoxigenin probe; ITS2 region of *A. terreus*
ASPEN-G	CCT CGA GCG TAT GGG GCT	15	5′-end-labeled digoxigenin probe; ITS2 region of *Aspergillus* and *Penicillium spp*
CA	AT TGC TTG CGG CGG TAA CGT CC	16	5′-end-labeled digoxigenin probe; ITS region of *C. albicans*
CT	AA CGC TTA TTT TGC TAG TGG CC	16	5′-end-labeled digoxigenin probe; ITS region of *C. tropicalis*
CP	AC AAA CTC CAA AAC TTC TTC CA	16	5′-end-labeled digoxigenin probe; ITS region of *C. parapsilosis*
CK	GG CCC GAG CGA ACT AGA CTT TT	16	5′-end-labeled digoxigenin probe; ITS region of *C. krusei*

PCR—ELISA, polymerase chain reaction—enzyme-linked immunosorbent assay; 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*.

were synthesized by TIB MOLBIOL (Berlin, Germany) and are listed in Table 1.[14,15,16]

To determine the sensitivity and to limit the assay to fungal pathogens in the blood, suspensions of EDTA-anticoagulated blood samples with Candida *(Candida albicans* and *Candida tropicalis*) and Aspergillus *(Aspergillus flavus* and *Aspergillus fumigatus)* conidia ($1—10^5$ conidia/ml) were diluted and removed for DNA extraction and amplification.[17]

Human DNA can obscure the detection of weak fungal DNA bands on agarose gels,[9] and the sensitivity of PCR—ELISA is 10—100 times that of ethidium bromide for monitoring DNA in blood samples. The collection of blood samples was performed on a weekly basis, during the first few weeks of infection, whenever the result of ethidium bromide for monitoring DNA was weak; this method was used for monitoring of DNA in samples with weak fungal DNA and for identification of species that are pathogens in humans.

To identify species of pathogenic fungi, another oligonucleotide (ITS3 universal probe) was used with specific probes of fungi as internal capture probes in the PCR—ELISA. This oligonucleotide was biotin labeled at the 5′ end. The PCR—ELISA was performed according to the manufacturer (PCR—ELISA DIG Detection Roche, Mannheim, Germany).

IFIs were defined as 'proven' or 'probable' as per the criteria proposed by the European Organization on Research and Treatment in Cancer and the Mycoses Study Group (EORTC/MSG).[18] The date of a proven diagnosis was the day on which the positive histopathological sample was obtained. The date of a probable diagnosis was defined as the day on which the sample that yielded fungi in culture was obtained. The date of a possible diagnosis was defined as the day of onset of clinical symptoms. PCR—ELISA precocity in diagnosing Systemic Candidiasis (SC) was assessed in comparison with the timing of the clinical suspicion of infection, the results of computed tomography (CT), and histological and microbiological criteria as defined by the EORTC/MSG.[18,19]

Following examination of all of the collected weekly blood samples from the 310 patients (7380 samples), patients who neither developed clinical signs of IFIs nor received empirical antifungal therapy as proposed by EORTC/MSG were taken to be 'controls'; the remainder were our 'subjects'.

The algorithm for the diagnosis of IFIs is described in Einsele et al.[9] Data were analyzed using the Chi-square test, post-hoc test, paired *t*-test, and regression stepwise test.

Results

Of the 409 patients who were admitted to the hospital between September 2005 and January 2007, 310 (120 renal recipients, 48 liver recipients, and 142 leukemic patients) entered our study and were followed for 6 months. The remaining 99 subjects were excluded, due to their failure

Table 2 Sites of body colonized with *Candida spp*

Site of isolate	Kidney recipients	
	Frequency	(%)
Mouth	109	35.2
Vagina	8	2.6
Urine	3	0.9
Mouth/vagina	29	9.4
Mouth/rectal swab	18	5.8
Mouth/urine	1	0.3
Mouth/vagina/rectum	10	3.2
Without colonization	132	42.6
Total	310	100.0

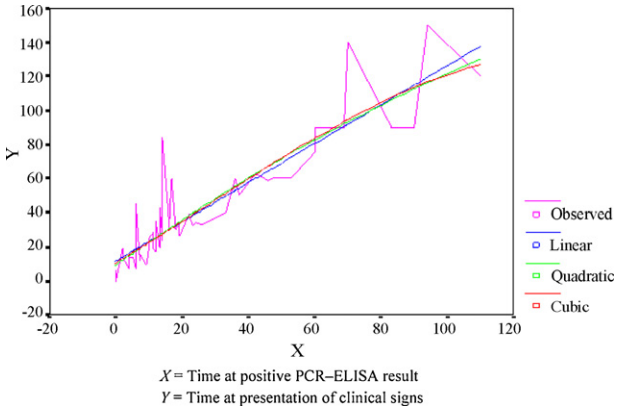

Figure 1 Relationship between time of infection and time of clinical signs by linear, cubic, and quadratic model.

in attending for the 6 months of follow-up (any patient who did not attend for the follow-up was excluded from the study). The female-to-male ratio was 120:190. The mean age of recipients was 33.6 years (range 3–73 years), mean hospitalization time was 23.8 days (range 14–150 days), and 178 patients (57.4%) had Candida colonization in different sites of their bodies on entering the study (Table 2). There was significant correlation between hospitalization time and IFIs. Also, we noticed a significant correlation between fungal colonization and IFIs ($p < 0.001$).

Clinical samples were examined for fungal infections by routine methods (direct examination and fungal culture), and 33 (10.6%) patients had positive cultures for fungal infections. Isolated fungi were *C. albicans* (57.6%), *A. flavus* (24.2%), *A. fumigatus* (6.1%), Mucor (3.0%), and *Alternaria spp* (9.1%). Five patients with IFIs had a positive blood culture result for *C. albicans*.

The lower limit of detection of the PCR assay was 2 cfu/ml of whole blood. For sensitivity and specificity of molecular assays, blood samples from the patients who neither developed clinical signs of IFIs nor received empirical antifungal treatment were examined, and 18 patients had positive PCR results for *Candida spp* and *Aspergillus spp* (false-positive test results). The sensitivity, specificity, and positive and negative predictive values of PCR–ELISA in our study with proven and probable IFIs were 84.6%, 92.7%, 75.3%, and 95.8%, respectively.

Molecular assays (panfungal PCR and PCR–ELISA) were positive in 55 (17.7%) patients. Fifteen patients had proven IFIs (histopathological evidence of severe esophagitis, invasive sinusitis, and central nervous system infection), 18 patients were probable (pneumonia with positive culture from bronchoalveolar lavage and bronchial washing with clinical and radiological criteria, abdominal magnetic resonance imaging showing abscess in the liver, positive culture from clinical specimens and related clinical signs), and 22 patients were possible (with clinical signs of infection), as per EORTC/MSG. The etiologic agents found with the molecular assay were *C. albicans* (67.3%), *A. flavus* (20.0%), *A. fumigatus* (7.3%), *C. tropicalis* (3.6%), and *Candida krusei* (1.8%). All patients with a positive fungal culture were also positive by PCR, except for one patient who was found with Mucor on histopathological examination.

Of 310 patients, 54 died; of the patients who died, 35 patients had IFIs. As our statistical analysis shows, there is a significant correlation between IFIs and patient death ($p < 0.0001$). Patients who were on fluconazole prophylaxis exhibited symptoms later than those who were not.

The relationship between the time of clinical manifestation and time of positivity of molecular test (as previously defined) was evaluated using the paired *t*-test. Results showed that the mean clinical manifestation time was 38.96 days (standard deviation (SD) 33.44) and the mean time of positivity of molecular test (time of infection) was 17.69 days (SD 17.18). The difference is significant ($p < 0.001$), and the mean difference was 21.27 days with an SD of 28.12, d.f. = 5, and $t = 5.45$.

To investigate the influential factor in the time of appearance of the infection, regression stepwise analysis was used. This study shows that providing all conditions are constant, cases with fungal colonization exhibited clinical signs 5.7 days earlier than non-colonized patients. Longer hospitalization resulted in earlier clinical manifestation, while use of prophylactic anti-fungal drugs did not prevent the manifestation of clinical signs and just delayed them. Investigation of the organs revealed that cases with lung and sinus infections exhibited clinical signs at the same time after positivity of PCR–ELISA, whereas, those with gastrointestinal infections showed the signs later.

Linear, quadratic, and cubic models for predicted infection time and clinical manifestation time are shown in Figure 1 and as follows:

$$\text{Linear model}: Y = 11.64 + 1.147X, \quad r^2 = 0.812,$$

$$\text{Quadratic model}: Y = 9.17 + 1.37X - 0.0025X^2, \quad r^2 = 0.815,$$

$$\text{Cubic model}: Y = 10.56 + 1.17X + 0.003X^2 - 4 \times 10^{-5}X^3, r^2 = 0.816,$$

where X = time of infection and Y = time of clinical signs.

In patients who were treated with anti-fungal agents, if the treatment was successful, the fungal PCR assay became negative after 14 days, and if the treatment failed, PCR–ELISA was positive until death.

Table 3 Predicted time for the manifestation of clinical signs after positive PCR—ELISA

	X	1	7	14	21	28
Y						
10—17		*				
18—25			*			
26—33				*		
34—41					*	
42—49						*

X = time at positive PCR—ELISA; *Y* = time at presentation with clinical signs.
*Positive PCR—ELISA on *X* and clinical manifestation times on *Y* as calculated by linear model.

Discussion

IFIs remain a significant cause of morbidity and mortality in immunocompromised patients. The most important parameter in determining the success of therapy for IFIs in these patients is the rapidity with which the diagnosis is made and treatment is initiated.

In immunocompromised patients, colonization of mucosal surfaces by endogenous *Candida* species is often followed by invasion of the vascular space, which carries a high risk of disseminated candidiasis. Candida colonization was observed in 57.4% of the total patients and 90% of the PCR-positive patients in this study. Preparing cultures of patient clinical samples is not a sensitive method for proper diagnosis, and in some infections such as chronic disseminated candidiasis (hepatosplenic candidiasis) supporting microbiological criteria are not required for the 'probable' category.[18] In this study, blood culture was positive in five patients. Detection of fungemia by means of fungal blood cultures is notoriously difficult and IFIs are diagnosed only at autopsy. However, it is possible that naked DNA be detected by PCR due to the presence of dead and degrading fungi within circulating phagocytes.[20]

In this study whole blood was used for DNA extraction from patients. The use of EDTA-whole blood samples allows for the detection of circulating conidia, spores, and hyphal fragments that have found their way into the blood stream.[10] As the volumes of serum used for DNA extraction tend to be smaller than the high volumes of whole blood, the sensitivity of the assay due to the lower fungal yield may decrease,[9] and the sensitivity of PCR in plasma is lower than that of the PCR performed on a whole blood sample.[21]

Morace et al. reported that molecular methods are significantly more sensitive than conventional fungal blood cultures and have a high negative predictive value (97.5%) for the development of disseminated candidiasis in neutropenic patients.[22]

Florent et al., on the basis of the analysis of 1205 serum samples from 167 patients, found that the sensitivity, specificity, and positive and negative predictive values of the PCR—ELISA for proven and probable invasive aspergillosis cases were 63.6%, 89.7%, 63.6%, and 89.7%, respectively, when samples with two consecutive positive results were used.[19] Other studies have recognized PCR—ELISA as a useful diagnostic tool for the detection and identification of fungal infections.[23,24] Comparing the results of this study with previously reported ones,[22—24] we found that the PCR—ELISA sensitivity and specificity values are within the same range.

Unlike in other studies[19,9,25] that reported that if a person had Candida colonization, molecular assay is not positive, in our study there were 10 patients with Candida colonization who had positive PCR—ELISA for *C. albicans*, and in follow-up they did not exhibit any clinical signs. This difference may be due to transient candidiasis in these patients or the very limited number of patients in previous studies.

The priority use of PCR over that of clinical and radiological signs has also been reported in other studies.[9,26] Hebart et al. reported PCR as the earliest indicator of invasive fungal infections, preceding clinical evidence with a mean of 5.75 days (range 0—14) in febrile neutropenic patients.[26] Florent et al. reported that PCR—ELISA positivity preceded mycological/histological diagnosis in eight patients (a total of 12 patients who had mycological/histological diagnoses) over a range of 1—110 days and preceded abnormalities on CT scan in 19 patients over a range of 1—84 days.[19] In our study, PCR-positive results were obtained within 21.27 days prior to the manifestation of clinical signs. This gap may be related to the different types of immunosuppressive drugs used, number of white blood cells (WBC count), and human cytomegalovirus infection. It is important to note that recovery from fungal infections is often primarily related to an improvement in the patient's neutrophils.

In this study, 63.6% of patients with IFIs died without any autopsy. We did not know if the patient deaths were due only to the progression of their background diseases or to the fungal infections, but we believe that if diagnosis and treatment are carried out as early as possible, the number of deaths will fall. In patients treated with antifungal therapy, the fungal PCR assay became negative after 14 days if the treatment was successful, and PCR—ELISA was positive until death if treatment failed.

To predict the manifestation of clinical signs by a linear model, we can draw a table (Table 3). According to this table if, for example, PCR was positive on day 1, clinical signs would appear within 10—17 days, or if PCR was positive on day 7, clinical signs would appear within 18—25 days. Since patients do not respond to treatment following a late diagnosis of fungal infection, and as even in some cases they have died, the need for an effective procedure for early diagnosis is urgent. This has been the goal of our study, which may make a contribution to the development of more efficient methods for the early diagnosis of IFIs. Further investigation into this matter is warranted.

Conclusions

It may be concluded that molecular assays could help to diagnose IFIs in the early stages of infection, preceding clinical manifestation. Immunocompromised patients at high risk should be checked weekly by this method for the early diagnosis of fungal infections. This method can also provide greater confidence in excluding a diagnosis of IFIs when negative results are obtained, and this method could be used for the monitoring of treatment, as well.

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Conflict of interest: No conflict of interest to declare.

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