Early Diagnosis of Systemic Candidiasis in Bone Marrow Transplant Recipients

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Abstract

Objectives: **Systemic candidiasis, are common infections during the neutropenic phase. The aim of this study was to identify quantitative *Candida* species ribosomal DNA using TaqMan technology for diagnosing candidiasis and monitoring them during hospitalization.**

Materials and Methods: **During the prospective, cross-sectional study, from September 2006 to September 2007, a total of 375 clinical blood specimens were collected from 35 patients with hematologic disorders once a week pretransplant and posttransplant. Patients were evaluated for systemic candidiasis during hospitalization. Cultures from the throat, urine, feces, and sputum, along with sonography and computerized tomographic scans, were done when patients were febrile and not having a response to antibiotics. All samples were cultured on Sabouraud dextrose agar with chloramphenicol, and direct, microscopic examination was performed. Blood samples were cultured by bedside inoculation into BACTEC medium at 35°C for 7 days. Clinical blood specimens were evaluated for *Candida* infections using the TaqMan-based PCR assay.**

Results: **Of the 35 recipients, 6 had multiple samples that were TaqMan-positive with *Candida* species probe, 3 had 1 PCR positive-result in their blood samples, and the 26 recipients showed negative results. Fungal rDNA was found in 2 patients before and after transplant. All 6 patients with systemic candidiasis had microbiologic and/or radiologic evidence of *Candida* infections.**

Conclusions: **It seems that TaqMan-based PCR assay can serve as an accurate method for diagnosing and monitoring *Candida* infections. This is the first report of its kind that shows *Candida* infections can be present in the blood of the bone marrow transplant candidates, so closer observation of the recipients who are neutropenic and receive immunosuppressive drugs seems warranted to improve their chances for survival.**

Key words: *TaqMan-PCR, Candida infections, neutropenia, Candida albicans, fungal DNA*

Among patients undergoing transplants or treatment for malignancies, more-intensive regimens have resulted in more profound levels of immunosuppression, for longer periods, and invasive fungal infections are more common in such patients. In neutropenic patients, these infections are the major causes of morbidity and mortality (1). *Candida (C) albicans* (60%) and *Candida* species (20%) are responsible for most fungal infections (2, 3). Given the rapidly fatal course of candidiasis, there is a need for improved methods for early diagnosis and subsequent initiation of antifungal therapy to have a significant impact on the death rates (2, 4). Current recommendations suggest that invasive fungal infections (eg, candidiasis and aspergillosis), should be treated empirically, because current methods of diagnoses are time-consuming and difficult to do (5, 6).

Traditional methods of diagnosis, including blood culture and biopsy, usually lack both sensitivity and specificity, or become positive late in the course of the infection because the culture regularly requires an incubation of 2 to 4 weeks before a definitive

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result. Blood culture systems may fail to detect 45% to 75% of the cases of disseminated candidiasis (7, 8); as a result, new nonculture-based methods are being developed. Molecular characterization of fungal DNA has been used to detect and differentiate fungi (9), which are faster, more stable, and less dependent on external factors than are morphologic methods (10-12). These assays, based on in vitro amplification and detection of fungal DNA, have been developed because of their high specificity and sensitivity. Real-time PCR assays dramatically decrease the risk of false-positive results, for the PCR and detection systems are coupled and conducted in a closed system, with no required, laborious, post-PCR analyses. A few of exonuclease-based TaqMan PCR assays can rapidly identify and specify *Candida* species (12).

This study sought to identify quantitative *Candida* DNA by real-time PCR assay in the blood samples of bone marrow transplant recipients for early diagnosis of *Candida* species infections, and monitor them during hospitalization.

Patients and Methods

During the prospective, cross-sectional study, from September 2006 to September 2007, a total of 375 clinical blood specimens were collected from 35 patients with hematologic disorders once a week pretransplant and posttransplant. Patients were evaluated for systemic candidiasis during their hospitalizations in Nemazi Hospital, affiliated with Shiraz University of Medical Sciences, Shiraz, Southern Iran. Patients received fluconazole as prophylaxis. Amphotericin B was added to the medications of those who were febrile and unresponsive to the administered antibacterials.

Cultures from the throat, urine, feces, and sputum, along with sonography and computerized tomographic scans, were done when patients were febrile and unresponsive to antibiotics. Broncho alveolar lavage or biopsy was not done, because patients had thrombocytopenic criteria. All samples were cultured on Sabouraud Dextrose Agar (Merck, Darmstadt, Germany) with chloramphenicol and direct microscopic examination was performed. Blood samples were cultured by bedside inoculation into BACTEC medium at 35°C for 7 days (Becton-Dickinson, Sparks, MD, USA).

Ethylenediaminetetraacetic acid (EDTA) anti-coagulant whole blood samples were collected prospectively once a week and stored at -20°C until examination. Erythrocytes, leucocytes, and fungal cell walls were lysed according to van Burik and associates (13). For spheroblast lyses, protein precipitation, and elution of DNA, QIAmp DNA Minikit (Qiagen, Hilden, Germany) was used in accord with the manufacturer's recommendations.

Attempts were made according to the established criteria to avoid contamination. Fungal amplification primers and the fluorogenic probes designed by Shin and associates (14) for all *Candida spp.* and *C. albicans, C. tropicalis, C. parapsilosis, C. glabrata,* and *C. krusei*. The primers and TaqMan probe were obtained from Metabion (Martinsried, Deutschland). DNA from *C. albicans* isolate (diagnosed with API) (API 32C [BioMerieux, France]) was amplified with both primers and inserted into pcr2.1 TA Cloning vector (Invitrogen Corporation, Carlsbad, CA, USA) was used according to the manufacturer's procedures; this plasmid was used as the standard. Quantification of *C. albicans* DNA was done with a serially diluted standard, in the range of 10^1 to 10^8 copies/well.

DNA and standard samples were analyzed using the Gene Amp 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA) with TaqMan universal PCR master mix (Roche, Branchburg, New Jersey, USA) and 0.2 μmol/L of each primer and 0.2 μmol/L of *Candida* species probe. Thermal cycling conditions consisted of heating at 94°C for 10 minutes, which preceded a 2-stage temperature profile of 30 seconds at 95°C, and 1 minute and 30 seconds at 60°C for 40 cycles (15). The threshold cycle valve was defined as the PCR cycle number at that point. Negative controls were tested using the same PCR reaction mixture under the amplification conditions described above without a template DNA but with distilled water. To determine the sensitivity of real-time, automated PCR for the detection of *C. albicans*, 100 copies/well of standard DNA were serially diluted with DNA and RNA free water and measured. European Organization on Research and Treatment in Cancer and the Mycoses Study Group (EORTC/MSG) guidelines were used to characterize the patients (16). Those who developed fever with no signs of fungal infections were classified as the controls.

Statistical analyses were performed with SPSS software for Windows (Statistical Product and Service Solutions, version 15.0, SSPS Inc, Chicago, IL, USA), and were subsequently analyzed using

Table 1. Characteristics of patients with positive PCR

Age/ year	Sex	Hospital stay/days	Primary disease	Diagnosis criteria	Time of clinic sign	Cloning	Type transplant	Maximum levels copies/mL	Positive PCR result on days	Out come
26	F	100	CML	C/ R/ M	+14	+	Allo graft	107	+5	Died
39	F	53	AML	C / R/ M	-2	+	Allo graft	103	-7	Survived
9	M	120	Coolys	C / R	+30	+	Allo graft	104	+8	Died
8	M	240	Coolys	C/ R/ M	+7	-	Allo graft	107	-3	Died
16	F	60	AML	C / R	+31	-	Autograft	103	+14	Survived
17	F	84	A. A.	C / R/ M	+36	-	Allo graft	104	+20	Survived

Abbreviations: AA, aplastic anemia; AML, acute myelocytic leukemia; C, clinical; CML, chronic myelocytic leukemia; M, microbiologic; MM, multiple myeloma; R, radiologic.
Positive numbers indicate days after transplant, and negative ones days before transplant

descriptive and cross-tabulation statistics. The Ethics Committee of the Clinical Microbiology Research Center at Shiraz University of Medical Sciences has reviewed and approved the study, noting the patients written, informed consent was provided before the study. The study protocol conforms to the ethical guidelines of the 1975 Helsinki Declaration.

Results

Thirty-five patients with hematologic disorders (bone marrow transplant candidates) were admitted to Nemazi Hospital, affiliated with Shiraz University of Medical Sciences, Shiraz, Iran. The female-to-male ratio was 12:23 (mean age, 22 years; range, 5-54 years), and the mean hospitalization was 70 days (29-207 days). Thirteen patients (37.1%) had thalassemia major followed by acute myelocytic leukemia (17.1%), chronic myelocytic leukemia (11.4%), aplastic anemia (8.6%), multiple myeloma (8.6%), acute lymphocytic leukemia (5.7%), and other hematologic disorders (11.5%).

Copy numbers of *Candida* DNA and threshold cycle values of the serial diluted standard, as well as the standard curve, are shown in Figures 1 and 2. The sensitivity of real-time PCR as a quantitative assay was 10 copies/well.

Of the 375 samples obtained from the 35 bone marrow transplant recipients, 50 were collected before bone marrow transplant and upon admission to the ward, and the rest (325) were prepared after transplant once a week. Total blood samples collected from 35 patients (375 samples) were examined, and those with no definite systemic candidiasis served as controls. Of the 35 recipients, 6 had multiple samples of TaqMan positive, with *C. albicans* (5 cases) and C. tropicalis probe (1 case), (2 proven cases with positive blood culture, 3 probable with clinical and mycologic

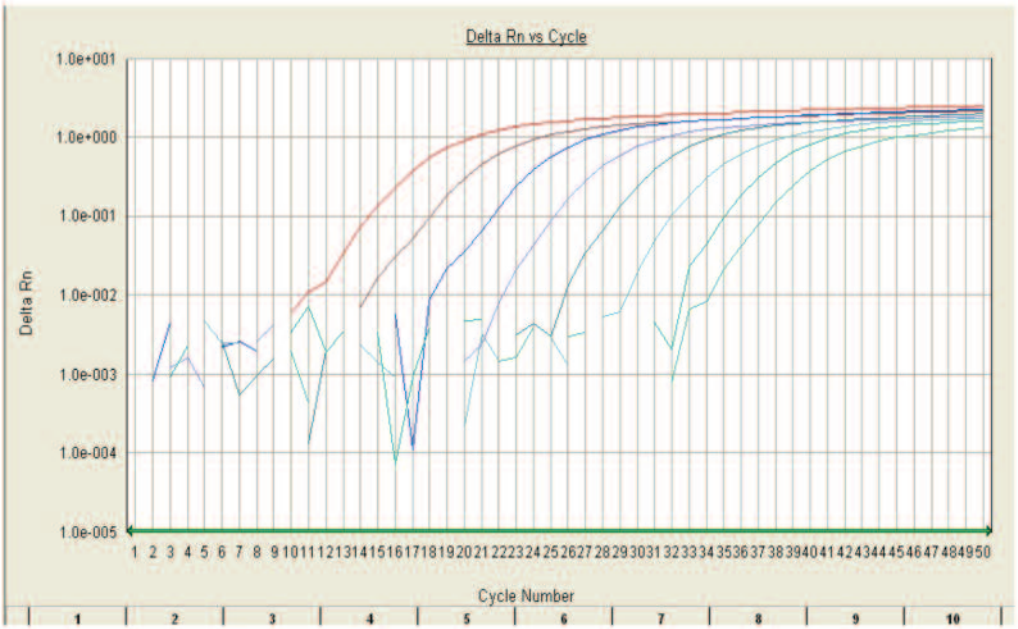

Figure 1. Amplification profile of standards for *C. albicans* real-time PCR; 10^1-10^8 copies/well

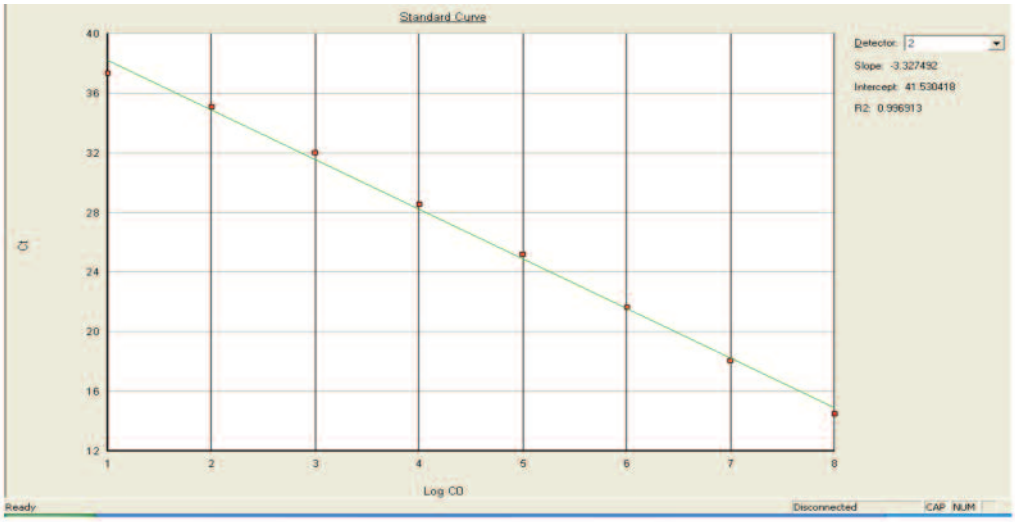

Figure 2. Standard curve for *C. albicans* real-time PCR. Threshold cycle values were plotted against various numbers of standard DNA copies. The slope was -3.327 cycles/log decade. The correlation coefficient was 0.997

criteria, and 1 probable with MRI from the abdomen and abscesses in the liver), of whom 2 patients' samples were positive PCR in the first week of admission to the ward before transplant. In these samples, the copy numbers of DNA were low in the first week, but increased once chemotherapy started and the patients presented clinical signs an average of 7 days after the first PCR results. The same features were true of the others, except that the average interval between the first PCR results and clinical manifestations was 16 days.

Upon the timely administration of antifungals, recipients exhibited good responses, and the number of DNA decreased weeks thereafter with healing signs, but when not timely administered, the recipients died with no decrease in DNA copy numbers. Characteristics of the patients with positive-PCR are shown in Table 1.

Three recipients had 1 PCR-positive result in their blood samples, and 24 controls had negative results. There was a significant difference ($P < .05$) in hospitalization between the fungal infected and noninfected patients. Seven recipients died, 3 of whom were infected with *Candida* species.

Discussion

Chronic, disseminated candidiasis has been recognized as a disease affecting patients with acute leukemia and the resulting neutropenia induced by the use of chemotherapeutic regimens (8). The pre-engraftment phase (0 to 30 days) is characterized by neutropenia, and breaks in the mucocutaneous barriers as a result of conditioning regimens and frequent vascular catheterization. During this phase, the most-prevalent pathogens causing infection are bacteria and *Candida* species (17). *Candida* infection is a major threat to patients receiving allogeneic transplant.

Real-time, TaqMan, PCR assay for quantization of *Candida* DNA allows accurate calculation of the amount of PCR product at a point in the early exponential phase of the reaction, and eliminates the concern that a reaction plateau will be reached at different cycles. This allows determining the initial fungal rDNA load from the amount of PCR product, by reference to an external standard (18). The sensitivity and specificity of primers and probe (14) on the assay were 100% and 97%. Data indicate that the TaqMan-based PCR assay can serve as an efficient method for the identification and quantization of candidiasis with a species-specific probe in blood samples.

Proven cases were defined by histologic evidence, and/or cultures from the blood, or other sterile sites (16), but because patients in the present study were thrombocytopenic, there was no histologic evidence; there were just 2 positive blood cultures for *C. albicans* (proven cases). Probable cases were defined by host factors, plus computed tomography/radiologic evidence, with supporting microbiologic signs, along with clinical signs of the infections (fever, unresponsiveness to antibacterial antibiotics ≥ 96 hours), and *Candida* colonization at multiple (> 2), noncontiguous, anatomic sites (16, 19). Real-time PCR is useful for systemic candidiasis

diagnoses, but as the present study shows, 3 patients had positive results at some point during the clinical course of their illnesses that might have been due to contamination, or transient candidemia, and components cleared by reticuloendothelial system. Positive results of PCR in 1 sample do not necessarily indicate the presence of systemic candidiasis. Real-time PCR may be helpful in differentiating true systemic candidiasis from transient candidiasis, because it can provide quantitative information on the *Candida* burden. Transient candidemia was evident for 1 week and with low burden, whereas true, systemic candidiasis was observed during the weeks following with increased copy numbers of DNA. It could be suggested that real-time PCR can diagnose the infection at early stages, and thereby promote management of patients and allow for early initiation of antifungal therapy, which is critical in reducing the high mortality among immunocompromised patients, particularly neutropenic cases. It seems that if antifungals are active against *Candida*, the burden of DNA in blood samples decreases, but in some cases, the burden of DNA increases until death.

Early detection of infection has a great affect on the clinical outcome of many infectious diseases. Two patients had *C. albicans* DNA in their blood before transplant with no clinical signs. In other studies (20, 21), it has been reported that molecular methods diagnose fungal infections before clinical manifestations; therefore, by extension, we hypothesize that in patients with hematologic disorders who received multiple chemotherapy and steroid treatments, and who were neutropenic at different stages of their illnesses, DNA can be identified before clinical manifestations, and fungal infection may be much more severe with critical signs. At that point, it is too late for recipients to show response to the antifungal therapy, and as a result, they must stay in the hospital much longer, or they may die. It is clinically significant and cost-effective to differentiate between recipients with, and those without, systemic candidiasis.

Conclusion

Considering the above findings, TaqMan-based PCR assay can serve as an accurate method for diagnosis and monitoring *Candida* infections. This is the first report of its kind showing *C. albicans* infections in the blood of the bone marrow transplant candidates, so closer observation of recipients who are neutropenic and receive immunosuppressive drugs seems warranted to enhance their survival. Meanwhile, further studies must be conducted into systemic candidiasis to promote survival among bone marrow transplant candidates and reduce both the cost and the length of hospitalization.

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