

A real time PCR assay on blood for diagnosis of invasive candidiasis in immunocompromised patient

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(Received: 7 June 2014; Revised: 25 July 2014; Accepted: 5 August 2014)

Abstract

Background and Purpose: Invasive candidiasis (IC) is a significant cause of morbidity and mortality in patients with hematologic disorders and bone marrow transplant recipients. Rapid, specific and sensitive test for the timely accuracy in immunocompromised patients to reduce mortality rates and prevent IC progress is necessary. We established a real-time PCR assay on blood for the diagnosis and differentiation of the causative *Candida* species.

Materials and Methods: Whole blood samples were collected twice, from 72 patients for Real Time PCR and blood culture assays. The primers and hybridization probes were designed to potentiate the specific sequence of 18S rRNA genes using Light Cycler system and Fluorescence Resonance Energy Transfer (FRET). The patients with hematologic malignancies and bone marrow transplant recipients were evaluated for IC based on the revised European Organization for Research and Treatment of Cancer/ Mycoses Study Group (EORTC/MSG) criteria.

Results: From 2009 to 2011, 72 patients with hematologic malignancies and bone marrow transplant recipients were evaluated for IC. The female to male ratio was 27:45; the mean age was 32.1 years. The most common malignancy in this patient was acute myeloid leukemia (AML) (27.8%) and acute lymphoblastic leukemia (ALL) (26.4%). Out of 72 patients, 11 patients (15.3%) had positive real time PCR /probe results. Based on the melting temperature (T_m) analysis, 5 (45.4%) *C. krusei*, 3 (27.2%) *C. tropicalis*, 2 (18.1%) *C. parapsilosis* and 1 *C. albicans* (9%) were identified. According to the revised EORTC/MSG, 1 patient (9%) and 10 patients (91%) were defined as proven and possible groups of IC, respectively. The mortality rate in proven and possible IC patient was found 54.5%.

Conclusion: The established Real-time PCR/FRET probe assay is an appropriate diagnostic tool for the detection of *Candida* species DNA and the management of patients suffering from hematologic malignancies and bone marrow recipient are at risk for IC.

Keywords: *Candida*, hematological malignancy, identification, invasive, Real-Time PCR

➤ How to cite this paper:

Ashrafi M, Nabili M, Shokohi T, Janbabaie G, Hedayati MT, Ali-Moghaddam K. A real time PCR assay on blood for diagnosis of invasive candidiasis in immunocompromised patient. Curr Med Mycol. 2015; 1(1): 35-41. DOI: [10.18869/acadpub.cmm.1.1.35](https://doi.org/10.18869/acadpub.cmm.1.1.35)

Introduction

The proportion of cancer patients with deep fungal infections has increased dramatically in recent decades [1]. Invasive candidiasis (IC) is a serious opportunistic infections that often in persons with impaired immunity, especially in the bone marrow and organ transplant recipients, or undergoing chemotherapy for the treatment of hematologic malignancies and severe neutropenia have occurred [2, 3] The mortality rates of IC have increased to 80%, which is currently the fourth cause of nosocomial bloodstream infection [4,5]. Despite the

emergence of new antifungal drugs, the mortality rate of IC in patients with acute leukemia and bone marrow transplant recipients is high (50% and 90% of cases, respectively) [6]. The well-known risk factors for IC include chemotherapy, immunosuppressive disease, hematopoietic stem cell or solid organ transplantation, parenteral nutrition, and indwelling catheters or prosthetic device [7]. Although *Candida albicans* is the most common cause of IC and two-thirds of all *Candida* are isolated from patients all over the world however, other *Candida* species such as

Candida krusei, *C. tropicalis* and *C. parapsilosis* are isolated from IC [8]. Current conventional diagnostic tools for IC include direct microscopy examination of tissue sections or bronchoalveolar lavage fluid, blood culture, detection of surface proteins (e.g. glucomannan or (1, 3)-beta-D-glucan) and detection of antibodies [9-12]. Early diagnosis of IC is difficult. Diagnosis based on clinical symptoms and cultures are non-specific and lack of sufficient sensitivity for the early diagnosis of IC. Serologic evaluation of IC by antibodies and circulating antigens show variable sensitivity and specificity [9,13]. Different molecular techniques for the early diagnosis of IC are present but these current PCR protocols have been criticized because of poor sensitivity, lack of standardization and excessive time and labor efforts. Some molecular analytical systems for the identification of various *Candida* species on blood samples were developed. Recently, LightCycler PCR Fluorescence Resonance Energy Transfer (FRET) techniques are increasingly being considered for early detection and identification. This technique has a high sensitivity and specificity [14]. LightCycler PCR technique is able to detect low fungal burden in blood and serum samples [15]. In this study, we aimed to extend a real-time PCR assay which was previously optimized for the detection of *C. albicans* on blood [16] for the diagnosis of invasive candidiasis in immunocompromised patients and subsequently differentiating causative *Candida* species using oligohybridization probe melting temperature (T_m) analysis.

Material and Methods

From 2009 to 2011, this descriptive study was designed to determine IC and candidemia in patients with hematologic malignancies in the oncology ward at Imam Khomeini Hospital in Sari, and bone marrow transplant recipient in Shariati Hospital in Tehran. Eighty patients met the inclusion criteria, 8 patients were excluded due to poor patient satisfaction and ultimately 72 patients were studied. The study protocol was approved by the Medical Research Ethics Committee of the Mazandaran University of

Medical Sciences (Ethical no. 89-5-13) and all patients gave their informed consent. The patients who were not willing to participate were excluded from the study.

Clinical samples

Clinical samples were obtained from patients thought to be at high risk from fungal infection based on EORTC/MSG [17]. These criteria included host factors, clinical signs and the mycological study of clinical samples in patients. Ten ml of whole blood, were taken twice a week, as well as 5ml for biphasic brain heart infusion medium bottle, 5ml for Ethylenediaminetetraacetic acid (EDTA) tubes. Blood culture bottles were incubated at 37 °C for two weeks and examined for fungal growth every day. All clinical samples were cultured on sabouraud dextrose agar with chloramphenicol and direct microscopic examination was performed. *Candida* obtained from blood culture were identified according to morphological (germ tube, chlamyospore test and CHROM agar *Candida* medium) and biochemical test using API 20C Aux *Candida* kits (Biomérieux, France).

DNA extraction

DNA extracted from whole blood was previously described [18,19]. Briefly, erythrocytes were hypotonically lysed in Red Cell Lysis Buffer (RCLB) (10mM Tris [PH=7.6], 5mM MgCl₂. 10mM NaCl) for 10 min at 37°C. The samples were pelleted at 5000 g for 10 min. The pellets were vortexed using glass beads (425-600µm, Sigma-Aldrich Corp, St. Louis, MO USA) for 3 min and the supernatant were treated with 180 U lyticase (Sigma-Aldrich Corp, St. Louis, MO USA) at 37 °C for 30 min. Two hundred microliter of treated supernatant extracted using the QIAamp DNA blood mini kit following the manufacturer's instruction and stored at - 20° C until PCR examination. To monitor the possible contamination in the DNA extractions process, saline or healthy control blood samples were included as well. Positive controls were included in each extraction to verify extraction efficiency.

Primer and Probe design

The primers and hybridization probes were designed to potentiate the specific sequence of 18S rRNA genes using LightCycler system and Fluorescence Resonance Energy Transfer (FRET). Primers and hybridization probes were designed specifically for the detection of 18S rRNA gene of *Candida* spp. [14, 20] (Table 1).

Table 1. Primers and probes used in DNA amplification

Primers:
CAN-F (5'-ATT GGA GGG CAA GTC TGG TG)
CAN-R (5'-ATC CCT TAG TCG GCA TAG-3')
(Eurofins MWG Operon, Ebersburg, Germany.)
Probes:
LC 640 5' TGG CGA ACC AGG ACT TTT ACT TTG A- phosphate
5' AGC CTT TCC TTC TGG GTA GCC ATT-LC fluorescein
(Tibmolbiol, Berlin, Germany)

Light Cycler-based PCR assay

Real-time PCR was performed on a Roche LightCycler (Roche Diagnostics, Mannheim, Germany) in a 20 µl reaction mix containing 2 µl of the extracted DNA, 15.4 µl Master mix (Light cycler DNA Master Mix, Roche Kit), 0.6 µl of HS prime Taq DNA polymerase 250 unit (GeNet Bio), 1 µl of each Primer (10 pmol/µl of primers, MWG-Operon), 1 µl of each hybridization probe (10 pmol/µl of probes, MWG-Operon). Seventy-two samples were run in parallel by performing 45 cycles of repeated denaturation (5s at 95°C), annealing (15 s at 62°C), and enzymatic chain extension (25 s at 72°C). The PCR was followed by melting temperature analysis cycle compromising 90°C for 20 s (TTR of 20°C s⁻¹), 45°C for 20 s (TTR of 20°C s⁻¹) and 90°C for zero s (TTR of 0.2°C s⁻¹) to check the specificity of the PCR product. And for the end stage, a cooling rate of 40°C to 30 s for one cycle was defined. To monitor contamination, aliquots of saline and human fibroblast DNA were prepared concurrently as negative extraction and amplification controls. All these controls were tested in the same procedure as described above.

Results

Of the 72 patients, 45 (62.5 %) males and 27 (37.5 %) were females. The age ranged between 2 years and 74 years. Their median age of patients in this study was 32.17 years. The mortality rate in present study was 33.3%. The highest mortality

rate in patients with Acute Lymphoblastic Leukemia (ALL) 29.7%, Acute Myeloid Leukemia (AML) 27%, non-Hodgkin's lymphoma and Hodgkin's lymphoma 11.3%, were seen. Sixty-six patients (91.7%) had neutropenia and (96.8%) patients were receiving chemotherapy. Regarding the clinical criteria, most of the symptoms include persistent high fever (86.1%), lethargy and weakness (56.9 %), cough (20.8%) and high fever and falling (12.5 %) were seen. One culture positive blood sample yielded *C. albicans* (1.4% of the patients) according to morphological and biochemical tests.

Antifungal agents were prescribed in 53 patients (87.5%). The most commonly used antifungal were fluconazole (34 patients; 54%) followed by amphotericin (10 patients; 40%), and combination therapy with amphotericin and fluconazole (4 patients; 5.6 %).

Quantification and Optimization of the assay

The determination sensitivity, specificity, reproducibility and assessment of detection limit of the assay of *Candida albicans* in whole blood were described in detailed previously by Nabili et.al, [16]. Briefly to determine specificity, the oligonucleotide specific for *C. albicans* hybridized only with DNA extracted from *C. albicans* cultures. All PCR reactions with DNA from other fungal species such as *Aspergillus* spp. and human genomic DNA were negative, indicating the specificity was 100% for this method. Melting temperature analysis of *C. albicans* was indicated in a single point of maximum temperature signal with the probe. T_m analysis subsequent to real time PCR amplification using standard strain revealed a specific melting peak signal with *C. albicans* probing setting and allowing the differentiation of *C. krusei*, *C. tropicalis*, *C. parapsilosis* and *C. albicans*. The specific melting temperature (T_m) patterns are shown in Table 2. Reproducibility of the FRET assay was evaluated by using inter-assay method previously [16]. Briefly, the amplification of serially diluted *C. albicans* (10⁰ to 10⁶ CFU) was repeated 6 times with DNA from the same extraction. Real-Time-PCR assay showed good reproducibility. The minimum amount of DNA, 1 yeast cell (10⁰ CFU/ml) equivalent (10 fg) was determined in our

previously published manuscript [16] for each PCR reaction, which indicates the high sensitivity of this method.

Real-Time-PCR results in clinical samples

We analyzed 110 blood samples from 72 patients, for testing Real-Time PCR. Of the 72 patients, 11 patients (15.3 %) were positive for the presence of *Candida* DNA on blood by Real-Time PCR/probe. Based on the melting temperature (T_m) analysis, 5 (45.4 %) *C. krusei*,

Table 2. Melting temperatures (T_m) for standard strains of *Candida* species with *Candida albicans* hybridization probe set

<i>Candida</i> species/ Standard Strain	T _m °C
<i>C. krusei</i> (CBS 573)	55.66
<i>C. tropicalis</i> (CBS 94)	63.15
<i>C. parapsilosis</i> (CBS 604)	62.47
<i>C. albicans</i> (CBS 562)	65.76

3 (27.2%) *C. tropicalis*, 2 (18.1 %) *C. parapsilosis* and 1 *C. albicans* (9%) were identified. According to host factors, clinical and mycology criteria on the revised EORTC/MSG, 1 patient (9%) and 10 patients (91%) were defined as proven and possible IC, respectively (Table 3). The mortality rate in proven and possible IC patient was found 54.5%.

Discussion

Candida species are the cause of nosocomial bloodstream infection in 8-9% patients admitted to the intensive care unit (ICU). Cancer patients are at a high risk of developing bloodstream infection [7,21, 22]. Serious risk factors for invasive candidiasis are chemotherapy in patients with hematologic malignancies and immunosuppressive therapy in patients receiving hematopoietic stem cell

Table 3. Demographic, characteristics and clinical data of eleven patients with proven or possible IC

patients	Age (yr)/ sex	Primary disease or risk factor	Neutropenic	Clinical symptom	Blood culture	IC classification	real time PCR	Agent (Melt curve analysis)	Outcome
1	47/M	HL	+	High f	-	Possible	+	<i>C. tropicalis</i>	Survival
2	74/M	CLL	+	High f/ Intraocular infection	-	Possible	+	<i>C. parapsilosis</i>	Death
3	58/M	AML	+	High F /C/S	-	Possible	+	<i>C. krusei</i>	Survival
4	59/M	AML	+	High F/D	+	proven	+	<i>C. albicans</i>	Death
5	2/M	ALL	+	High F	-	Possible	+	<i>C. krusei</i>	Survival
6	50/F	ALL	+	High F	-	Possible	+	<i>C. krusei</i>	Death
7	60/M	MM	+	High F/ S/ D	-	Possible	+	<i>C. tropicalis</i>	Death
8	15/M	T	+	High F	-	Possible	+	<i>C. krusei</i>	Survival
9	45/M	L	+	High F	-	Possible	+	<i>C. parapsilosis</i>	Death
10	37/M	HL	+	High F	-	Possible	+	<i>C. tropicalis</i>	Death
11	12/M	ALL	+	High F	-	Possible	+	<i>C. krusei</i>	Survival

ALL: Acute lymphoblastic leukemia, AML: Acute myeloid leukemia, HL: Hodgkin's lymphoma, L: Lymphoma, D: Dyspnea, F: Fever, S: Sputum, C: Cough, MM: Multiple Myeloma, T: Thalassemia

transplant [23]. Several conventional microbiological techniques such as blood cultures for the diagnosis of invasive candidiasis are used which has a very low sensitivity. The detection of immunological and molecular markers has provided an alternative for early diagnosis of invasive candidiasis [21]. Several PCR based techniques have been developed that their sensitivity and specificity are above 90% [24]. The 18S rRNA genes are highly conserved regions of the genome and used to detect *Candida* species [25]. Real time PCR in conjugation with fluorescent detection of amplicon using FRET has several advantages to culture-based method for the diagnosis of invasive candidiasis. This method is highly sensitive, rapid, and allows differentiating various species of *Candida* and quantifying the fungal load in blood samples [14]. In the present study, the same gene region was used in real time PCR technique for the detection of *Candida* species. This method has high sensitivity and able to detect at least two copies of the genome *C. albicans* in serum samples [26]. In Klingspor's study [27], the regions 18S rDNA and in Hsu study [28], the regions 28S rDNA were used as target for detection. The researchers showed that these two gene regions have a high sensitivity for detection of *Candida* species [27, 28]. In the present study, to assess the sensitivity of the technique using FRET, prepared from serial dilutions (10^0 to 10^6 *C. albicans* standard strain) were mixed with the blood of healthy volunteers. Serial dilutions of all samples successfully amplified with the increase in fluorescence signal probes were amplified by real time PCR method. In our study, the minimum amount of DNA, 10 CFU was determined. In Loffler et al. study, PCR-ELISA assay was performed at the detection limit of the 5 CFU *Candida* in 1 ml whole blood [29]. In a study using two fluorogenic probes in a TaqMan-based PCR assay, the low limit of detection of 1-20 CFU/ml blood have been reported [30]. Horn et al. in a study conducted in 2011 by PCR, the sensitivity of 10 CFU/ml was reported [31]. Schabereiter-Gurtner et al., in a study conducted in 2007, using SYBR Green

technique, the sensitivity of 5 to 10 CFU/ml blood for detecting *Candida* in whole blood was reported, which is consistent with the current study [32]. The differences of sensitivity depend on to certain factors such as applying different methods of DNA extraction, using different primers and probes, using different devices and techniques of the implementation.

The study also revealed that the FRET technique has high specificity and good reproducibility. In this study, Melting temperatures (T_m) for *C. albicans* 65.76 °C, *C. krusei* 55.66 °C, *C. tropicalis* 63.15 °C and *C. parapsilosis* 62.47 °C was identified. Fricke et al. in a study conducted in 2010 with the FRET technique to analyze the melting temperature of *C. albicans* 67 °C, *C. tropicalis* 63.9 °C, *C. parapsilosis* 61°C and *C. glabrata* 62.3°C [33]. The differences between these studies may result from different materials, device and operating system. The prevalence of invasive candidiasis in patients with hematologic malignancy and bone marrow transplant recipients, by real time PCR/FRET method was 15.2 %, which indicated a high prevalence. Hachem et al. reported, the prevalence of the disease to be as 12 % in patients with hematologic malignancies in [34]. In our study, based on melting curve analysis *C. krusei* in 5 cases (45.4%), *C. tropicalis* in 3 cases (27.2 %), *C. parapsilosis* in 2 cases (18.1 %) and *C. albicans* in 1 case (9.3 %) were identified. The majority of *Candida* species isolated from patients was non-albicans *Candida* species (90.7%). Abi-said et al. [35] demonstrated that *C. albicans*, *C. tropicalis* and *C. parapsilosis* were the most common species obtained from blood samples of patients with hematological malignancies and transplant recipients whereas in our study, *C. albicans* is the less common causative agent.

Real time PCR method has advantages of speed; reduce exogenous contamination, and capability for quantification the fungal burden. Designing a standard and simple molecular method for surveillance of at risk patient is crucial and can result in earlier starting suitable antifungal agents. Since the early diagnosis and differentiation of *Candida* species is beneficial,

before starting treatment to prevent drug interactions, is important because non- *albicans* species of *Candida* often is resistant to azoles and polyene antifungal drugs.

Conclusion

The established Real-time PCR/FRET probe assay is an appropriate diagnostic tool for the detection of *Candida* species DNA and the management of patients suffering from hematologic malignancies and bone marrow recipients are at risk for IC.

Acknowledgements

We are grateful to Dr. Shahram Samiei for his excellent real time PCR support.

Authors' contributions

T.S. designed and managed the research, wrote the draft manuscript and edited the final manuscript. G.J. and K.A.M. referred the patients, M.A., M.N. applied all tests and wrote the draft, and M.T.H. managed the research.

Conflicts of interest

The authors state no conflict of interest.

Financial Disclosure

No financial interests related to the material of this manuscript have been declared.

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