

Prevalence of candidemia and associated *candida* subtypes following severe sepsis in non-neutropenic critically ill patients

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Summary. *Background:* Invasive candidiasis management through the rapid initiation of appropriate antifungal therapy has been shown to be associated with the better prognosis, improved clinical outcome and reduced mortality in critically ill patients. Therefore, selection of an appropriate antifungal therapy should be based on the distribution of candida species and the pattern of antifungal resistance. This study aimed to assess the prevalence of candidemia and associated subtypes following severe sepsis in non-neutropenic critically ill patients. *Methods:* This study was a cross-sectional study that was conducted on severe sepsis patients stayed at least seven days in intensive care unit. Patients less than 18 years old, pregnant and breastfeeding patients, immunocompromised patients, neutropenic patients, patients with concurrent use of antifungal medicines and cytotoxic agents were excluded. To assess the candidemia, one milliliter of patients' blood sample was collected. Sample analysis was performed by Real-Time PCR and high resolution melting curve analysis method. *Results:* Thirty-one critically ill patients were recruited in this study over 12-month period. Candidemia with a detection limit of 100 pg per 0.2 ml blood sample was not recognized in any of the included patients. *Conclusion:* The present result indicates low incidence of candidemia in the targeted intensive care units, but other factors such as small sample size, exclusion of patients with compromised immune system and the low fungal load at the time of sampling may also account for our observation. (www.actabiomedica.it)

Key words: candidemia, severe sepsis, ICU, Real-Time PCR

Introduction

Over the past decades, invasive fungal infections have emerged as an important cause of sepsis and subsequent morbidity and mortality in critically ill patients (1). Historically, *Candida* species are considered

to be the predominant agents responsible for fungal sepsis, and account for 10.15% of nosocomial infections (about 5% of all cases of septic shock and severe sepsis) (2). The Sepsis Occurrence in Acutely Ill Patients (SOAP) study reported a fungal infection rate of 17% in patients with sepsis in European intensive care

units (ICUs) (3). Candidemia is an important cause of mortality in critically ill patients (25-38%), and the crude mortality rate of *Candida* infections is about 40-75% (4, 5). The economic burden of these infections is significant as candidemia is associated with increased costs of care and prolonged hospitalization (6). The incidence of *Candida* infections is greater in patients with a history of broad-spectrum antibiotic use, diabetes, central line catheter use, burn, extensive surgery specially intra-abdominal surgery, immunosuppression, renal failure and parenteral nutrition (7). Prophylaxis and preemptive measures with empirical and targeted therapies are currently applied antifungal strategies in high-risk patients. The epidemiology of fungal infections and resistance patterns of ICU infections as well as the pharmacokinetic, drug interactions, safety profile, spectrum of activity, and pharmacy acquisition cost are the factors that must be considered when the appropriate antifungal therapy is necessary. Resistance to fluconazole especially in *C. glabrata* and *C. krusei* isolates represents a major obstacle against successful empirical therapeutic and prophylactic strategies (8). Moreover, timely source control and appropriate management of antifungal infections can improve clinical outcomes (9). Notably, several scoring system such as *Candida* score and *Candida* colonization index have been reported to have a predictive value for early fungal treatment (10, 11).

Candida albicans is considered to be the most common cause of candidemia. However, some studies have mentioned the increased rate of non-*albicans* candidemia that leads to higher rate of mortality and antifungal drug resistance (12, 13). In order to obtain a more precise information on the epidemiology of candidemia, identification of isolates to the species level is crucial. For instance, an increasing prevalence of candidemia in Iceland was reported for the period between 2000 and 2011, but the results were completely different in Switzerland, where a national surveillance study concluded that the incidence rate of candidemia had remained unchanged during the period of 1991 to 2000. In a multi-center study which was performed in the United States between 2004 and 2008, 54% of samples represented non-*albicans Candida* infections and 46% represented *C. albicans* infections (14). *C. glabrata* was responsible for 26% of all samples of candidemia, followed by *C.*

parapsilosis, *C. tropicalis*, and *C. krusei* explaining 16%, 8% and 3% of samples, respectively. Another retrospective study which was done in China showed that there is a trend change in candidemia incidence in which *C. tropicalis* (28.6%), *C. albicans* (23.3%) and *C. parapsilosis* (19.5%) are the causative agents (15). This demonstrates that the results reported in studies done in one institution or country cannot be exactly generalized to other countries or institutions.

Early diagnosis of invasive candidiasis is critically in intensive care units. Blood culture is accepted as a gold standard for diagnosis of invasive fungal infections. Identification of *Candida* isolated in positive blood cultures with conventional biochemical and phenotypic methods requires commonly two or three days. Therefore, the availability of a rapid test for identification of *Candida* spp, more specifically *C. glabrata* and *C. krusei* could prevent delays in the administration of appropriate therapy (16). Over the past decades, development of non-culture based method for the diagnosis of candidemia such as antibody assay, beta-D-glucan and other antigen assay and polymerase chain reaction (PCR) has been occurred (17). The diagnosis of invasive candidiasis with PCR method have a low threshold of fungal cell detection which is comparable to the detection threshold of blood cultures (18-22). In a study analyzing candidemia cases from 1987 through 1991 mentioned that more than half of candidemia cases involve <1 cell/mL (23). Therefore, one of the main challenges of PCR for candida infection detection is to overcome this burden. PCR-based method have increased sensitivity, especially in cases of invasive candidiasis for which blood culture results are negative (17, 24, 25). Earlier diagnosis and the possibility of monitoring the persistence or resolution of infection are the other advantages of this method (26). A recent advance in PCR-based assays is high-resolution DNA melting (HRM) analysis (27, 28). Some recent studies used HRM to differentiate clinical *Candida* species (27-29). HRM is a modern technique for the identification of genetic variants in Real-Time PCR via comparison of melting curves of different amplicons. It is potentially a novel solution for genotyping, sequence matching and mutation scanning (30).

To sum up, invasive candidiasis management through the rapid initiation of appropriate antifungal

therapy has been shown to be associated with a better prognosis, improved clinical outcome and reduced mortality in critically ill patients. Therefore, selection of an appropriate antifungal therapy should be based on the distribution of *Candida* species and the pattern of antifungal resistance. Considering these issues, the aim of this study was to assess the prevalence of candidemia and associated subtypes following severe sepsis in non-neutropenic critically ill patients.

Materials and methods

This study was a cross-sectional study that was conducted on severe sepsis patients admitted to general and emergency ICU of Sina Hospital affiliated to Tehran University of Medical Sciences. Patients with severe sepsis stayed at least seven days in intensive care unit were included. Patients less than 18 years old, pregnant and breastfeeding patients, immunocompromised patients (congenital or acquired immunodeficiency, human immunodeficiency virus infection, chronic renal failure, nephrotic syndrome, lymphoma, leukemia, hodgkin disease, generalised malignancy, iatrogenic immunosuppression, solid organ transplantation and multiple myeloma), neutropenic patients, patients with concurrent use of antifungal medicines and cytotoxic agents were excluded. This study was approved by the institutional Ethics Committee and performed in accordance with the principles of the Declaration of Helsinki.

To assess the candidemia and associated subtypes; APACHE-II, SAPS-II and SOFA in the first day of ICU admission and SAPS-II; SOFA and Candida Score were measured on the sampling day. Changes in SOFA and SAPS-II score over time were analyzed by performing paired-samples T-Test in SPSS 16 software. Statistical significance was defined at P values <0.05. Length of ICU and hospital stay and 28 day mortality, duration of mechanical ventilation were recorded and the type of antifungal treatment was recorded if patients received antifungal therapy after sampling. One milliliter of blood sample from patient's CV-line was collected. Samples were maintained at -80°C until analysis time.

Candida DNA Extraction. Five yeast strains were obtained from Iranian Biological Resource Center and Persian Type Culture Collection used as reference strains to test by Real-Time PCR system. These strains included *C. albicans*(PTCC 5027), *C. parapsilosis*(IBRC-M 30005), *C. krusei*(PTCC 5295), *C. guilliermondii*(IBRC-M 30071) and *C. glabrata*(PTCC 5297). *Candida* strains were grown on YM media for about 18-24 h. Genomic DNA was extracted via Bio Basic fungal genomic DNA isolation kit according to the manufacturer's instruction. Briefly, 300 mg of cell pellets collected from centrifugation of 3 ml fungi liquid culture was grounded in liquid nitrogen. The grounded sample was then transferred to a microcentrifuge tube. 400 µL of the universal digestion buffer was added to the sample. The sample was incubated at 65°C for 30 min. Subsequently, 200 µl of washing buffer was added and incubated at -20°C for 5 minutes and centrifuged at 12000×g for 5 minutes. The supernatant was transferred to a clean 1.5ml tube. Then, 0.5 ml of isopropanol was added and incubated at room temperature for 5 minutes and centrifuged at 12000×g for 5 minutes. The supernatant was discarded. Afterwards, 1ml of pre-cooled 75% ethanol was added and centrifuged at 12000×g for 5 minutes. The supernatant was discarded and the sample was incubated at room temperature for 5 minutes. Finally, 50 µl of elution buffer was applied to the tube and incubated for 5 min at 25°C. The ratio of absorbance at 260 nm and 280 nm of purified DNA was measured using spectrophotometer. About 2ng of purified DNA of all yeast strains was added to the PCR mixture for Real-Time PCR.

Patient's Blood Samples. When blood from patients were tested, 200 µl of blood was treated with a QIAamp DNA Mini kit as suggested by the manufacturer. Moreover, 200 µl of non-infected healthy individual blood was processed for DNA extraction and considered as negative control. 20 µl of Proteinase K was added to 200 µl of blood and vortexed for 15 s. Subsequently, 200 µl of lysis buffer was added, vortexed again and incubated for 10 min at 56°C. Then 200 µl of 96% ethanol was added, transferred to a Qiagen column and spun down for 1 min at 6000 ×g. Afterwards, 500 µl of washing buffer was pipetted on to the column and spun down for 1 min at 6000 ×g. The

washing procedure was repeated and buffer was centrifuged for 3 min at 20000 $\times g$. Finally, the column was placed in a microcentrifuge tube and 50 μ l of elution buffer was applied to the column and incubated for 5 min at 25°C. 5 μ l was assayed in Real-Time PCR.

Real-Time PCR. Sample analysis was conducted by Real-Time PCR and high resolution melting curve analysis method. Primers had been described in previous studies (31) and were able to amplify the internal transcribed spacer regions 1 and 2 including the 5.8S rDNA of a broad range of fungi including candida species. Forward primer CAN-F (5'-GGT CAA ACT TGG TCA TTT AG-3') and reverse primer CAN-R(5'-TCT TTT CCT CCG CTT ATT G-3') were used as universal primers in this study. Amplification of the target sequences and HRM analysis was carried out using the Step-One-Plus Apparatus (Applied Biosystems). Real-Time PCR was performed in a total volume of 20 μ l containing 10 μ l MeltDoctor™ HRM Master Mix, 3.4 μ l of distilled water, 0.8 μ l of each primer(10 pM) and 5 μ l of extracted specimens. After an initial step of 10 min at 95°C, 40 cycles were performed for 30 sec at 95°C, 30 sec at 54°C, 1 min at 72°C and 15 sec at 80°C. The HRM analysis was performed following PCR amplification and the melting curve was generated with the following protocol: 15 s at 95°C, 1 min at 75°C and 15 s at 93°C. Fluorescence data were measured at every 0.3°C change from 75°C to 93°C and HRM curves were analysed using HRM Software (version 3.0.1, Applied Biosystems). At the end of the Real-Time PCR-HRM run (3 h 10 min), fluorescence was normalized and the limit of the analysis was set by setting the pre- and post-melt slider from 75-76°C to 90.9-91.3°C for Candida species identification. Normalized, difference and derivative melting plots were used in the HRM analysis.

Sensitivity. The limit of detection of the Real-Time PCR assay were tested by preparing 10-fold serial dilutions from 2 ng to 20 fg of *C. krusei* genomic DNA

Then 2 μ l of each concentrations were assayed in Real-Time PCR and the sensitivity of QIAamp DNA Mini kit were tested by spiking 200 μ l of blood from a non-infected healthy individual with 5 μ l of serial dilutions of *C. krusei* DNA ranging from 20 ng/ μ l to

200 fg/ μ l. The DNA from all samples was extracted at the concentrations ranging from 2 ng/ μ l to 20 fg/ μ l of genomic DNA. Then 2 μ l of each concentrations were assayed in Real-Time PCR. All reactions were run in duplicate and negative controls were run with each experiment.

Results

31 critically ill patients were recruited in this study over 12-month period. 21 patients were male and 10 patients were female. The cause of ICU admission in patients was different and is summarized in Table 1. The mean age of included patients was 57.6 \pm 20.5 years. The average duration of ICU stay was 19.3 \pm 9.4 day and the mean duration of hospital stay was 27.4 \pm 13.3 day. The average duration of patient's mechanical ventilation before sampling was 8 \pm 6.7 day. The patients overall 28 day mortality was 32.3%. The mean measured candida score was 2.8 \pm 0.7 following by 58% have candida score more than 3, 6.5% ranged from 2.5 to 3 and 35.5% ranged from 2 to 2.5. The average patient's APACHE-II score in the first day of ICU admission was 15.5 \pm 6.4. The average patient's SOFA score in the first day of ICU admission was 5.5 \pm 2.4 and the mean SOFA score in the sampling day was 6.2 \pm 2.3. There was no statistically significant difference between the mean SOFA score of the first day and the sampling day (P Value=0.078). The average patients SAPS-II score in the first day of ICU admission was 39.5 \pm 12.9 and the mean SAPS-II score on the sampling day was 38.3 \pm 12.9. There was no significant reduction in the average of first day SAPS-II score compared with the sampling day (P Value=0.558).

Table 1. The causes of patient's admission to ICU.

Underlying	Disease Number
Surgery (Abdominal surgery/other)	17 (7/10)
Pneumonia	5
Cerebrovascular Accident	2
Acute Coronary Syndrome	2
Multiple Trauma	2
Toxic Epidermal Necrolysis	1
Multiple Sclerosis	1
Electrical Injury	1

11 patients were treated with antifungal therapy after sampling. 8 patients received amphotericin B and 3 patients received fluconazole.

Genomic DNA of five *Candida* species was extracted via Bio Basic fungal genomic DNA isolation kit. The purified *Candida* species DNA 260 nm/280 nm ratio ranged from 1.7 to 1.8. DNA extraction from blood samples was performed by QIAamp DNA Mini kit as recommended by the manufacturer and the 260 nm/280 nm ratio of DNA extracted from clinical samples using spectrophotometer ranged from 1.6 to 1.8.

All tests were conducted in one working day. About 20 ng of genomic DNA of *C. albicans*, *C. parapsilosis*, *C. guilliermondii*, *C. krusei* and *C. glabrata* were considered as standard samples and 5 µl of 31 extracted specimens in duplicate were assayed by the Real-Time PCR method. Then, High-resolution melting curve analysis was performed. Normalized and derivative HRM curves analysis revealed that each of the five *Candida* species generated a distinct melting curve that was characteristic of its species (Fig 1). The five *Candida* species generated major, minor or shoulder

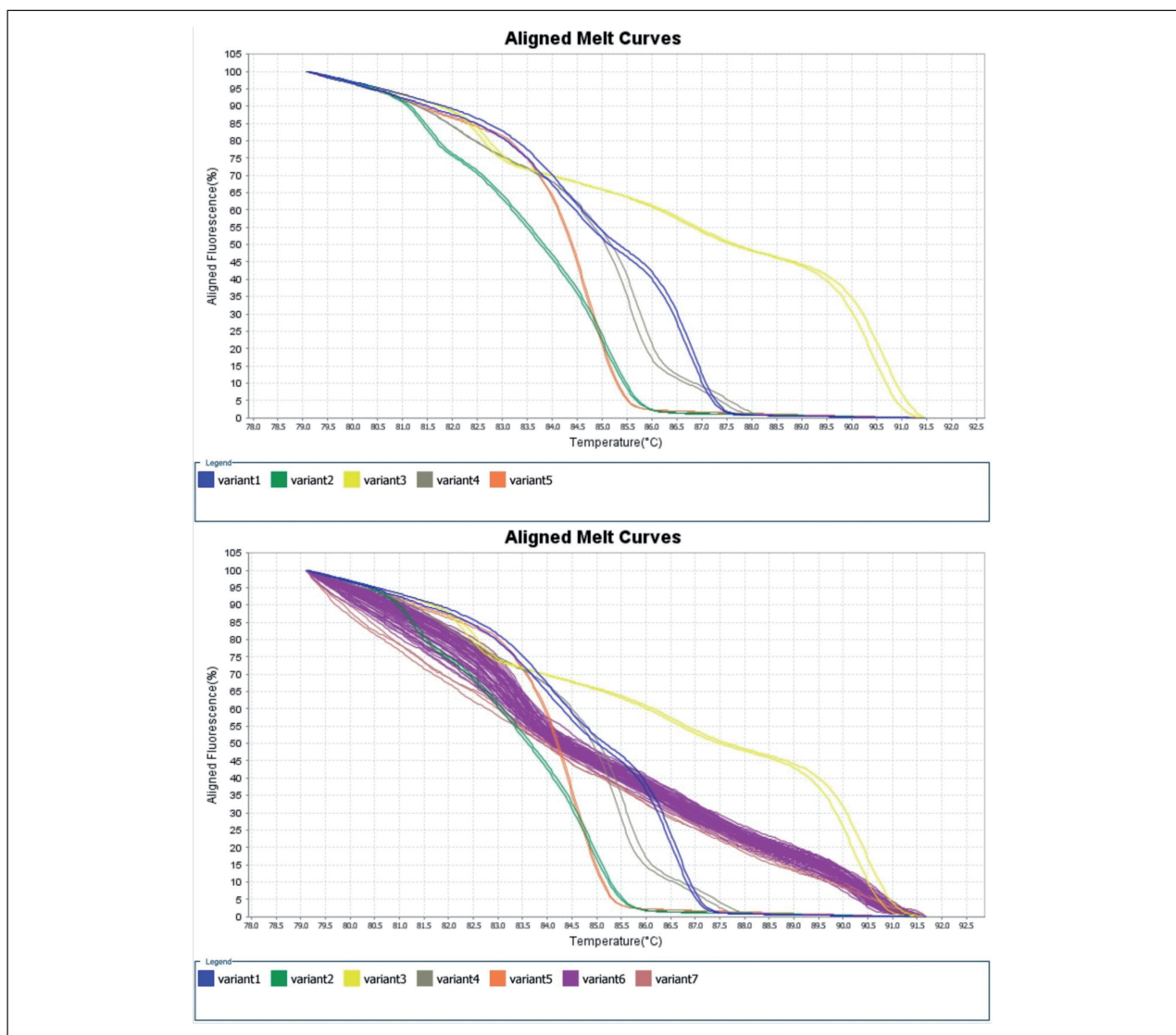


Figure 1. Normalized high resolution melting curves of type reference candida species and patient's clinical samples. Variant 1: *C. albicans*, variant 2: *C. parapsilosis*, variant 3: *C. krusei*, variant 4: *C. glabrata*, variant 5: *C. guilliermondii*, variant 6,7: Patient's clinical samples showing different normalized high resolution melting curves to those of the corresponding reference *Candida* species

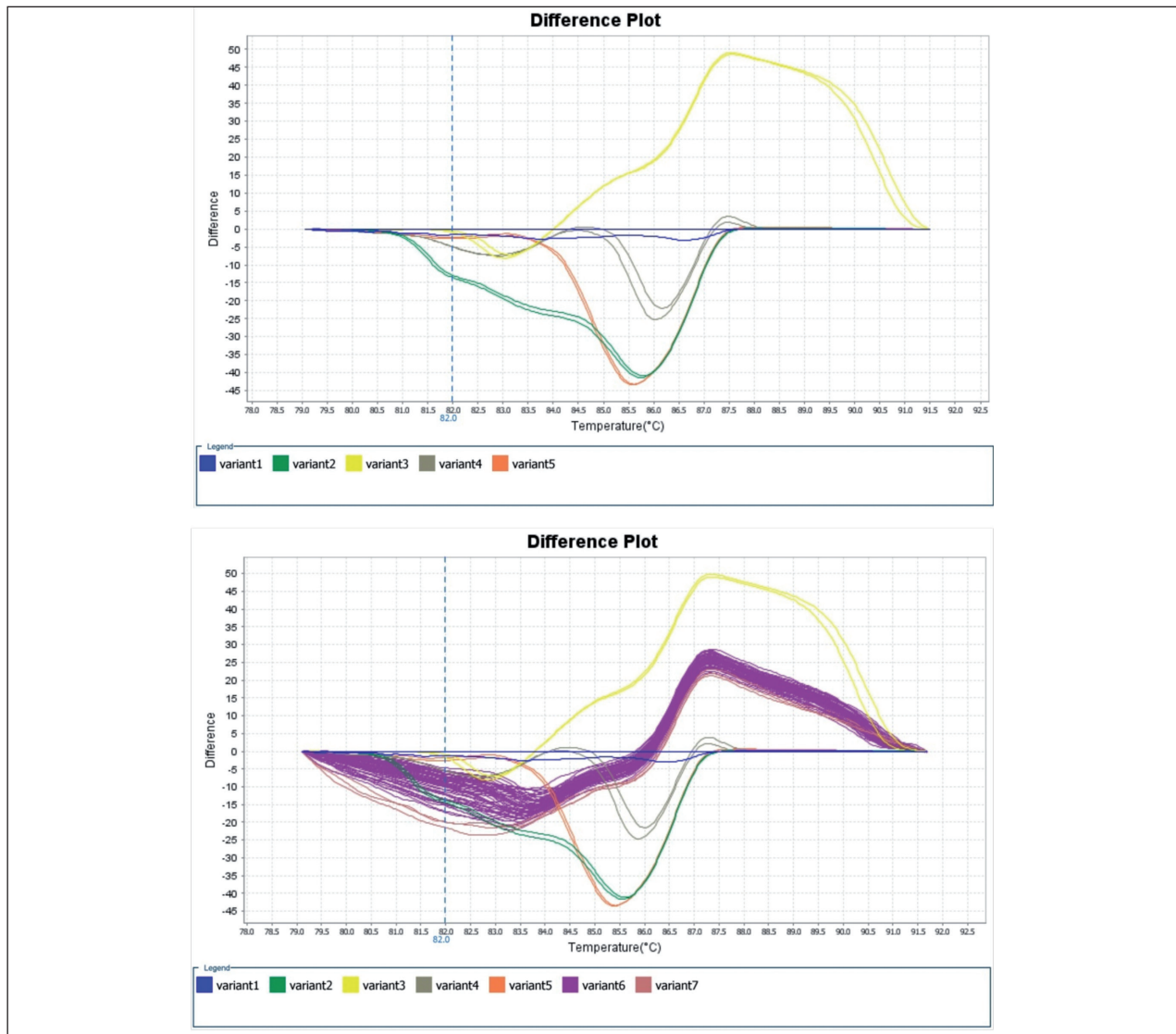


Figure 2. Normalized high resolution melting curves of type reference *Candida* species and patient's clinical samples. Variant 1: *C. albicans*, variant 2: *C. parapsilosis*, variant 3: *C. krusei*, variant 4: *C. glabrata*, variant 5: *C. guilliermondii*, variant 6,7: Patient's clinical samples showing different normalized high resolution melting curves to those of the corresponding reference *Candida* species

peaks at the following temperatures: *C. albicans* had one major peak with one shoulder between 83°C and 89°C; *C. parapsilosis* had one major peak with one shoulder and one minor peak between 80°C and 86°C; *C. krusei* had one major and two minor peaks between 81°C and 92°C; *C. glabrata* had two minor peaks and one major peak between 81°C and 88° and *C. guilliermondii* had one minor peak and one major peak between 80°C and 86°C. When data for different *Candida* species were plotted against one reference strain

(*C. albicans*), difference curve analysis showed five distinct profiles representing the five *Candida* species with the clinical samples showing different profiles in comparison with standard strains (Fig. 2). According to HRM analysis, candidemia was not found in any of patient's blood samples.

The standard curve was obtained by preparing 10-fold serial dilutions from 2ng to 20 fg of *C. krusei* genomic DNA. The sensitivity of the assay was found to be 4 pg of *C. krusei* genomic DNA (Fig. 3).

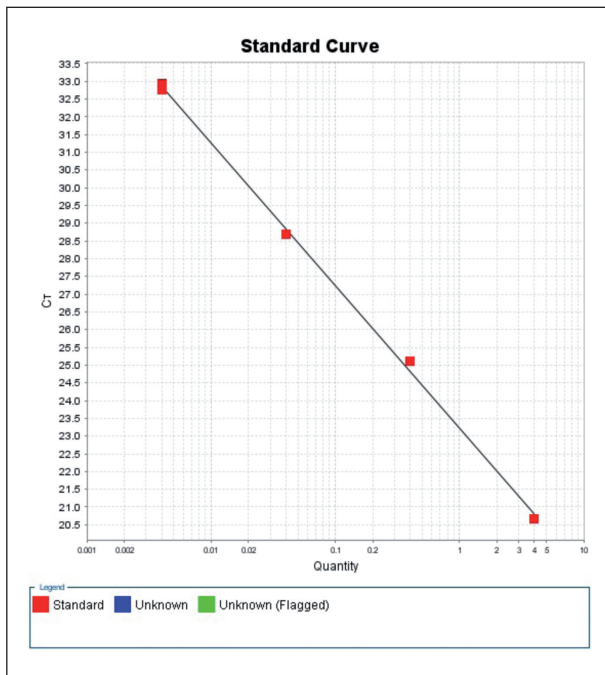


Figure 3. The Real-Time PCR standard curve of *C. krusei* genomic DNA

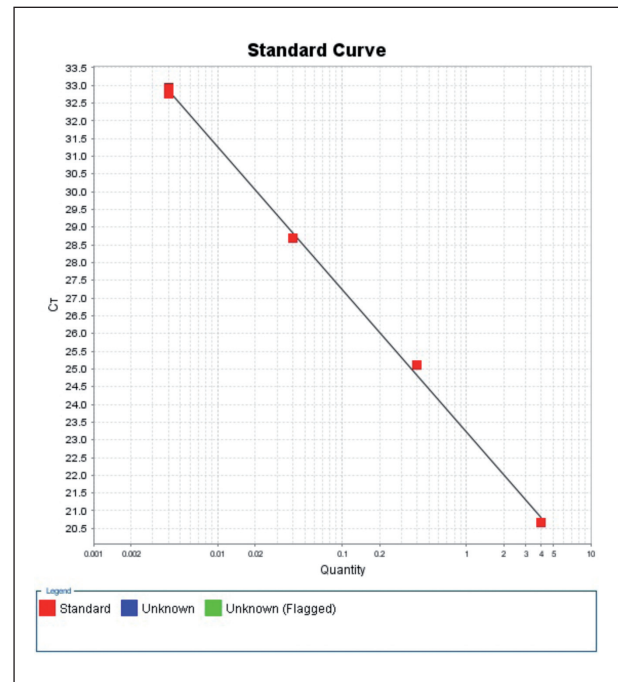


Figure 4. The Real-Time PCR standard curve of spiking *C. krusei* genomic DNA

To analyze the sensitivity of QIAamp DNA Mini kit, blood from a non-infected healthy individual was spiked with serial dilutions of *C. krusei* genomic DNA. The DNA from all samples was extracted using the kit. Then, Real-Time PCR was performed. After Real-Time PCR amplification of all genomic DNA, as few as 100 pg of purified *C. krusei* genomic DNA could be detected in the 0.2 ml of blood samples (Fig. 4).

Discussion

According to the findings of previous studies, invasive candidiasis management through rapid initiation of appropriate antifungal therapy can lead to better prognosis, improved clinical outcome and reduced mortality in critically ill patients. On the other hand, inappropriate antifungal therapy is associated with treatment failure, development of antimicrobial resistance and increased costs of care. Therefore, selection of an appropriate antifungal therapy should be based on the distribution of *Candida* species and the pattern of antifungal resistance. Relying on these facts, this study

was designed to assess the prevalence of candidemia and associated subtypes following severe sepsis as a major risk factor for candidemia. In order to create uniformity in the included patients population, taking into account different patterns of invasive candidiasis between immunocompetent and immunocompromised patients, immunocompromised patients who comprise a large number of ICU admissions with high morbidity of fungal infections were excluded. Sample analysis was conducted using Real-Time PCR and high-resolution melting curve analysis and candidemia was not found in any of the patient's blood samples. Different reasons can be considered to justify the results. The low incidence of candidemia in the intensive care unit of targeted hospital can be taken into account. The Sepsis Occurrence in Acutely Ill Patients (SOAP) study which included 3147 patients from 198 European ICUs to evaluate the sepsis pattern in intensive care unit of the European countries, reported a *Candida* infection rate of 17% in patients with sepsis in European ICUs. *C. albicans* was responsible for 13% of candida infection cases while 4% of all candidemia cases were found to be infected with non-albicans species (3). In another

study which was performed in 24 intensive care units in France within one year to assess the incidence of candidemia and candiduria in critically ill patients, the average incidence of 6.7 per 1000 ICU admission was reported (32). Despite the large enough sample size of previous studies, low incidence rate of candidemia in critically ill patients was demonstrated. The low sample size of current study compared with the previous studies may have an effect on the results obtained.

According to the previous studies, candida score is considered to have a predictive value for early diagnosis of invasive candidiasis. Leory et al conducted an observational cohort study to evaluate the candida score in ICU. They concluded that a candida score > 3 has predictive value for early antifungal therapy in severe septic patients (33). In another study which was performed by Leon et al, candida score >2.5 was considered to be associated with high risk for invasive candidiasis development with the specificity of 74% and the sensitivity of 81% (34). On the other hand, in a cohort study of colonized non-neutropenic patients with a candida score <3, the incidence rate of candidiasis was 2.3% and the maximal candida score of 5 was associated with an incidence rate of 23.6%. Therefore, the accuracy of candida score for positive prediction of invasive candidiasis was not approved (35). Moreover, in this study, the candida score was measured in all patients on the day of sampling and 58% of included patients had candida score more than 3 but we could not find any candidemia in the blood samples. So, the results of our study also do not confirm the candida score's true value for early detection of invasive candidiasis.

Blood culture is the current gold standard for the diagnosis of invasive candidiasis which takes 24 to 48 h to show positive samples while detection of the specific *Candida* isolates might take even longer (36). On the other hand, non-culture-based diagnostic methods such as PCR, have been developed to allow diagnosis of candida infections as early as 6 h and thereby an earlier initiation of appropriate species-oriented therapy that could lead to a reduced incidence rate of mortality (22). The capacity of PCR-based methods to detect candida species DNA even in case of negative blood cultures have also been shown in Kourkoumpetis et al study (24). Furthermore, High-resolution

DNA melting analysis is a novel technique used for candida species genotyping in some recent studies (28, 30). Despite the previous studies which performed HRM analysis on candida isolates, for the first time, in this study the direct blood samples were used for candida species genotyping and as few as 100 pg of purified candida genomic DNA was detected in the 0.2 ml of blood samples. The sensitivity of the diagnostic method used for detection of invasive candidiasis in clinical samples has been considered as an important factor in several studies. The short half-life of circulating DNA, the intermittent hepatic clearance of candida cells from blood and the low fungal load at the time of sampling may have contributed to the lower sensitivity. In a study which was conducted by Lau et al using MT-PCR method, the level of circulating fungal cells in the blood was estimated to be less than 10 CFU/ml with 25% of cases having less than 1 CFU/ml (37). Pfeiffer et al also demonstrated that over half of the positive *Candida* blood cultures in the first episodes of candidemia contained less than 1 CFU/ml of candida species which is below the threshold which can be reliably detected by currently available DNA-based diagnostic tests (23). Hence, finding a standard diagnostic test for early diagnosis of invasive candidiasis especially in the first episodes of invasive candidiasis is still challenging. In this study, sampling from patients for early treatment of candida infections was performed in the early episodes of candidemia. So, this is an important justification for unrecognized candidemia.

Conclusions

Based on the present findings, candidemia with a detection limit of 100 pg per 0.2 ml of blood samples was not recognized in any of the included patients with the diagnosis of severe sepsis who were confined in ICU for at least seven day. Some issues should be taken into consideration before interpreting the present results, such as the low incidence of candidemia in the targeted intensive care units, small sample size, excluding patients with compromised immune system and the low fungal load at the time of sampling. This result needs to be verified in large-scale multicenter

studies inclusive of immunocompromised patients. Because of low fungal load, sampling in the first episodes of candida infection is not recommended. Finally, PCR optimization to achieve a detection limit less than 1 CFU/ml is highly recommended in future studies to allow diagnosis of undetectable candidemia

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