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Full Length Research Paper

Real time polymerase chain reaction for identification of *Candida* species isolated from blood stream infections in non-neutropenic cancer patients

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This work aimed at identifying *Candida* species isolated from blood stream infection in non-neutropenic cancer patients by conventional and molecular methods. Out of 200 blood samples from cancer patients with blood stream infection, 89 were positive for *Candida* which were identified using chromogenic medium (CHROMagar *Candida*), and real-time polymerase chain reaction (RT-PCR) assays targeting the fungal ITS2 region using a LightCycler instrument. The present study revealed that non-*albicans Candida* species caused most of the cases of candidaemia in the cancer patients. A total of 89 *Candida* isolates were obtained, *Candida albicans* represent 15/89 patients (16.85%) and non-*albicans Candida* species accounts for 74/89 patients (83.1%) of which *Candida tropicalis* represent 32.6% followed by *Candida parapsiliosis* 23.6%, and then *Candida rugosa* (21,4%) and the lowest percentage was in *Candida glabrata* (5.6%). The results of the study revealed that real time-PCR could detect 81 out of 89 cases of candidaemia with sensitivity of 91.01%, specificity of 95.6%, PPV of 94.18% and NPV of 92.30%. The high prevalence of non-*albicans* candidaemia should be considered prior to antimicrobical therapy in non-neutropenic cancer patients with candidaemia. Real-time PCR represents a rapid and accurate method for diagnosis of candidaemia.

Key words: Candidaemia, *Candida albicans*, real-time polymerase chain reaction (RT-PCR).

INTRODUCTION

Candida species are currently the fourth most common cause of bloodstream infections worldwide, and the third most common cause of bloodstream infections in patients with malignancy (Chung et al., 2005). Candidiasis is not only associated with mortality of about 30to 40% in cancer patients, but also extends the duration of hospital stay and increases the cost of medical care (Naglik et al., 2004). The clinical presentations of patients with blood stream infection caused by *albicans* and *non-albicans Candida* species are indistinguishable. However, *non-albicans candida* species are often less susceptible to fluconazole than *C. albicans* and may require a greater dosage of antifungals to cure clinically (Chakrabarti et al., 2008). Early diagnosis of invasive fungal infections is important in the management of invasive fungal infection and in improving the outcome and preventing relapse of the underlying malignancy (Verma et al., 2003).

Previous studies have revealed blood stream infection rates ranging from 4 to 22% due to *Candida* species in cancer patients. The rise in incidence of non-*albicans*

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Table 1. Sequence and concentration of the primers, probe and probe specific T_m in real time PCR assays (Hebart et al., 2000).

Probre or primer	Sequence (concn)	Probe specific T_m (°C)
***Candida*-specific primers**	CCTGTTTGAGCGTCRTTT (0.15 µM) TCCTCCGCTTATTGATAT (0.5 µM)	
***Candida*-specific probe**		
C.alb-S	Cy5-CATTGCTTGCGGCGGTA-biotin (0.2 µM)	66 or 55
C.glab-S	Cy5GTTTTACCAACTCGGTGTTGAT-biotin (0.2 µM)	65
C.trop-S	Cy5-GGCCACCACAATTTATTTCA-biotin (0.2 µM)	63
C.rugosa-S	Cy5-CGAGCGAACTAGACTTT-biotin (0.2 µM)	60
C.para-S	Cy5-GAAAGGCGGAGTATAAAC-biotin (0.2 µM)	58

candidaemia and the emergence of antifungal resistance have further fuelled the need to carry out such a study (Halliday et al., 2005).

Azoles, antifungal agents have therapeutic activity against different *Candida* species. Among the azoles drugs, fluconazole shows that good tolerance has appeared and antifungal drug resistance is quickly becoming a major problem especially in immuno-compro-mised patients (Xu et al., 2000). This resistance also favors the emergence of *C. krusei* and *C. glabrata* (Cirak et al., 2003).

Conventional fungal identification methods may require 48 to 72 h or longer in order to yield definitive identifications, leading to significant morbidity and mortality that is associated with invasive *Candida* infections (Boyanton et al., 2008). Real time-polymerase chain reaction (PCR) technology can directly detect the presence of fungi in hours with high level of sensitivity and specificity *(*Chen et al., 2000; Loeffler et al., 2000).

The identification of species via melting curve analysis with species-specific hybridization probes further increases specificity. The fast turnaround time of less than 2 h is another advantage of the real-time PCR technology (Verweij and Meis, 2000). The aim of this study was to detect the incidence of *Candida* spp. in non-neutropenic cancer patients and evaluate the role of real time PCR in diagnosis of these cases.

MATERIALS AND METHODS

An approval was gotten from the ethical committee in Tanta Faculty of Medicine and a written consent from all participants in this study was performed over 12 months from March 2012 to March 2013 on cancer patients complaining of blood stream infection caused by *Candida* spp. (candidemia). An episode of candidaemia was defined as the isolation of *Candida* species from a single positive blood culture. 6 ml blood was taken from each patient; 3 ml were used for microbiological study using standard cultivation methods and 3 ml with citrate anticoagulant stored at −20°C until it was used for DNA extraction and real time-PCR.

Blood culture and organism identification

For diagnosis of candidaemia, 3 ml blood was collected in BacT/ALERT FA aerobic blood culture bottles (bioMérieux). Blood culture bottles were subcultured onto Sabouraud's dextrose agar. *Candida* cultures were considered positive if a 10 CFU appeared on the plate. Identification of these isolates was carried out by growth on the chromogenic medium CHROMagar by colony morphology and pigmentation according to the manufacturer's instructions and biochemical reactions (Forbes et al., 2002).

Real time-PCR analysis

From blood samples, DNA was extracted with a High Pure PCR template preparation kit by following the instructions of the manufacturer. DNA was eluted with 100 µl elution buffer and stored at -20°C until use **(**Hebart et al., 2000).

The 20 µl real-time PCR mixtures were prepared with 2 µl of LightCycler-FastStart DNA master SYBR green I (Roche Molecular Biochemicals, Mannheim, Germany), 4 mM $MgCl_2$, primers and probes as shown in Table 1, and 3 µl of DNA extract made up to 20 µl with water. PCR was performed in a LightCycler instrument with preliminary denaturation for 10 min at 95°C, followed by 60 amplification cycles (with a temperature transition rate of 20°C/s) of denaturation at 95°C for 8 s, annealing at 55°C for 10 s, and primer extension at 72°C for 10 s, with a single fluorescence acquisition step at the end of the extension. This was followed by a melting analysis of the probe-PCR product consisting of 95°C for 30 s and then cooling to 35°C for 60 s before the temperature was raised to 98°C at a rate of 0.2°C/s with continuous fluorescence acquisition. A final cooling step was performed at 40°C for 10 s. Samples were considered positive for *Candida* species upon the presence of a biprobe-specific melting peak. Specific T_m values of the biprobes are shown in Table 1.

RESULTS

During a 12 months period (from March 2012 to March 2013), out of 200 cancer patients with blood stream infection admitted to Oncology and Internal Medicine Departments in Tanta University Hospital, 89 were diagnosed with candidaemia were eligible for the study. 43 patients (48.3%) out of 89 were males and 46 (51.6%) were females, ranging in age from 15-79 (mean ± SD, 49 ± 3.49) years. Gastrointestinal and breast cancer were the most frequent among the studied group accounting for 35.9 and 26.9%, respectively. The mean of the hospital stay was 3.58 (range; 1-9) days. Tables 2 and 3 present demographic and clinical characteristics of cancer patients with candidaemia.

Table 2. Demographic characteristics of cancer patients with candidiasis.

Patient characteristic	Number (%)
sex	
Male	43 (48.3)
Female	46 (51.6)
Age (years)	
Range	15-79
mean±SD	49 ±3.49
Days admitted in hospital (days)	
Range	1-9
Mean±SD	3 ±4.45
Cancer type	
GIT	32 (35.9)
breast	24 (26.9)
lung	10 (11.23)
Head and neck	12 (13.48)
Others	11 (12.35)
total	89 (100)

Table 3. Clinical criteria of patients with candidaemia.

Patients with candidaemia	*C. albicans*	Non *C. albicans*
No. 89	No.15 (16.85%)	No.74 (83.15%)
Age (years)	36.07±17.4	34±17.02
Sex male/female	7/8	35/29
Steroid therapy	1 (6.66%)	15 (20.27%)
Broad spectrum AB	15 (100.00%)	74 (100.00%)
Mortality	5 (33.33%)	30 (40.54%)

Table 4. Frequency of isolation of *Candida* spp.from 89 cancer patients with candidaemia.

Candida spp.	Number (%)
Candida albicans	15 (16.9)
C.tropicalis	29 (32.6)
C. parapsilosis	21 (23.6)
C. rugosa	19 (21.4)
C. glabrata	5 (5.6)
Total	89 (100.00)

Considering the etiology of candidaemia, *Candida albicans* was the cause in 15/89 patients (16.9%) in contrast to non-*albicans Candida* species in 74/89 patients (83.1%). Of the non-*albicans* species, *Candida tropicalis* was responsible for the maximum number of episodes (29/89, 32.6%), followed by *Candida parapsilosis* (21/89, 23.6%), *Candida rugosa* (19/89, 21.4%) and *Candida glabrata* (5/89, 5.6%) (Table 4). The crude mortality in patients suffering from non-*albicans* and *albicans* candidaemia was 40.54 and 33.33%, respectively, the difference was not statistically significant. Distribution of *Candida* spp. in relation to cancer type is shown in Table 5.

As regard the results of real time-PCR, it was found that out of 89 blood samples which were positive for *Candida* spp. By conventional culture method 81 (91.01%) were positive for *Candida* spp. by real time-PCR and out of 111 negative blood samples by conventional culture methods 96 blood samples were negative for *candida spp.* by real time-PCR. Table 6 shows the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of real time-PCR when compared with conventional culture methods.

DISCUSSION

This study described bloodstream infections caused by *Candida* spp. in cancer patients using conventional methods

Table 5. Distribution of *Candida* spp. in relation to cancer type.

Diagnosis	*C. albicans*	*C. tropicalis*	*C. parapsilosis*	*C. rugosa*	*C. glabrata*
GIT	4 (4.49%)	7(7.86%)	5(5.61%)	3(3.37%)	1(1.12%)
breast	3 (3.37%)	7(7.86%)	2 (2.24%)	6(6.74%)	2(2.24%)
lung	1 (1.12%)	5 (5.61%)	3(3.37%)	4(4.49%)	-(00.00%)
Head and neck	6 (6.74%)	7(7.86%)	7(7.86%)	4(4.49%)	2(2.24%)
Others	1(1.12%)	3(3.37%)	4(4.49%)	2**(**2.24%)	- (00.00%)
Total 89 (100.00%)	15(16.9%)	29(32.6%)	21(23.6%)	19(21.4%)	55 (5.6%)

Table 6. Results of conventional culture and real time-PCR.

Sample	*BC results for *Candiada* spp.	PCR +ve	PCR -ve	Total	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Blood (*n*=200)	Positive (89)	81	8	89	91.01	95.6	94.18	92.30
	Negative (111)	5	96	111				

*BC: Blood culture.

and real time PCR to provide information on the epidemiology of candidaemia in this patient population by conventional culture methods; the results of this study showed that *Candida* spp. could be isolated from 89 (44.5%) cases out of 200 cases of blood stream infection from oncology unit in Tanta university hospital in a period of 10 months (from March 2012 to March 2012). These results are different from the results of Borzotta and Beardsley (1999) that reported the rate of candidaemia in cancer patients to be 1.5%. This difference may be attributed to the difference of the regimen of chemotherapy or the rules of infection controls in each institution.

In this study, non-*albicans Candida* species accounted for the majority of the episodes of candidaemia in 74/89 patients (83.1%). Previous studies have shown high rates of carriage of these species on the hands of healthcare workers *(*Rangel-Frausto et al., 2006). Thus, the hands of healthcare workers could be one possible source of transmission; these results are in agreement with the two studies of Chakrabarti et al. (2009) and Xess et al. (2007), respectively that could isolate *non-Candidal* strains from 75 and 66% population of patients. In the present study, *C. tropicalis* was isolated from 29 (32.6%) cases, these results came in agreement with the results of Pfaller et al. (2010), who reported that worldwide, the frequency of bloodstream infections due to *C. tropicalis* has increased. In India, *C. tropicalis* ranks first among non-*albicans Candida* species in causing candidaemia (Chakrabarti et al., 2009; Xess et al., 2007).

In this study, *C. glabrata* could be isolated from 5 (5.6%) cases and *C. krusei* was not detected in the isolated strains, the results are in accordance with that of Vollmer et al (2008) that could isolate only one strain of *C. krusei* from different clinical specimens in immunocompromised patients.

In the present study, *C. tropicalis* and *C. parapsilosis* were the two most commonly isolated species from cases of candidaemia. Thus, the species distribution in this study closely matched that of previous studies from North India (Xess et al., 2007). However, points in which this study differed from others were the lower isolation rate of *C. glabrata* (5.6%) and the absence of any case of candidaemia due to *C. krusei*. These findings probably reflect the absence of selective pressure exerted by azole antifungal prophylaxis at our institutions, which would have led to isolation of species that are known to be inherently resistant to fluconazole such as *C. krusei.*

In the present study, *C. rugosa*, could be isolated from a large number of cases of fungaemia (19/89, 21.4%), the result which is in agreement with that of Minces et al. (2009) who isolated *C. rugosa* from 25% of cases. Minces *e*t al. (2009) also was in agreement with our study in that blood stream infections with *Candida* spp. are especially common among patients in Intensive Care Units and associated with typical risk factors such as indwelling central vascular catheters, broad-spectrum antibiotics and steroid therapy. These results are in agreement with that of Colombo et al. (2006) who reported a large number of episodes of fungaemia due to *C. rugosa*. In addition, an outbreak of fungaemia due to *C. rugosa* was reported by Colombo et al. (2003).

Real-time PCR, is an approach that enables culture-independent screening of *Candida* infections within only a few hours. Rapid diagnosis would improve the survival rate in cancer patients. In this study, real-time PCR facilitated a rapid screening for simultaneous differentiation of the isolated 5 medically important *Candida* species causing blood stream infection in cancer patients in the same LightCycler run.

In the present study, real-time PCR showed mostly high

sensitivity. In some samples, PCR detected a greater number of different *Candida* species than culture. In these cases, patients had received antifungal therapy before sampling, which may be a reason for these discrepancies. Furthermore, whenever *C. albicans* was detected in addition to the cultured *Candida* (*C. glabrata* and *C. tropicalis*) species, *C. albicans* might have been overgrown by the other fungi and therefore missed by culture. On the other hand, culture detected a greater number of different species than real-time PCR in five samples, which have been already observed in previous studies (Willinger et al., 2003)

The present study revealed that blood culture is considered to be an important tool for the detection of systemic infection but it is time consuming and may lead to increase in the mortality of cancer patients with candidaemia. This is in contrast with Einsele et al. (1997) and Pfaller et al. (2010) who reported that the culture was positive in less than 50% of patients with disseminated candidiasis.

Conclusion

The high prevalence of non-*albicans* candidaemia should be considered prior to antimicrobical therapy in non-neutropenic cancer patients with candidaemia. Real-time PCR represents a rapid and accurate method for diagnosis of candidaemia.

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