EARLY DETECTION OF CANDIDA ALBICANS IN NEONATAL INTENSIVE CARE UNITS

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ABSTRACT

Background and objective: Candidemia is a one of the most common causes of late-onset sepsis in the neonatal intensive care unit (NICU) and is associated with significant morbidity and mortality. The aim of this work is to evaluate the value of Real-Time PCR for early detection of blood stream Candida albicans infection (candidemia) in NICU. ***Materials and Methods***: The study included 60 newborn admitted in NICU of Al-Zhraa hospital, Al-Azhar University Hospital for girls, Egypt, during the period from January 2010 to January 2011, who had one or more risk factors for candidemia. A total of 60 blood samples were submitted to testing by blood culture and real-time PCR. 20 cases were diagnosed as candidemia by blood culture method, the other 40 neonates were negative for candidemia by blood culture method considered as control group. ***Results***: the blood culture was positive in 20/60 samples and Candida albicans was the predominant cause of candidemia 15/20 (75 %) followed by C. tropicalis 4/20 (20 %) and C. glabrata 1/20 (5 %). Real-time PCR for Candida albicans was positive in 14/15 and gave only one false-positive and false-negative result. It had a sensitivity, specificity and accuracy of 93.3 %, 97.8 % and 96.7% respectively. Central venous catheter (CVC) was the most frequent risk factor among candidemic cases 15/20 (75 %), followed by mechanical ventilation (MV), endotracheal tube (ETT) and admission > 10 days in NICU (45 %) for each. Out of 20 patients, 17 had more than one risk factor (Odd Ratio [OR] 22.7; 95%CI 5.31, 96.77; $p<0.0001$). ***Conclusion***: Real-Time PCR can be applied as a rapid diagnostic method for candidemia and can aid in early antifungal therapy and in minimizing the several drawbacks of candidemia. Identification of risk factors is more important in predicting the neonates who are at risk for candidemia.

INTRODUCTION

Candidemia is the fourth most common nosocomial bloodstream infection [1]. Neonatal candidemia accounts for 12% to 15% of late-onset sepsis episodes in the NICU [2]. Neonates diagnosed with candidiasis are at significant risk for mortality (20%) and morbidity [3, 4, 5]. Pediatric patients in critical condition, particularly neonates, are especially vulnerable

to invasive Candida infections (ICI), partly because of their age and severe underlying disease and partly because of the invasive procedures used [6]. Risk factors such as preterm babies with low body weight, staying for long-term periods at intensive care units, subjected to invasive procedures, receiving one or more antibiotic schemes are considered to pose a risk for the development of infections by opportunistic agents [7]. Although C. albicans remains the most common fungal pathogen isolated from blood and body tissue, recent literature shows an increased prevalence of non-candida species [8, 9, 10]. The diagnosis of invasive candidiasis is difficult because there are no specific clinical manifestations of the disease and colonization and infection are difficult to distinguish. In the last decade, much effort has been made to develop reliable tests for rapid diagnosis but non of them found widespread clinical use [11]. Traditional diagnostic methods based on culture techniques and biochemical tests require 2 or more days and could be inaccurate [12], although a blood culture positive for candida was considered the gold standard for diagnosis of candidemia [13]. A variety of PCR assays based on the detection of fungal DNA in sterile human body fluids or tissue samples to allow early diagnosis of fungal infections and to improve the survival rate of patients suffering from invasive infections has been described. However, none of the developed PCR assays have been standardized, resulting in diverging results [13]. The introduction of real-time PCR technology in the detection of fungal infections has increased reliability of PCR results compared to results obtained by conventional PCR methods. Real-time PCR sharply decreases the risk of false-positive results due to PCR product carry-over during gel electrophoresis or enzyme linked immunoassays to check the specificity of the conventional PCR product [14]. The aim of this work is to evaluate the value of risk factors and real-time PCR for early detection of candidemia in NICU patients before development of clinical sepsis.

MATERIALS AND METHODS

Clinical samples were obtained from Sixty newborn admitted in NICU of Al-Zhraa hospital, Al-Azhar University Hospital for girls, Egypt, during the period from January 2010 to January 2011, who had one or more of the risk factors for candidemia. All samples were evaluated by blood culture and real-time PCR.

Approximately 2.5 mL of fresh blood obtained from patients was inoculated aseptically into a blood culture bottle containing 25 mL of Sabouraud's broth (Sigma-Aldrich Chemie GmbH) and incubated at 37°C for 14 days maximum. After 24 hours incubation subculture was done on: Sabouraud's dextrose agar (SDA) with chloramphenicol (Oxoid, UK): plates were incubated aerobically at 37°C for 48 hours. All plates were examined after the given periods for any growth. If no growth was found, subcultures were done every three days for three successive times and if still no growth was seen, cultured plates were considered negative and excluded. Phenotypic identification of Candida species was based on germ tube assessment and CHROMagar Candida (Hardy Diagnostics Inc.).

Detection of Candida albicans by quantitative Real-time PCR: The clinical specimens were tested using Candida albicans-specific real-time PCR assay. The amplified PCR products were identified with Light Cycler SYBR Green I dye fluorescent based on LightCycler FastStart DNA MasterPLUS SYBR Green I (Roche).

DNA extraction: DNA was extracted from 200 µL of serum by MagNA Pure Compact Nucleic Acid Isolation Kit I. using MagNA Pure Compact instrument for automated purification of DNA [15]. DNA extraction from a clinical sample is a critical step for molecular diagnosis. DNA extraction in the present study was done by an automated system using MagNA Pure Compact nucleic acid isolation kit I with the MagNA Pure Compact instrument. Total time for the automated purification of DNA from 8 samples is about 25 minutes [15]. Automated extraction systems have the advantage over manual extraction in that recovery of nucleic acids is consistent and reproducible, also it keep sample manipulation to a minimum and hence reducing the risk for cross contamination. Finally, once these systems have been validated and proper maintenance procedures are in place, quality control monitoring is less intensive than that required for manual extraction [16].

Candida albicans-specific primers: Primers for C. albicans are specific for the ITS (Internal Transcribed Spacer) of the fungus, with amplicon size 273 bp and melting temperature of 85.7±0.7 °C: Forward: (5`→3`) TTTATCAAC TTGTCACACCAGA, gene bank accession no. L47111, Reverse:

(5`→3`) ATCCCGCCTT ACCACTACCG, gene bank accession no. L28817 [17].

PCR Protocol: The C. albicans DNA extracted from serum samples was subjected to a consensus primer mediated PCR method using the LightCycler FastStart DNA MasterPLUS SYBR Green I (Roche). The standard PCRs were carried out in 20 ul. The samples were cycled according to the LightCycler protocol. There was an activation step of Taq polymerase at 95 °C for 10 minutes, followed by 45 cycles of denaturation at 95 °C for 10 s , annealing at 65 °C for 5 s and extension at 72 °C for 10 s. This was followed by melting analysis consisting of 95 °C for 30 s. and then cooling to 35 °C for 60 s. before 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 [17].

Results were interpreted as positive or negative by the presence or absence of the melting temperature peak of 85.7 ± 0.7 °C and by the effectiveness of quantitative score where the data was saved and processed by the software of the analyzer both can increase the specificity and sensitivity of amplification reactions. The real-time PCR assay analytical detection threshold was 200 copies of C. albicans amplicon / ml.

STATISTICAL ANALYSIS

Analysis of the collected data was done using SPSS version 11.0. Data were presented as mean ± Standard deviations for continuous variables. For qualitative variables, frequency data were summarized as number and percentage. Chi square (X2), Odd Ratio [OR] and 95% confidence intervals (CI) were applied for comparison of qualitative data. p value considered significant if <0.05. Sensitivity (true positive ⁄true positive + false negative), specificity (true negative ⁄true negative + false positive), positive predictive value (true positive⁄true positive + false positive), negative predictive value (true negative ⁄true negative + false negative) and accuracy (true positive + true negative ⁄ true positive + false positive + false negative + true negative).

RESULTS

Samples were obtained from 60 neonatal patients at risk for candidemia, 29 were males and 31 were females. A total of 60 samples were analyzed by blood culture and real-time PCR assays. Out of these, 20 showed positive blood cultures. Table 1 shows: the demographic and the distribution

of risk factors in cases and control. Table 2 shows: The risk factors within this population of patients with candidemia. All patients had at least one predisposing risk factor. The most common risk factor was the use of central venous catheter (75%), followed by mechanical ventilation, endotracheal tube, Admission in NICU > 10 days (45%) for each. Table 3 shows: Most of the patients had multiple risk factors. Out of the 20 patients, 12 had three different risk factors and 5 had four risk factors. Only, 3 cases had one risk factor. Table 4 shows: The distribution of Candida species among the blood culture positive patients. Candida albicans, Candida tropicalis and Candida glabrata caused 75%, 20%, 5% of the candidemia respectively. Table 5 shows: Real-time PCR was positive in 14 samples and gave only one false-positive and false-negative result, its diagnostic indices are detailed in Table 5.

Table (1): Demographic characteristics and risk factors in cases and control.

Variables	Cases (No = 20)		Control (No = 40)	
	No	%	No	%
CVC	15	75.0%	11	27.5%
MV	9	45.0%	8	20.0%
ETT	8	40.0%	6	15.0%
Postnatal Steroids	4	20.0%	8	20.0%
Antibiotics	4	20.0%	8	20.0%
Admission > 10 days	9	45.0%	3	7.5%
GA < 32 weeks	5	25.0%	2	5.0%
VLBW <1500 gram	5	25.0%	2	5.0%
Multiple risk factors	17	85.0%	8	20.0%
Males	5	25.0%	24	60.0%
Age in days (on admission)	3.95±2.39		4.83 ±2.34	

CVC; Central venous catheter, MV; Mechanical ventilation, ETT; Endotracheal tube, VLBW; Very low birth weight, GA: gestational age.

Table (2): The risk factors for predicting cases of candidemia in NICU patients..

Variables	Candidemia (N=20)		P-value	OR	95% Confidence Interval	
	N	%			Lower	Upper
CVC	15	75	0.00	7.91	2.32	26.98
MV	9	45	0.04	3.27	1.01	10.57
ETT	8	40	0.04	3.78	1.09	13.13
Postnatal Steroid	4	20	0.64	1.00	0.26	3.82
Antibiotics	4	20	0.54	1.18	0.30	4.61
Admission >10 days	9	45	0.001	10.09	2.32	43.88
GA < 32 weeks	5	25	0.04	6.33	1.11	36.27
VLBW < 1500 grams	5	25	0.04	6.33	1.11	36.27

CVC; Central venous catheter, MV; Mechanical ventilation, ETT; Endotracheal tube, VLBW; Very low birth weight, GA: gestational age, OR; Odd ratio, *p* significant if < 0.5.

Table (3): Multiple risk factors for predicting cases of candidemia in NICU patients.

Multiple risk factors	Candidemia (N=20)		P	OR	95% Confidence Interval	
	N	%			Lower	Upper
CVC, MV and ETT	8	40	<0.0001	22.7	5.31	96.77
CVC, VLBW, GA< 32w and Admission > 10 days	5	25				
Steroids, Antibiotics and Admission > 10 days	4	20				
Total	**17**	**85**				

CVC; Central venous catheter, MV; Mechanical ventilation, ETT; Endotracheal tube, VLBW; Very low birth weight, GA: gestational age, OR; Odd ratio, *p* significant if < 0.5.

Table (4): Distribution of various Candida species isolated from blood in cases of neonatal candidemia.

Species	Blood culture	
	No	%
C. albicans	15	75.0 %
C. tropicalis	4	20.0 %
C. glabrata	1	5.0 %
Total	20	100.0 %

Table (5): Sensitivity and Specificity of real-time PCR for diagnosis of Candida albicans among studied groups.

Test		Culture		Sensitivity	Specificity	Predictive value		Accuracy
		+ve	-ve			+ve	-ve	
PCR	+ve	14	1	93.8 %	97.8%	93.9%	97.8%	96.7 %
	-ve	1	44					

PCR; Polymerase chain reaction, +ve; Positive, -ve; Negative

DISCUSSION

The present study emphasizes the importance of candidemia among neonatal intensive care unit (NICU). Candidemia is not only associated with a significant mortality but also extends the duration of hospital stay and increases the cost of medical care [18]. All these drawbacks are related to many factors among them; most of clinicians do not pay attention to risk factors till clinical sepsis develop or even after that. A septic-appearing newborn routinely receives antibacterial agents at the time of clinical presentation. In contrast, many clinicians will only start antifungal therapy when a positive culture is reported or when the patient fails to improve after a

period of antibacterial agents. On the other hand, the routine blood cultures for candidal identification up to species level and susceptibility till reporting results take also a long time that can aggravate these drawbacks. This study, aimed to evaluate the value of risk factors and real-time PCR in early diagnosis of candidemia before appearing of clinical sepsis.

Numerous studies have identified risk factors, among them are: receiving broad-spectrum antibiotic therapy and steroids [19, 20]. Infants with positive or negative culture showed no difference between receiving broad-spectrum antibiotic therapy ($p = 0.54$), and steroids ($p = 0.64$) (table 1).

Our Statistical analysis revealed the following significant risk factors associated with Candida spp. isolation: central intravascular catheter (CVC), mechanical ventilation (MV), endotracheal intubation (ETT), prolonged hospitalization, VLBW and gestational age (GA) < 32 ($p < 0.05$) (table 2). All of these extensively reported as major factors for candidemia. [19, 21-23]. CVC was the most frequent risk factor in the positive blood culture cases (75%). It was the only risk factor in two positive cases, while was associated with other risk factors in 13 cases (table 3). We observed that most of patients with positive blood cultures had multiple risk factors ($P<0.001$, OR =22.7, 95% CI =5.31 – 96.77) (table 3). Several other studies have illustrated the link between the presence of multiple risk factors (e.g. duration of intubation and length of NICU hospitalization or antibiotics and steroids) and emergence of fungal sepsis [19, 24- 26].

Candida albicans was the most common isolate (75%) as reported by others [27]. Over the years, non-albicans Candida infections have become more frequent as major causes of neonatal candidemia [28 - 30]. Candida parapsilosis was reported to be most common isolate in several studies [23, 31- 33]. Our data shows Candida tropicalis to be the second most common agent (20%). Marı´a t al., reported that Candida tropicalis represented 10% of patients with candidemia in his study, which may be acquired by horizontal transmission [34]. Its ability to produce clusters has been one of its major pathogenic components. However, C. tropicalis has not been thoroughly studied and could potentially play an important role in nosocomial infections [35, 36]. Our data revealed that Candida glabrata was the least cause of

neonatal candidemia (5%). Although it was considered to be less pathogenic but studies have shown no significant differences in morbidity among species [37].

The technique used in this study was the real-Time PCR for Candida albicans. So, the comparison was applied according to whether the case was positive or negative for Candida albicans by blood culture while other Candida species were not included to make this comparison possible.

Several PCR assays have been developed for the diagnosis of candidemia, in this study, we evaluated the efficiency of real-time PCR for the diagnosis of candida albicans as a cause of candidemia in NICU patients at high-risk for candidemia. Real-Time PCR assay was directly performed from serum samples obtained from these patients. To date, the optimal biological specimen for the molecular detection of candidemia is a matter of debate. Some authors have used serum [38, 39], whole blood samples [40, 41] or blood culture samples [42] for the detection and identification of Candida spp. for diagnosis of candidemia.

The real-time PCR assay identified 14 of 15 positive specimens detected by blood culture with one false-negative and one false-positive results. Negative PCR in ultimately culture-positive samples represents a serious problem in making a diagnosis and may return to a deficient DNA extraction, small amounts of target DNA in the sample, as well as the small amount of sample submitted to PCR, or PCR inhibitors. The melting curve analysis designed in this study detected Candida albicans DNA from 14 patients with a positive blood culture and gave only one false-positive result when compared with the reference standard, blood culture with a sensitivity, specificity, PPV, NPV and accuracy of 93.9% 97.8% 93.9% 97.8% and 96.7% respectively. It should also be noted that the inexpensive blood culture system used in this study may have a lower sensitivity than widely used automated culture systems moreover, the ability of the automated system (MagNA Pure Compact) used for extraction to recover minute amounts of DNA in an efficient manner may thus preferentially inflate the false-positive rate or represented true false-positive results caused by PCR contamination.

Lack of standardization in the molecular diagnosis of candidemia made many discrepancies in sensitivity and specificity of several studies [27]. Our results

were matched with some studies [43- 45] but did not match with others [13, 42, 46- 48]. In general, the introduction of real-time PCR technology together with automated extraction systems in the detection of fungal infections have increased the reliability of PCR results compared to results obtained by conventional PCR methods. The identification of species via melting curve analysis with species-specific hybridization probes further increases specificity, as one mismatch in the probe binding site would lead to an altered melting temperature (Tm). The fast turnaround time of less than 2 h is another advantage of the real-time PCR technology [49].

Our study was focused on Candida albicans because it was the most common pathogen isolated during routine work as processed by others [50]. The differentiation between clinically relevant Candida species is of great value in guiding antifungal therapy [51]. Van Burik et al. and White et al. described panfungal PCR assays that can only detect the genus Candida [52, 53]. Recently, Schabereiter-Gurtner et al. [54] described a Light Cycler assay that allows the simultaneous detection and identification of various Candida and Aspergillus species; its clinical efficiency needs to be assessed in prospective studies.

CONCLUSION

In conclusion: Real-Time PCR can be applied as a rapid diagnostic method for candidemia and can aid in early antifungal therapy and in minimizing the several drawbacks of candidemia. Identification of risk factors is more important in predicting the neonates who are at risk for candidemia.

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الكشف المبكر عن فطر الكانديدا البيكانز في مرضى العناية المركزة للأطفال حديثي الولادة

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يعتبر وجود فطر الكانديدا بالدم واحدا من أهم الأسباب التي تؤدى ظهور تسمم الدم المتأخر فى الأطفال حديثي الولادة وقد تسببت فى حدوث نسبة عالية من الاعتلال و الوفيات.

والهدف من هذا البحث : هو تقييم طريقة تفاعل البلمرة المتسلسل فى الزمن الحقيقي للتشخيص المبكر لعدوى الكانديدا البيكانز بالدم في مرضى العناية المركزة للأطفال حديثي الولادة.

وقد شملت هذه الدراسة ستون مريضا من أطفال أدخلوا للعناية المركزة للأطفال حديثي الولادة وكان يوجد عندهم سببا واحدا أو أكثر يؤدى للإصابة بفطر الكانديدا بالدم. ستون عينة دم خضعت للفحص باستخدام طريقة مزارع الدم التقليدية وبطريقة تفاعل البلمرة المتسلسل فى الزمن الحقيقي لاكتشاف العدوى . وأصفر البحث عن وجود عشرون حالة مصابة بفطر الكانديدا بالدم وأعتبر الأربعون حالة الأخرى كمجموعة ضابطة.

و قد أوضحت هذه الدراسة أن : وجد أن مزارع الدم التقليدية كانت إيجابية فى عشرون عينة من الستون عينه محل البحث وكان فطر الكانديدا البيكانز الأكثر شيوعا بين حالات المصابة الكانديدا بالدم (75%) ويليه فطر الكانديدا تروبيكالز (20%) ثم

فطر الكانديدا جلابراتا (5%). تفاعل البلمرة المتسلسل فى الزمن الحقيقي للكشف عن فطر الكانديدا البيكانز كان إيجابيا فى عينة أربعة عشر عينة من الخمسة عشر عينة التي تم كشفها بطريقة مزارع الدم التقليدية كما أوضح البحث أن هذه الطريقة أعطت حالة إيجابية كاذبة وأخرى سالبة أوضحت الدراسة أن حساسية هذه الطريقة 93.3% ودرجة تحديدها 97.8% والدقة 96.7%. ووجود القسطرة الوريدية من أشهر عوامل الخطورة للإصابة بهذه العدوى ويليها التنفس الصناعي والأنبوبة الحنجرية والحجز أكثر من عشرة أيام بالمحضن (45%) لكل عامل. من العشرين مريضا المصابين بالكانديدا فى الدم كان هناك سبعة عشر مريضا لديهم أكثر من عامل من عوامل الخطورة المسببة للعدوى بالكانديدا فى الدم (22.7).

ونستنتج من هذه الدراسة : أن معرفة عوامل الخطورة هو الأهم فى توقع الأطفال الأكثر عرضة لحدوث عدوى الكانديدا فى الدم بالرغم من أن مزارع الدم تعتبر هي المقياس الذهبي أو الطريقة الأكيدة لتشخيص عدوى الكانديدا بالدم ولكن حساسيتها ودقتها فى تشخيص عدوى الكانديدا بالدم منخفضة ومضيعة للوقت. أن تفاعل البلمرة المتسلسل فى الزمن الحقيقي هو اختبار ذو حساسية ودرجة تحديد ودقة عالية فى تشخيص عدوى الكانديدا بالدم وطبقا للدراسة الحالية فإن تفاعل البلمرة المتسلسل فى الزمن الحقيقي تستخدم كطريقة سريعة فى تشخيص الكانديدا بالدم وتساعد على العلاج المبكر لاستخدام مضادات الكانديدا وتقلل الآثار الجانبية العديدة للكانديدا بالدم.