PCR in Comparison with Culture Methods for The Diagnosis of Candida albicans Responsible for Candidemia in Leukemic Patients

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Abstract

Background: Currently, candidemia infections represent an increasing cause of morbidity and mortality in seriously ill hospitalized patients. Because the accurate diagnosis of candidiasis remains difficult, fast and reliable assay for characterization of fungal pathogens is critical for the early initiation of adequate antifungal therapy and/or for introduction of preventive measures.

Objective: To detect candidemia in leukemic patients by molecular methods in comparing with golden standard method (culture method).

Materials and methods: A total of 60 leukemic patients were included in this study. Clinical type and other demographic data were recorded. Blood samples were taken from each patient, culture; germ tube formation and carbohydrate fermentation were done for each sample. DNA extraction and polymerase chain reaction (PCR) were used for detection of *Candida albicans*in cultured bottles. This study was conducted on leukemic patients admitted to four different hospitals in Baghdad city from September 2010 to March 2011. Sixty patients suffering from acute lymphoid (ALL) and myeloid (AML) leukemia were included in this study. The age of patients were ranging between 3-46 years old. Twenty five apparently healthy individuals were enrolled in this study as control group. Three milliliters of blood were collected from each patient; 1.5 ml was inoculated in 20 ml Brain heart infusion broth (Cruikshank. 1975). The rest of blood (1.5ml) was stored in -20ᴼC for further analysis. Blood cultures were incubated at 37°C for 10 days, and examined daily for growth. DNA purification kit was purchased from QIAGENE®Company. This method was used for the purification of genomic DNA from fresh or frozen samples of 1 ml overnight yeast cultures by using the GentraPuregene Yeast/Bact. Kit. PCR was performed to detect *Candida albicans* species through the amplification of specific gene (αINT1)

Results: Only one positive culture result out of 60 samples was obtained for *Candida sp.,* (1.7%). PCR results showed that there were only three out of sixty were positive for *C. albicans* (5%). In this study we obtained only one positive sample according to culture, while three samples only gave positive results according to PCR method. These results suggest that molecular analysis of candidemia is more sensitive and less time consuming than culture and other conventional methods.

Conclusions: we concluded the following: The rate of candidemia was 1.7% among leukemic patients, according to culture results, only 5% of blood cultures was positive according to PCR. Results showed 100% sensitivity and 96.6% specificity and it is rapid, easy, reliable and also applicable in clinical laboratory for identification of medically important *Candida spp*

Key words: Candidemia, PCR, *Candida albicans*.

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Introduction

Candida albicans is a member of the normal microbial flora colonizing human gastrointestinal and vaginal tracts (Odds, 1988). In healthy human hosts, it may only cause a range of mild superficial infections [1]. But in immunocompromised patients, life-threatening systemic candidiasis may develop [2, 3].

Candida species are the fourth common cause of blood stream infections (BSI) in hospitalized patients, and the most commonly isolated species include *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. parapsilosis* [4].

However, routine identification procedures from positive blood cultures requires at least one day for the pure culture and germ tube test. An additional 2-4 days for chlamydiospore formation and assimilation tests, especially for the germ tube negative isolates, which take up to 5 days or longer [5].

The current "gold standard" for detection of candidemia is blood culture. However, not only is it a time consuming method, but its sensitivity for early detection of infection has been reported to be as low as 50% [6,7].

However, diagnosis remains difficult and more importantly, rapid identification of infecting *Candida* species is necessary for early effective and appropriate antifungal therapy [8,9]. Molecular techniques are targeted to detect *Candida* species in a short period of time, with a high sensitivity and specificity.

Traditional methods that are used to identify clinical isolates of *Candida* species are time-consuming and not appropriate for rapid, accurate and reliable identification. So, this study aims to identify of *Candida albicans* in leukemic patients by standard gold method and biochemical tests and Molecular identification *Candida albicans* from blood culture samples by Polymerase Chain Reaction (PCR).

Materials and Methods

This study was conducted on leukemic patients admitted to four different hospitals in Baghdad city from September 2010 to March 2011 are: Al-Kadhemiya Teaching Hospital, (Medicine ward), City of Medicine, (Medicine and Hematology ward), Central Teaching Hospital for Pediatrics, (Medicine ward) and Al-Yarmook Hospital / (Medicine ward).

Sixty patients suffering from acute lymphoid (ALL) and myeloid (AML) leukemia were included in this study. The age of patients were ranging from 3-46 years old. Twenty five apparently healthy individuals were enrolled in this study as control group.

Three milliliters of blood were collected from each patient; 1.5 ml was inoculated in 20 ml Brain heart infusion broth (Cruikshank. 1975). The rest of blood (1.5ml) was stored in -20°C for further analysis.

Isolation and Identification of Candida spp.:

Blood cultures were incubated at 37°C for 10 days, and examined daily for growth. Examination was done by direct method (Staining with Lactophenol cotton blue stain), and indirect method by culture Sugar fermentation test and Germ tube test.

DNA Purification by Using the GentraPuregene Yeast/Bact. Kit:

DNA purification kit was purchased from QIAGENE®Company. This method was used for the purification of genomic DNA from

fresh or frozen samples of 1 ml overnight yeast cultures by using the GentraPuregene Yeast/Bact. Kit.

DNA quantification by spectrophotometer:

Determination of the DNA purity and quantity by spectrophotometer from the relative absorbance of DNA at 260nm and 280nm.

Polymerase chain reaction:

Candida albicans 500/730 IC is an *in vitro* nucleic acid amplification test for qualitative detection of *Candida albicans*in. PCR was performed to detect *Candida albicans* species through the amplification of specific gene (αINT1) which were derived from integrin – like protein alpha – INT1p. Synthetic oligonucleotides primer used were LH1 (5'-AGC CAC AAC AACAACAACAAC TCT) and LH2 (5'-TTG AGA AGG ATC TTT CCA TTG ATG).

Candida albicans 500/730 IC Test is based on three major processes: sample preparation, nucleic acid amplification of DNA using specific *Candida albicans* primers and detection of the amplified products on the agarose gel. The kit contains the Internal Control which can be used in the isolation procedure and serves as an amplification control for each individuall processed specimen and to identify possible reaction inhibition.

The following amplification program was started as: Thermal cycling conditions of PCR were as follows according to instructions of the kit manual. Denaturation; samples were initially denaturized at 95C^{o} for 5 minutes, and subjected to (42) cycles, each cycle consists of denaturation at 95C^{o} for 1 minute, annealing at 63C^{o} for 1 minute, extension at 72C^{o} for 1 minute, sample maintained at 72C^{o} for 1 minute. To avoid the risk of contamination of PCR samples, the precautions and guidelines advocated by kwok and Higuchi 1989 were followed. PCR products were resolved by horizental gel electrophoresis as follows: 10 ul of samples were separated on 1.5% agarose gel containing 0.5 ug/ml of Ehidium bromide in 1X TBE (pH 8.0) buffer visualized by UV illumination, photographed by digital camera.

Statistical Analysis

Statistical analysis was performed according to the statistical package for social sciences (SPSS) 19.01and Microsoft Excell 2010 for configuration of data, tables and figures. Categorical data were described as frequency and percentage; comparison done by Chi-square test. P-value of ≤ 0.05 was used as the level of significance.

Results

A total of 60 patients suffering from Acute lymphoid leukemia (ALL) and acute myeloid leukemia (AML) were enrolled in this study. Generally, their age were ranged from 4 to 53 years old, with mean age of 27.15 year.

Identification of *Candida* species:

Only one positive culture result out of 60 samples was obtained for *Candida sp.,* (1.7%). This isolate was belonged to patient with acute lymphoid leukemia. Carbohydrate fermentation test and germ tube formation test were applied for determination of *Candida* spp.

DNA extraction results:

DNA was extracted from inoculated brain heart infusion broth with specialized DNA extraction kit for yeasts. The results showed that there were only three positive cases out of sixty(5%), the size of the DNA fragments separated compared to the DNA ladder and appeared to have 11,000 bp, with DNA concentration 1.3µg/ml and DNA purity was 1.6 (Figure 1).

Two of the positive results were belonged patients with ALL (one of them was culture positive) and the other belonged to AML. There was no statistical significant difference for DNA extraction results and the type of leukemia p=0.635 (table 1, figure 1).

Table (1): Correlation between DNA extraction results and the type of leukemia.

		DNA extraction results		Total
		Negative	Positive	
Type of leukemia	ALL	30	2	32
	AML	27	1	28
Total		57	3	60

P value=0.635.

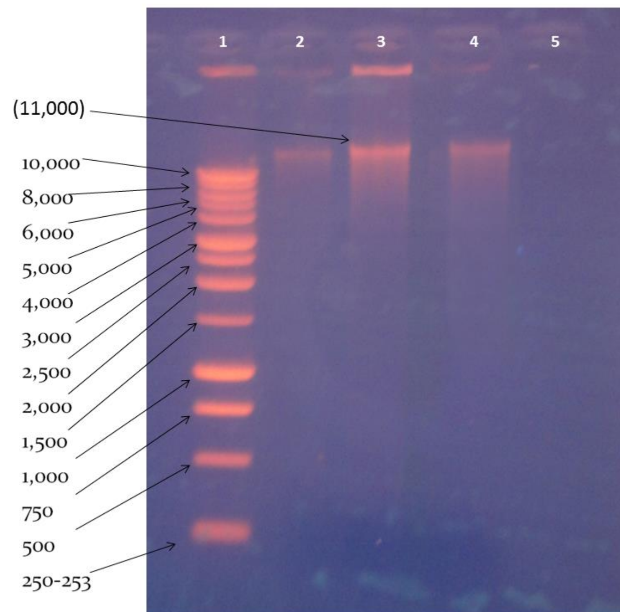

Figure (1): Agarose gel (1%) showing DNA bands extracted from blood culture on SA.
Lane 1: DNA ladder 1kb.
Lane 2, 3 and 4: DNA extracted from patients' blood culture samples.
Lane 5: negative control.

Polymerase chain reaction of *Candida albicans*:

PCR results showed that only three out of sixty were positive for *C. albicans* (5%).

Two of those patients were males children with ALL and only one adult female with AML.

The value of kappa index of the DNA extraction results and PCR results was equal to 1, this value means that there was a complete agreement between these tests. While there was a partial agreement between PCR results and this obtained from Sabauroud's agar, When kappa index was equal to 0.487 (table 2, figure 2).

Table 2: Correlation between PCR results, culture results and DNA extraction.

		PCR results			P value	Sensitivity	Specificity	PPV	NPV	Kappa index
		Negative	Positive	Total						
Culture results	Negative	57 (96.6%)	2 (3.4%)	59	0.011	100%	96.6%	33.3%	100%	0.487
	Positive	0 (0.0%)	1 (100.0%)	1						
DNA extraction results	Negative	57 (100%)	0 (0.0%)	57	≤0.001	100%	100%	100%	100%	1
	Positive	0 (0.0%)	3 (100.0%)	3						

PPV= positive predictive value
NPV= negative predictive value

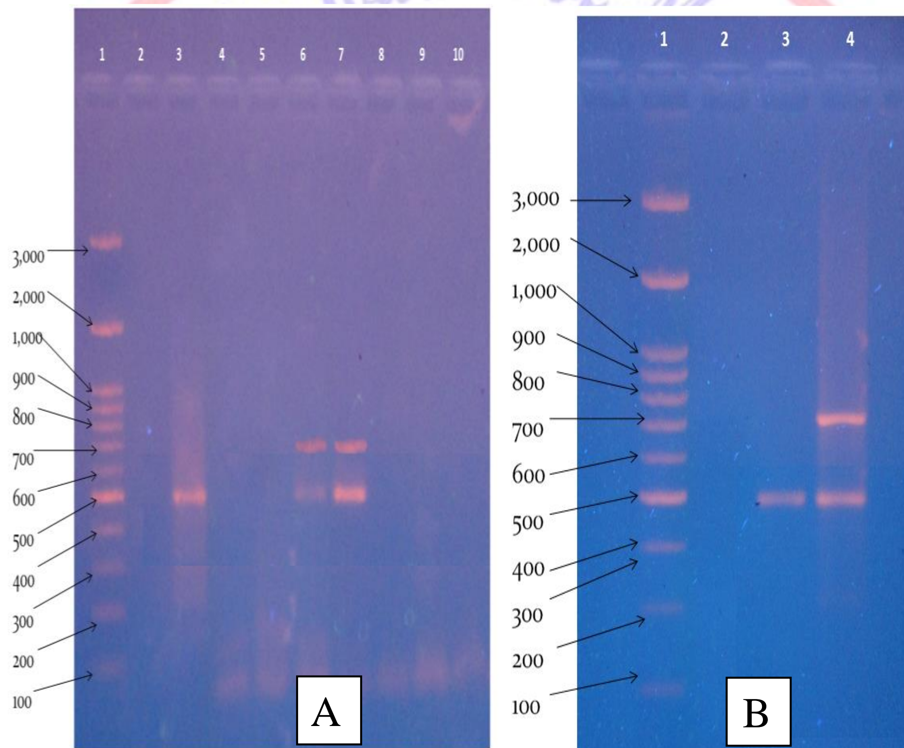

Figure (2): 1.5%agarose gel showing products of PCR of blood culture samples.

A
Lane 1: 100bp DNA ladder.
Lane 2: negative control.
Lane 3: positive control (500bp).
Lane 6 and 7: *Candida albicans* positive (500 bp).
Lane 4, 5, 8, 9 and 10: *Candida albicans* negative.

B
Lane 1: 100bp DNA ladder.
Lane 2: negative control.
Lane 3: positive control (500bp).
Lane 4: *Candida albicans* positive (500 bp) and 730 bp Internal control.

Discussion

Inability or delay in diagnosing of fungal infection defers the administration of appropriate therapy. This has grave implications for the prognosis of the patient: reliable and rapid diagnostic tests for systemic mycoses are imperative to improve rates of patient survival [10].

PCR-based assay was used to identify *Candida albicans* directly from blood culture bottles without further subculturing. Also, the growth of *Candida* cells in bottles during culture of blood increases the concentration of target DNA available for extraction and PCR amplification. The 1:5 dilution of patient blood with blood culture medium may also effectively dilute out PCR-inhibitory factors commonly found in the whole blood [11], making simple DNA extraction methods feasible without the need for additional cumbersome purification steps.

Further decreases in the time needed for detection and identification may be achieved by testing aliquots of blood culture bottles early, prior to detection of growth by the blood culture instrument.

In this study, we reported that 3 cases out of 60 were PCR positive (5%) including only one positive blood culture sample. This gave an inclination that PCR is the most sensitive procedure to detect *Candida albicans* from all purified DNA obtained from blood cultures. This implication was the first in our country to detect candidemia.

PCR method showed 100% sensitivity and 100% specificity to detect all blood culture positive cases. Furthermore, PCR have lower positive predictive value 33.3% and 100% negative predictive value for blood culture.

This technique was better than of Lau, *et al.,* 2010 that showed lower sensitivity (75%) and negative productive value of (85%) which were reported in this study. The single MT-PCR-positive/culture-negative result for *C. albicans* (97% specificity) was not necessarily false-positive since nonviable cells or insufficient numbers of cells may have been found in the blood samples. Furthermore, the sensitivities of blood culture systems are known to be poor even when viable cells are inoculated into the bottle, which may due to the insufficient number of the agent [12].

Conclusions

From this study, we concluded the following:

1. The rate of candidemia was 1.7% among leukemic patients, according to culture results.
2. Only 5% of blood cultures was positive according to PCR.
3. PCR results showed 100% sensitivity and 96.6% specificity and it is easy, reliable and also applicable in clinical laboratory for identification of medically important *Candida spp.*

Recommendations

We recommend the following:

1. Wide coverage of area including center (Baghdad) and other localities to estimate the rate of candidemia among leukemic patients.
2. Using more primers to detect other possible species of *Candida*.
3. Further studies by using new methods such as RNA extraction and amplification and post PCR analysis for further analysis.

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