

Investigation of Candidemia Diagnosis by PCR Method in Febrile Neutropenic Patients with Hematological Malignancies

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ABSTRACT

Objective: Candidiasis is the predominant fungal infection that occurs invasively and opportunistically in individuals with compromised immune systems. Febrile neutropenia patients receiving cytotoxic chemotherapy due to malignancy, especially acute leukemia and stem cell transplantation are at greater risk among many others. In this study, it was aimed to search for Candida DNA to make an early diagnosis by using PCR method

Materials & Method: This prospective study was conducted in Pediatric Hematology Transplantation Clinic of Akdeniz University. The study enrolled 78 patients with malignancies and candidiasis infection who were experiencing febrile neutropenia. The febrile neutropenic episodes were identified prospectively by monitoring the daily leukocyte counts and fever symptoms of the patients.

Results: Candida was grown in the blood cultures of seven (9%) of the febrile neutropenic patients included in the study. All of the breeding Candidas were C.albicans. The underlying primary diagnosis of 6 (85.7%) of the febrile neutropenic patients with reproduction was hematological malignancy, while the underlying primary diagnosis of one (12.5%) was solid malignancy (Neuroblastoma). PCR detected Candida DNA in 9% of Febrile Neutropenic patients. C.albicans was grown in the blood cultures of all patients with Candida DNA.

Conclusion: As a result, The conclusion drawn was that in cases where candidemia is suspected, particularly in febrile neutropenic patients with hematological malignancies who are immuno-compromised and at high risk of mortality, rapid and accurate diagnosis using Candida PCR with high sensitivity and specificity is necessary.

Keywords: Candidiasis, febrile neutropenia, malignancy, PCR, immuno-compromised

Research Article

Received 23-01-2023

Accepted 15-02-2023

Available Online: 16-02-2023

Published 28-02-2023

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INTRODUCTION

Candida species are common fungi in yeast morphology in nature. They can be found in the skin, respiratory system, gastro-intestinal tractus and female genital flora (1). They are opportunistic pathogens and in the presence of predisposing factors can cause local or widespread, superficial or deep, acute or chronic infections called as candidiasis (2, 3).

Candidiasis is the most common opportunistic and invasive fungal infection in immuno-compromised individuals. Febrile neutropenia patients receiving cytotoxic chemotherapy due to malignancy, especially acute leukemia and stem cell transplantation (SCT) are at greater risk among many others (3). Candidemia may be a fatal risk factor in neutropenic patients because they are difficult to treat and can cause serious morbidity and mortality in these immuno-compromised patient groups (4).

Candida can usually be isolated in blood cultures taken at the advanced stage of infection, when treatment options have less chance of success in immuno-compromised patients. However, early diagnosis of candidemia and initiation of specific anti-fungal therapy before fungal masses grow, especially in patients with hematological malignancies, prolongs life expectancy (5).

Despite all the developments up to date, infections are the most common cause of death in patients with hematological malignancies (4). While mortality rates in febrile neutropenia were around 30% in the 1970s, this rate decreased to 2-10% with advances in antibiotic therapy and early antimicrobial therapy (5). In the United States (USA), approximately one person dies from hematologic malignancy every 9 minutes (6). Such high mortality rates show the importance of pre-emptive approach to these patients. International guidelines recommend taking blood cultures from patients presenting with neutropenic fever and starting antimicrobial therapy within 60 minutes (7). However, some researchers state that treatment should be started within thirty minutes. During septic shock, survival is reduced by 8% for each hour of delay to initiation of therapy (8).

Febrile neutropenia is an infectious disease emergency and a disease with high mortality and morbidity. Fever may be almost the only manifestation of infections in patients with hematological malignancies. Mental status changes, hypotension, hypoxia, and similar findings can be counted among the signs of infection, especially in elderly patients. Due to the qualitative and quantitative insufficiency of the neutrophil functions of the patients, typical signs and symptoms of inflammation are not exhibited. For this reason, deaths can be prevented by making a rapid diagnosis, taking appropriate cultures from possible foci of infection and initiating empirical treatment quickly (9, 10).

Study Hypothesis

In this study, it was aimed to search for *Candida* DNA in order to make an early diagnosis by using PCR (Polymerase Chain Reaction) method in venous blood samples taken from febrile neutropenia patients with malignancy and candidiasis in the first hours when the fever started to rise, and to compare the applied PCR method with the conventional automated blood culture system in terms of quality and quantity.

Since the success of DNA extraction by PCR will affect the result, two different extraction kits were compared in terms of compatibility, analytical sensitivity and time. At the same time, the compatibility of blood culture and PCR results with findings supporting the diagnosis of systemic candidiasis, and the degree of neutropenia and blood culture and PCR positivity were compared.

MATERIAL and METHODS

This study was conducted prospectively in the Pediatric Hematology Transplantation Clinic of Akdeniz University, involving a total of 78 patients with malignancies and candidiasis infection who experienced febrile neutropenia. The study received ethical approval from the committee with the protocol number 11-6067, and the study followed the principles of the Declaration of Helsinki with informed consent obtained from all participants. The febrile neutropenic episodes were determined by monitoring the daily leukocyte counts and fever symptoms of individuals. Patients with neutrophil levels below 500/mm³ or between 500-1000/mm³ but expected to decrease below 500/mm³ within 48 hours and having fever above 38.0 °C or 38.2 °C were defined as having febrile neutropenia. During the isolation of *Candida* DNA from blood, the study followed the

previous protocol studies published by Loeffler et al. and utilized automated Magna Pure LC and manual Qiagen kits developed by Loeffler.

Sample Collection and Study Procedure

Blood samples were taken in the first hours of fever started to rise and inoculated into EDTA tubes (Ethylene Diamine Tetra Acetic Acid) for PCR processing and blood culture bottles containing soybean casein broth for blood cultures (for adult patients; Plus, Aerobic/F – for pediatric patients; pēds plus/F Becton Dickinson). Approximately 3 – 5 ml blood samples taken into EDTA tubes were transferred to Eppendorf using filtered pipette tips purified from RNA and DNA and kept at -70 °C until purification of *Candida* DNA.

Blood culture vials were placed in a Bactec 9240 (BD Bactec-Becton Dickinson Diagnostic Instrument Systems, Cockeysville, Md) blood culture device. It was incubated in the device for one week until the growth signal was obtained. Following this they were inoculated on blood agar and SDA (Sabouroud Dextrose Agar) and then incubated at 37 °C for 24 hours. In addition, urine, catheter, stool, and throat samples were also taken simultaneously. Blood cultures and colonization specimen (urine, catheter, feces, and throat) were detected for species differentiation. Germ tube test was applied to *Candida* fungi observed in yeast morphology as a result of microscopic examination. Physiological and biochemical properties of germ tube-forming *Candida* were examined and identified at the species level.

In order to detect the differentiation of non-germ-tuberos *Candida*, species identification was performed in Mini API (BioMérieux) fully automated device with the ID 32C (BioMérieux) kit, which gives results based on fermentation and assimilation reactions. Purification, amplification, and imaging of *Candida* DNA were carried out in our institution. There was no difference in analytical sensitivity (10 CFU/ml) of *Candida* DNA extracted with the two extraction methods we used (Magna Pure LC DNA Isolation Kit III-Roche, modified manual (Lyticase + QIAamp DNA Mini Kit-Qiagen). However, Magna Pure LC system should be preferred as it provides 100% time saving compared to the modified manual (Lyticase + QIAamp DNA Mini Kit-Qiagen).

Determination of Analytical Sensitivity of PCR Methods

Positive Control: In this study, ATCC 90028 standard strain of *C. albicans* obtained from the American culture collection (ATCC) was utilized as a positive control. The strain was grown in the medium by incubating in SDA at 35 °C for 24 hours. With *Candida* obtained from the culture, McFarland value was read in a densimeter device in a phosphate buffer with a turbidity equivalent to 106 *Candida* cells in 0.5 McFarland. Suspensions of 105, 104, 103, 102, 101 *Candida*/ml were made and diluted in healthy human blood with EDTA obtained from the blood bank. The analytical sensitivity of the test was determined with these suspensions at different dilutions.

Negative Control: Two controls, sterile deionized water and healthy human EDTA blood obtained from blood bank, were utilized for negative control.

Statistical analysis: SPSS 20 package program was used for the statistical analysis of the data. Data are presented as the mean $\pm$ SD, as percentages of the mean, or as the distribution of the frequency. Fisher's exact test was used where appropriate to test for frequency differences between groups, Mann-Whitney U was used to compare numerical variables between two groups, and One-Way ANOVA was used to compare numerical variables between more than two groups. Values of $p < 0.05$ were considered to indicate a statistically significant result.

RESULTS

A majority (69%) of the individuals ($n=54/78$) were hospitalized in Internal Medicine/Hematology, and 20% were hospitalized in the Pediatrics/Hematology-Oncology services, and 10% ($n=8/78$) were in the Stem Cell Transplantation (SCT) unit. The gender distribution was as follows: 60.3% ($n=47$) were male and 39.7% ($n=31$) were female. Of the four pediatric patients, one was female, and three were male. Four adult patients were male.

Acute myeloid leukemia (AML) has been diagnosed in 37% ($n=20/54$) of the patients in the Internal Medicine/Hematology service and acute lymphoblastic leukemia (ALL) was the most common primary diagnosis in 37.5% ($n=6/16$) of patients in the Pediatrics/Hematology-Oncology service and in 62.5% ($n=5/8$) of patients in the CHT unit. The primary diagnoses of four patients hospitalized in the pediatric hematology-oncology service were Burkitt's Lymphoma, ALL, Neuroblastoma, and NHL, while the primary diagnoses of three internal medicine hematology patients were AML, HL (hodgkin lymphoma), and NHL (non-hodgkin lymphoma).

Of the seven patients with PCR positive result and *C.albicans* growth in blood culture; four were hospitalized in the Department of Pediatrics/Hematology-Oncology, and three were hospitalized in the Department of Internal Medicine/Hematology. Three of the four patients hospitalized in the Pediatrics/Hematology-Oncology department were male, and the mean age (age $\pm$ SD) was 9.33 ± 5.31 years, while the mean age of three patients hospitalized in the Internal Medicine/Hematology department was 69.33 ± 4.49 years.

Candida growth was not observed in the cultures of 50 of 71 patients. There has been colonization at least one of the samples of 21 patients (culture growth in urine cultures of five patients, catheters in five patients, stool cultures in seven patients, and throat cultures of three patients).

Candida was grown in the blood cultures of seven (9%) of the febrile neutropenic patients included in the study. All of the breeding *Candidas* were *C.albicans*. The underlying primary diagnosis of 6 (85.7%) of the febrile neutropenic patients with reproduction was hematological malignancy, while the underlying primary diagnosis of one (12.5%) was solid malignancy (Neuroblastoma). PCR detected *Candida* DNA in 9% of Febrile Neutropenic patients. *C.albicans* was grown in the blood cultures of all patients with *Candida* DNA.

Two of the seven patients with *C.albicans* growth in blood culture had *C.albicans* colonization in urine, catheter, and feces in the throat cultures of three patients. In patients with growth in blood culture, there was colonization in the urine in two patients, in the stool in one patient, in the stool and throat in one patient, in the urine and throat in one patient, and in the catheter and throat in one patient. No concomitant growth was observed in urine, catheter, stool, and throat cultures.

The neutropenia level of all patients with positive blood culture and PCR was $< 500/\mu\text{l}$, and the neutropenia level of 14 (19.7%) patients with negative blood culture and PCR was $< 500/\mu\text{l}$. The difference between these two groups was statistically significant ($p=0.000044$). The median neutropenia duration of the patients with positive blood culture and PCR was determined as 9 days, while the median neutropenia period of the patients with negative blood culture and PCR was 7 days. The difference between these two groups was statistically significant ($p= 0.001$). The duration of neutropenia was longer in those with positive blood culture and PCR results than in those with negative blood culture and PCR results, indicating a statistically significant difference.

Although the cost of blood culture was cheaper than the PCR method, the result was obtained in 96 hours with the blood culture method in disseminated candidiasis is delayed. Early diagnosis will increase the chances of treatment success. With the PCR method, the diagnosis of candidemia can be made in a short period of 5 hours, 2 hours for extraction, 3 hours for amplification and imaging.

Table 1. Malignities accompanying febrile neutropenia patients

Primary Diagnosis	Internal Medicine/ Hematology		Pediatrics/Hematology Oncology		Stem Cell Transplantation	
	n	%	n	%	n	%
AML	20	37	2	12.5		
ALL	5	9.25	6	37.5	5	62.5
NHL	8	14.81	3	18.75	1	12.5
HL	10	18.50				
MM	4	7.40				
AA	2	3.70	1	6.25		
MDS	3	5.55				
T. Major					2	25
Burkitt Lymphoma			1	6.25		
CLL	1	1.85				
CML	1	1.85				
Neuroblastoma			1	6.25		
Total	54	100	16	100	8	100

Table 2. Comparison of the results of blood culture and PCR methods

Mehods	Positive		Result Negative		Total	
	n	%	n	%	n	%
Blood Culture	7	9	71	91	78	100
PCR	7	9	71	91	78	100

Table 3. In Candida DNA extraction, modified manual (Lyticase + QIAamp DNA mini kit-Qiagen) and automated MagNA Pure LC (Roche) protocols; comparison of required blood volume, extraction time, analytical sensitivity and cost parameters.

Parameters	Modified manual (Lyticase + QIAamp DNA Mini Kit) extraction protocol	Automated MagNA Pure LC extraction protocol
Required blood volume	3 ml	100 µl
Average for eight patients extraction time (hours)	4 10	2 10

Table 4. Risk factors for candida infection in study population

	PCR and Blood Culture Positive Patients						
	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Multiple Antibiotic Use	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Urinary Catheter	Yes					Yes	Yes
Diabetes Mellitus (DM)		Yes					
Candiduria Story	Yes			Yes			
Central Venous Catheter		Yes					
Kidney Failure / Dialysis		Yes					
Corticosteroid Use	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Abdominal/Cardiac Surgery							
Attempt							
Burn		Yes					

DISCUSSION

Candida species are the first fungi among the invasive fungal agents that cause opportunistic fungal infections in immuno-compromised patients. Disseminated candidiasis is frequently seen in patients with hematological malignancies receiving intensive cytotoxic chemotherapy due to prolonged granulocytopenia. Candidemia is a feared risk factor in neutropenic patients as they are difficult to treat and an expensive infection that can lead to serious morbidity and mortality. In febrile neutropenic patients receiving cytotoxic chemotherapy due to their malignancy, especially in patients with immuno-suppressed acute leukemia and CHT, *Candida* occupies first among opportunistic and invasive fungal infections (13, 14). Due to its rapid and sensitive nature, PCR is preferred in diagnosing candidemia in neutropenic patients with hematological malignancies with suppressed immune systems. Early diagnosis of candidemia is delayed by conventional methods such as blood culture (13).

In the study of Işık et al. in immune-compromised pediatric patients with leukemia, two out of 50 febrile neutropenic patients (8%) found *Candida* detected. *Candida krusei* was isolated in the blood culture of one of the two cases, and *C. albicans* and *C. krusei* were isolated in the blood culture of the other case. The study indicated that the primary diagnosis for the first case was ALL, and for the other case, it was Fanconi anemia (15). In the studies conducted by Pagano L. et al., which investigated febrile neutropenic patients with candidemia and hematological malignancies, it was reported that the most frequent primary diagnosis was AML (16).

In a study investigating febrile neutropenia attacks in patients with hematological malignancies followed for 15 years at Cerrahpaşa Medical Faculty, the median neutropenia duration was 7.5 days (17).

Dinubile et al. reported that the neutropenia level was $<500/\mu\text{l}$ in the majority of patients (77.8%) with hematological malignancies in their study (18). Neutropenia level was $<500/\mu\text{l}$ in all seven patients with *C. albicans* growth in blood culture and positive *Candida* PCR in blood samples. Neutropenia level was $<500/\mu\text{l}$ in 14 (19.7%) of our 71 patients whose *Candida* PCR was negative and *C. albicans* did not grow in the blood culture. The level of neutropenia was found to be statistically significantly different between these two groups ($p=0.001$).

In a multicenter prospective study conducted by the Invasive Fungal Infection (IFIG) group of the European Organization for Research and Treatment of Cancer (EORTC), the most frequently isolated *Candida* species was *C. albicans* (55%) among a total of 249 cancer patients with candidemia, of which 90 had solid tumors and 159 had hematological malignancies (19). In a study conducted at Ege University, *C. albicans* (54%) was reported as the most frequently isolated *Candida* species among febrile neutropenic patients diagnosed with hematological malignancy (20).

Pagano L. et al. stated that *C. albicans* (41%) was the most frequently isolated species among candidemia agent in febrile neutropenia patients with hematological malignancies (16). In our study, seven (9%) of 78 malignant and febrile neutropenic were found to be *Candida* DNA positive by PCR, and

C. albicans was isolated from the blood cultures of the same seven patients.

Diagnosis of invasive *Candida* infections is made by isolation of the fungus by culture, serological investigation of antigens and antibodies, histopathological examination and/or radiological imaging of invasive tissue, and demonstration of *Candida* nucleic acids using molecular methods. The diagnosis of the disease is difficult due to the absence of specific clinical signs and symptoms. However, by molecular methods, *Candida* growth in blood cultures and proof of the presence of *Candida* DNA are data that will make invasive candidiasis acceptable (21). Microbiological confirmation in disseminated candidiasis is difficult because blood cultures are negative in up to 50% of deep candidiasis cases proven by biopsy and autopsy and become positive in late infections (21).

After incubation of febrile neutropenic patients in blood cultures, *Candida* spp. The average time it takes to say yes is between 4-5 days. Thanks to newly developed techniques such as lysis centrifugation method, blood culture sensitivity has increased. However, this method has encountered a high rate of false positives. For these reasons, the early diagnosis of candidemia is delayed by conventional methods such as blood culture (21).

Effective treatment of candidemia is delayed with empirical antibiotics administered to the patient and antifungal drugs administered at prophylactic doses over the course of time. Empiric treatment with multiple antibiotics in febrile neutropenic patients changes the balance of the endogenous flora, and colonization and progression to deep tissues are followed by blood-borne spread. *Candida* can usually be isolated in blood cultures taken at the advanced stage of infection, when treatment options have less chance of success in immune-compromised patients. However, early diagnosis of candidemia and initiation of specific antifungal therapy before fungal masses grow, especially in patients with hematological malignancies, prolongs survival. For these reasons, rapid molecular methods are needed (22).

Molecular diagnostic applications provide the rapid genotypic determination of microorganisms by replacing the biological production of microorganisms in culture media. Molecular diagnostic methods emerge as necessary in cases where *Candida*s reproduce slowly or cannot be produced, and in cases of immune unresponsiveness where serological diagnosis cannot be performed. In a study by Maaroufi et al., they showed that PCR-based diagnosis of candidemia was more sensitive (100%), specific (97%) and reproducible (96 – 99%) than conventional blood cultures (23). In the study of Kalkancı et al., a PCR protocol using species-specific primers (Pcon 1, Pcon 2) was utilized to detect *Candida* species in blood samples of 32 neutropenic patients. While four out of 32 (12.5%) patients were positive by PCR, only one of these four patients was found to be positive by blood culture. As a result, it was emphasized that PCR is a very sensitive technique for demonstrating *Candida* species from blood and its use in diagnosing cases with suspected candidemia would be beneficial (24).

In *Candida* PCR studies, lower analytical sensitivities (<10 CFU/ml) were determined than our study; nPCR, snPCR or RT-PCR methods were used, and the imaging was observed

that the imaging techniques performed with the ELISA method could detect lower limits. If blood culture positivity was accepted as the gold standard in terms of sensitivity and specificity in the diagnosis of candidemia, blood culture was found to be positive in all patients with positive PCR. In contrast, blood culture was found to be negative in all patients with negative PCR (25).

In the venous blood samples taken from the patients in the first hours when the fever started, before the antifungal treatment started; *Candida* DNA was searched for early diagnosis using PCR method. The applied PCR method was compared with the conventional automated blood culture system in terms of quality and quantity. The concordance of neutropenia degree with blood culture and PCR positivity was determined. Early candidemia diagnosis and specific antifungal therapy initiation before fungal masses grow in patients with hematological malignancies prolongs survival. PCR should be performed in patients as it provides a very specific, sensitive, and rapid diagnostic result, especially in febrile neutropenic patients with an underlying hematological malignancy and who do not have a fever despite long-term use of multiple antibiotics, on suspicion of candidemia with a high mortality risk.

CONCLUSION

As a result, *Candida* PCR with high sensitivity and specificity and rapid diagnosis is necessary when candidemia is suspected, especially in febrile neutropenic patients with hematological malignancies with a high risk of mortality and immuno-compromised febrile neutropenia.

Acknowledgments: None

Conflict of interest: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. This research did not receive and a specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author Contributions: **MY, BÖ, GM:** Study conceptualization, protocol planning, clinical data collection, clinical data analysis and supervision. **MY:** manuscript writing/editing.

Ethical approval: Ethical approval: All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and/or with the Helsinki Declaration of 1964 and later versions. Informed consent or substitute for it was obtained from all patients for being included in the study. Akdeniz University Faculty of Medicine Clinical Research Ethics Committee approval has been granted with protocol number 11 – 6067.

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