Using PCR and Real-Time PCR (LightCycler) for Diagnosis and Follow up of Invasive Fungal Infections in Patients with Hematological Malignancies and Transplantation

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ABSTRACT

In modern medicine, for early diagnosis of infections, tests that have high specificity and sensitivity should be preferred. For this reason, especially for patients with hematological malignancies and transplantation, that have high mortality and morbidity ratios, some molecular biological techniques are coming into use today for differential diagnosis and follow up of invasive fungal infections. In the coming years it will become easier to early diagnose and also to plan optimal treatments, by using these techniques for diagnosis of fungal infections. In this study, we made fungal DNA isolation from blood samples, which were taken from patients, in immunodeficiency state, with hematological malignancies and transplantation. For polymerase chain reaction (PCR), we used universal fungal primers. By using real-time PCR samples from 20 patients, which were found positive by PCR, the amount of DNA was measured. In real-time PCR, we used *Aspergillus* colonies as our standard, and for this purpose, SDA petri dishes were incubated for 72 hours at 30°C and; 10^1-10^6 cfu/mL serial dilutions were made by using hemocytometry. We performed DNA isolation and PCR from these dilutions. Fungal species specific products giving one band of 500 bp, were fixed in 2% agarose gel. We measured the DNA amounts by real-time PCR by using Sybr Green I. Standard dilution series and extraction samples were studied at the same time by real-time PCR. Measurement of the DNA quantities in the separate samples, one at the time of first day and the other after 15 days were interpreted by the LightCycler system. Results of real-time PCR in these two different samples were compared; it was noted that fungal DNA was increased in 5, decreased in 6 and equivalent in 9 patients. These findings showed that real-time PCR is a new, specific and a sensitive method among the other quantitative PCR systems. This method was also quicker than the other quantitative PCR systems. Currently, the investigators prefer this method because, it gives early idea about patient condition, for early diagnosis of infections and treatment patients who are under risk of some kind of infection, and for follow up treatment results. Additionally for diagnosis of fungal infections serial samples in PCR, together with other conventional diagnostic methods, should used.

Key Words: Real-time PCR, Invasive fungal infections, Polymerase chain reaction.

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ÖZET

Hematolojik Maligniteli Olup Nakil Yapılan Hastalarda Fungal İnfeksiyonların Tanı ve İzlenmesinde PCR ve Real-Time PCR (LightCycler) Kullanımı

Yüksek mortalite ve morbiditeye sahip olan hematolojik maligniteli ve nakil yapılmış hastalarda, invaziv fungal infeksiyonların tanısı ve izlenmesi için yüksek duyarlılık ve özgüllüğe sahip moleküler biyolojik tekniklerin kullanılması gündemdedir. Bu çalışmada hastalardan immünyetmezlik dönemde alınan kan örneklerinden fungal DNA izole edildi. PCR için üniversal fungal primerler kullanıldı. PCR ile pozitif bulunan 20 olguda real-time PCR ile DNA miktarı ölçüldü. Real-time PCR'de *Aspergillus* kolonileri kontrol olarak kullanıldı. DNA miktar ölçümü Sybr Green I ile yapıldı. Standart dilüsyon serileri ve ekstraksiyon örnekleri real-time PCR ile aynı zamanda ölçüldü. DNA miktar ölçümleri, birinci gün ve 15. gün örneklerinde LightCycler ile yapıldı. Bu iki örnekteki real-time PCR sonuçları karşılaştırıldı. Fungal DNA'nın beş olguda arttığı, altı olguda azaldığı dokuz olguda aynı kaldığı görüldü. Bu bulgular real-time PCR'nin diğer kantitatif PCR yöntemlerine göre daha duyarlı ve özgül olduğunu ve diğer sistemlerden hızlı olduğunu göstermektedir. Halen araştırmacılar bu yöntemi hastanın durumu hakkında erken bilgi vermesi, infeksiyonların erken tanısı ve tedavinin izlenmesinde kullanılabilmesi nedeni ile tercih etmektedir. Fungal infeksiyonların tanısında seri PCR örneklerinin izlenmesi ve diğer konvansiyonel yöntemlerin de kullanılması yararlı olacaktır.

Anahtar Kelimeler: Real-time PCR, İnvaziv fungal infeksiyon, Polimeraz zincir reaksiyonu.

INTRODUCTION

Present evolution of molecular biology has led researchers clone and organize process of nucleic acid from individual genes. These procedures can also be applied to fungal genes. Development of PCR technology made it possible to rapidly identify different types of pathogenes in clinical mycology[1]. PCR is one of the most significant techniques, which come over the problems of low sensitivity and specificity[2]. Deep fungal infections cannot usually be diagnosed until the late stage especially in patients with immunosuppression, haematological malignancy or transplantation. At this stage all therapeutic modalities have little chance of success[3]. However, in case of rapid identification of fungal infections with highly sensitive and specific techniques with molecular biology it will not be necessary for the clinician to wait for culture results or start treatment with a presumptive clinical indication. Our aim in this study was determined the reliability and value of PCR in diagnosis of invasive fungal infections. Using real-time PCR (Light-Cycler) we have quantitatively identified the amount of fungal DNA in blood samples obtained from patients with proven fungal infection at the time of diagnosis and 15 days later.

MATERIALS and METHODS

In this study, we used a well-working PCR technique and modified real-time PCR protocols with using Sybr-Green which were established by Einsele et al 120-blood samples anticoagulated with EDTA, which were taken from patients, immunosuppressed, with hematological malignancies and transplantation[4,5]. We selected the patients whose were under a risk of developing fungal infections. DNA isolation was done using by QIA-amp tissue kit (Qiagen). After that, at thermal cycler, 35 cycles (30 sec at 94°C, 1 min at 62°C, 2 min at 72°C) amplification was made by using 18S rRNA (5'-ATT GGA GGG CAA GTC TGG TG and 5'-CGG ATC CCT AGT CGG CAT AG) as universal fungal primers which were more suitable primer pairs for fungal PCR[5]. Sterile distile water was used as negative control for PCR. *Aspergillus fumigatus* were cultured on Sabouraud Dextrose agar (SDA) for 72 hours at 30°C and DNA was extracted from *A. fumigatus* colonies and this DNA isolates were used as positive control for PCR. In addition to this, in real-time PCR, we used *Aspergillus* colonies as our standard, and for this purpose, SDA petri dishes were incubated and; 10^1-10^6 cfu/mL serial dilutions were made by using hemocytometry. After PCR, all products were shown at 2% agarose gel fungal species specific products giving one band of 500 bp, were fixed in agarose gel, represented as positive, samples which were found positive by PCR, the amount of DNA was measured by using real-time PCR[6,7]. Measurement of the DNA quantities in the separate samples of the same patients, one at

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the time of diagnosis and the other after 15 days were repeated, results were compared to each other and interpreted by the real-time PCR system. Real-time PCR was performed in glass capillaries which ensures rapid equilibration between the air and the reaction components because of the high surface to volume ratio of the capillaries. So that, results were taken in short time. There was a system in which every amplification step was shown. In this system, special probes and same fluorescent dyes were used, as we done especially Sybr-Green I was preferred. In this system, there is also an optic reader, which measures the rising of fluorescent dye with increasing amount of DNA in each cycle of amplification. Sybr-Green I is double-stranded DNA selective fluorescent dye, provides rapid way to detect increase in DNA amount. In tubes, in every cycle of amplification, when amount of double stranded DNA increases, binding to this and amount of dye which gives fluorescence also increases. By means of melting curve analysis, we decided whether amplified DNA is target area or not. After amplification completed, tubes which contain PCR products were heated slowly, while fluorescence observed. At certain degree (melting point), DNA strands apart from each other and give fluorescent dye free and fluorescence decreases immediately. This melting point depends on length of amplified DNA and GC/AT ratios. If this ratio is high, there are more hydrogen bands between two strands. So temperature, which is needed to convert the double stranded DNA to single stranded DNA increases proportionally with this ratio. Every product has specific melting point, because every product has specific length and genome content. It is understood that if this product was wanted or not, by looking at melting points of products. In this study, results are taken from printers as cfu/mL, after 30 min following the 35 cycles amplification program[4]. After PCR, products were observed at agarose gel.

RESULTS

Fungal infections were detected in twenty of 120 blood samples of patients with immunodeficiency state by PCR after extraction. After 15 days another blood sample were taken from the same patients and amount of DNA was measured by real-time PCR. Results of only four positive patients samples by PCR and real-time PCR at 2% agarose gel are shown in Figure 1. All patients' results were found positive by culture bet-

ween 3-20 days. We confirmed positive samples by using culture and to sequencing. We sequenced [by ABI Prism 377 (Perkin Elmer)] positive samples and all had given *A. fumigatus* specific base pairs. All positive patients had taken antifungal therapy following the first day PCR result. After antifungal treatment only five patients who had increased amount of DNA whose were three of them died and two of them decreased DNA amount after 20 days later to taking antifungal therapy. Results of measurement of the DNA amounts, by real-time PCR in separate samples of 20 patients with fungal infection, one at the time of diagnosis and other after 15 days were shown in Table 1. Real-time PCR standard curve results, in which positive sample results were analyzed are shown in Figure 2. According to first sample results fungal DNA amounts for five patients were found 10^5-10^6 cfu/mL; for ten patients, 10^4-10^5 cfu/mL; for four patients, 10^3-10^4 cfu/mL; and for one patient, 10^1-10^2 cfu/mL. The results for the samples taken after 15 days for two patients were >10^6 cfu/mL, for two patients, 10^5-10^6 cfu/mL; for eleven patients, 10^4-10^5 cfu/mL and for five patients 10^3-10^4 cfu/mL.

Finally, if we compare results of first samples and

Orders: M: Molecular weight standard (PCR Marker: Sigma P9577)

1 and 9: First sample,

2 and 10: Second sample,

3 and 11: Third sample,

4 and 12: Fourth sample,

5 and 8: Negative control (sterile distilled water),

6 and 7: Positive control (10^5-10^6 cfu/mL A. fumigatus).
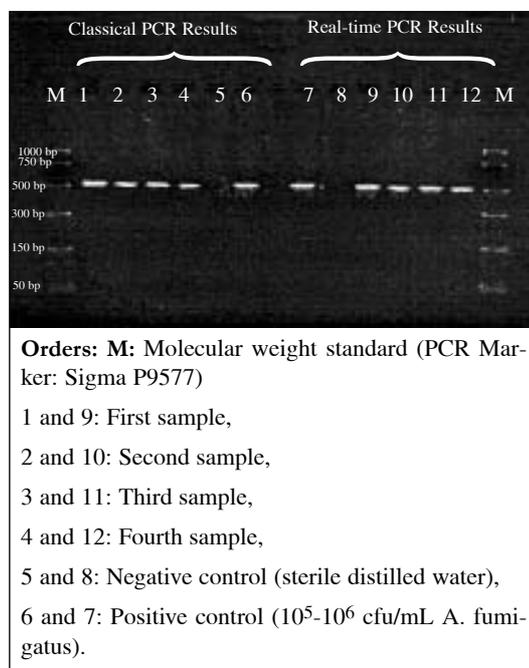
Figure 1. PCR and real-time PCR results for four patient samples.

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samples taken after 15 days; real-time PCR of amounts of fungal DNA increased for five patients, decreased for six patients and remained the same for nine patients. So that, while considering DNA levels, antifungal treatment protocols were replanned.

DISCUSSION

Incidence of invasive fungal infection has increased especially in immunodeficient patients. This is true for patients who receive chemotherapy for hematologic malignancies, patients having immunosuppressive treatment after organ transplantation, patients with AIDS or nosocomial infections as well[8-10]. Such patients with immunosuppression are the ones who need most the molecular methods for nucleic acid analysis. Fungal infections in such patients show rapid progression and cause highly lethal complications if proper treatment cannot be offered. This is why we need rapid and highly sensitive diagnostic methods[11,12]. Methods like PCR and real-time PCR (which has quantitative property as well) are developed in recent years. Loeffler et al reported on using real-time PCR for the diagnosis of fungal infections and determining the amount of DNA[4]. Furthermore real-time PCR is also used for diagnosis of many viral or bacterial infections[13,14]. There are many studies on PCR for early diagnosis of invasive fungal infections. Conclusion was that PCR, compared to blood cultures and ELISA, is much more sensitive and specific for determining fungal antigens and antibodies[15-17]. In this study, we have used PCR and real-time PCR for the early diagnosis of invasive fungal infections in 120 samples obtained from patients with transplantation or hematologic malignancies. PCR was positive in 20 patients whose initial samples and samples taken 15 days later were further analyzed by real-time PCR for determining the amount of DNA. When results of real-time PCR in these two different samples were compared; it was noted that fungal DNA was increased in five (three of them died and two of them decreased DNA amount after 20 days later to taking antifungal therapy), decreased in six and equivalent in nine patients. According to these results antifun-

Table 1. Real-time PCR results (cfu/mL)

Patients	Real-time PCR result I (first sample results)	Real-time PCR result II (sample results after 15 days)	Comparison of real-time PCR result I and II
1	10^3-10^4	10^5-10^6	↑
2	10^4-10^5	10^4-10^5	→
3	10^4-10^5	> 10^6	↑
4	10^4-10^5	10^4-10^5	→
5	10^4-10^5	10^4-10^5	→
6	10^3-10^4	10^4-10^5	↑
7	10^3-10^4	10^3-10^4	→
8	10^4-10^5	10^4-10^5	→
9	10^4-10^5	10^4-10^5	→
10	10^3-10^4	10^3-10^4	→
11	10^4-10^5	10^3-10^4	↓
12	10^5-10^6	10^3-10^4	↓
13	10^5-10^6	10^4-10^5	↓
14	10^4-10^5	10^5-10^6	↑
15	10^4-10^5	10^4-10^5	→
16	10^5-10^6	10^3-10^4	↓
17	10^5-10^6	10^4-10^5	↓
18	10^4-10^5	10^4-10^5	→
19	10^5-10^6	10^4-10^5	↓
20	10^1-10^2	> 10^6	↑

↑: Increase, ↓: Decrease, →: Same level.

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A: For first samples (10^1-10^6 cfu/mL)
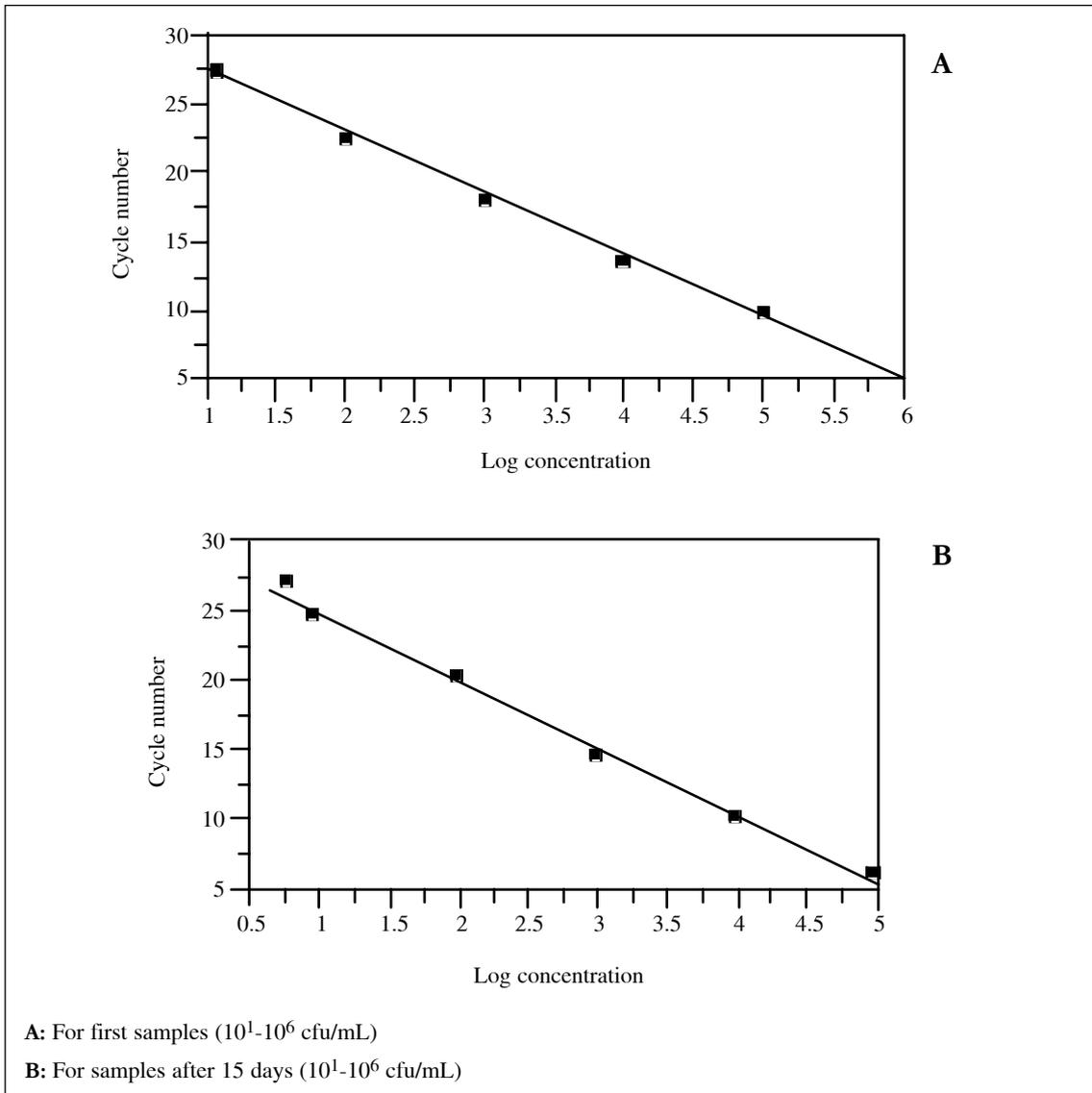
B: For samples after 15 days (10^1-10^6 cfu/mL)

Figure 2. Real-time PCR standard curves in which positive results were analyzed.

gal treatment protocols were reestablished. As a result early diagnosis in these patients provided proper treatment and follow up. Comparing to both sample results, treatment protocols replanned according to amount of DNA detected in samples taken after 15 days.

As a conclusion, PCR and real-time PCR are the newest, specific and sensitive techniques. Compared to others both provide rapid results and give early idea about patient's condition whose are under a risk of fungal infections. On the other hand, PCR and real-time PCR should be performed for serial samples and together with conventional diagnostic methods.

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