Original Article

Detection of *Aspergillus* species in bone marrow transplant patients

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Abstract

Introduction: Invasive aspergillosis is a severe complication of cytotoxic chemotherapies and bone marrow transplantation (BMT). The aim of this study was to assess the utility of a real-time PCR assay for the early diagnosis of *Aspergillus* species in blood samples from BMT patients.

Methodology: Blood specimens (n = 993) from patients (n = 82) scheduled for BMT were collected prior to transplant and for 100 days post transplantation. The specimens were later tested using an *Aspergillus*-specific real-time PCR assay. Cultures of clinical samples, along with sonography and computerized tomographic scans, were performed as standard of care.

Results: *Aspergillus* DNA was positive in 94 sequential blood samples from 13 patients with clinical and radiological signs of infection. Samples from three of these patients were PCR-positive for *Aspergillus* in the first week of admission, prior to transplantation. Four patients with aspergillosis were cured with antifungal agents and nine died. An additional 12 patients without clinical signs of infection were PCR-positive on one occasion each, while two patients with clinical signs of infection were PCR-negative. Compared to routine methods of aspergillosis diagnosis, the respective sensitivity, specificity, negative, and positive predictive values of the PCR method by patient were 86.6%, 82%, 96.5% and 52%.

Conclusions: The results show that *Aspergillus* infections in the blood of bone marrow transplant patients can be dectected by PCR methods. Early detection of *Aspergillus* infections by PCR has the potential to positively impact patient mortality rate and provide cost savings to hospitals.

Key words: invasive aspergillosis, bone marrow recipients, transplantation, real-time PCR

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Introduction

Invasive aspergillosis is a major cause of morbidity and mortality in patients with hematologic disorders, occurring in 15.1% of the allogeneic hematopoietic stem cell transplant (HSCT) recipients [1] and in 2% of those receiving autologous HSCT [2]. The mortality rate can exceed 90% in bone marrow transplant recipients (BMTR) [3]. Survival of such patients depends on early diagnosis and prompt therapy. Unfortunately, blood culture is not sufficiently sensitive to diagnose these infections, and culture of bronchoalveolar lavage (BAL) fluids is positive in only 50% of the proven invasive aspergillosis cases at autopsy [4]. Mycological and histopathological methods are suboptimal owing to the need for invasive procedures to obtain specimen [5]. Only the galactomannan test has reached a satisfactory level of confidence, in some studies, to be included as the diagnostic tool of invasive aspergillosis in hematology, despite a high level of false positive results (~10-15%) [6,7,8].

Rapid and sensitive molecular detection assays for *Aspergillus* infection have been recently developed utilizing the power of PCR and real-time PCR [9,10]. The aim of this study was to assess the utility of a real-time PCR assay for the early diagnosis of *Aspergillus* species in blood samples of bone marrow transplantation (BMT) recipients for early diagnosis.

Materials and methods

During the prospective cross-sectional study period, from September 2006 to March 2009, 82 patients with hematologic disorders (BMT candidates) were admitted to Nemazi Hospital, affiliated with Shiraz University of Medical Sciences, Shiraz, Iran, and evaluated for aspergillosis for a period of 100 days. Thirty-one patients (37.8%) had thalassemia major, followed by acute myelocytic leukemia (14 cases, 17%); chronic myelocytic leukemia (9 cases, 10.9%); aplastic anemia (8 cases. 9.8%); multiple myeloma (7 cases, 8.5%); acute lymphocytic leukemia (5 cases, 6.0%); and other

hematologic disorders (8 cases, 10.0%). The female-to-male ratio was 35:47, the mean age was 25 years (range 4-57 years), and the mean hospitalization period was 78 days (35-227 days). The study hospital is the major centre performing BMTs in southern Iran. Patients received fluconazole as prophylaxis and amphotericin B was added to the medications of those who were febrile and not responsive to the administered antimicrobials (> 96 hours). True cases of invasive aspergillosis were defined as those patients with clinical and/or radiological signs of infection according to the guidelines of the European Organization on Research and Treatment in Cancer and the Mycoses Study Group (EORTC/MSG) [14]. The results of PCR testing were not utilized for clinical decision making purposes and the study authors were blinded to patient data during the testing of specimens.

Cultures of samples from throat, urine, feces, and sputum, along with sonography and computerized tomographic scans, were also performed when the patients were febrile and not responsive to the received antibiotics. Bronchoalveolar lavage or biopsy was not conducted because patients had thrombocytopenic criteria. All the samples were cultured on Sabouraud Dextrose Agar (Merck, Darmstadt, Germany) with chloramphenicol and following growth were examined by direct microscopy.

EDTA anticoagulated whole blood samples (3-5 ml) were collected once a week and stored at –20°C until PCR testing was performed. All blood samples collected from the recipients were tested. As negative controls, blood sampling was also performed on 60 volunteer patients with negative criteria [14]. Blood specimens were lysed according to the method of van Burik *et al.* [11]. DNA was purified using the QIAmp DNA Minikit (Qiagen, Hilden, Germany) in accordance with the manufacturer's recommendations.

The primers, TaqMan probe (Metabion Martinsried, Germany), and thermal cycling conditions for the *Aspergillus* real-time PCR assay were previously described [12,13]. The fungal amplification primers and probes target *Aspergillus*-specific sequences of the fungal 18S rRNA gene. The forward primer was 5'-TTG GTG GAG TGA TTT GTC TGC T-3' and the reverse primer was 5'-CTA AGG GCA TCA CAG ACC TG 3' (Metabion, Martinsried, Germany). The TaqMan probe was FAM-5'-TCG GCC CTT AAA TAG CCC GGT CCG C-3'-TAMRA. Samples were analyzed using the GeneAmp 7500 sequence detection system (Applied Biosystems, Foster City, California, USA) with TaqMan Universal PCR Master Mix (Roche, Branchburg, New Jersey, USA). Negative controls without template DNA were included in each PCR run. *Aspergillos niger*, *Aspergillos fumigatus* and *Aspergillos flavus* conidia were detected in pretesting using the described protocol.

To develop a PCR amplification standard, PCR fragments from an *A. fumigatus* isolate were amplified and inserted into pcr2.1 TA Cloning vector (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. To determine the sensitivity of real-time PCR assay for the detection of *A. fumigatus*, 100 copies/well of standard DNA were serially diluted with DNA- and RNA-free water and measured. The lower limit of detection of the assay was calculated to be ~10 copies/PCR reaction. Although the assay is capable of providing quantitative values on *Aspergillos* load, these data are not presented and results are rather described in a qualitative format. Data were entered into SPSS version 15 and were subsequently analyzed using descriptive and cross tabulation statistics.

Ethical considerations

The ethics committee of the Clinical Microbiology Research Center at Shiraz University of Medical Sciences reviewed and approved the study. Written informed consent was obtained from the patients prior to the study.

Results

A total of 993 blood samples collected from 82 patients were evaluated for *Aspergillus* infection. Of the 993 samples, 91 were collected prior to bone marrow transplant and upon admission to the ward, and the remaining were collected following transplantation once a week, for 100 days. *Aspergillus* DNA was positive serially in 94 blood samples from 13 transplant recipients ($n \geq 4$ specimens/patient) with clinical and radiological signs of aspergillosis (eight probable with clinical, radiological and mycological criteria; two probable with MRI from the abdomen showing abscesses in the liver, and three possible with clinical signs). Twelve recipients without clinical signs had one PCR-positive result in their blood samples and were considered to be false-positive by PCR. Only two recipients with possible clinical signs and a response to antifungal agents had negative PCR results and were considered to be PCR false-negative. All 60

negative patient controls showed negative PCR results. Sensitivity and specificity by patient were 86.6% and 82%, respectively, while the respective negative predictive value and positive predictive value of the PCR method were found to be 96.5% and 52%.

Samples in three patients had positive PCR results in the first week of admission to the ward prior to transplantation. In these samples the copy numbers of DNA were low in the first week but increased once chemotherapy was started. On average, these 3 patients presented clinical signs within 9 days of a positive PCR result, while for the remaining 10 infected patients the average length of time was 21 days after the first positive PCR results.

Upon the timely administration of antifungal agents, 4 of the 13 infected patients exhibited good responses and the numbers of *Aspergillus* DNA in their tests decreased during the weeks thereafter, but the other 9 patients died with no decrease in DNA copy numbers. Characteristics of the 13 invasive aspergillosis patients are shown in Table 1. Of note, a high proportion (69.2%) of the invasive aspergillosis infected recipients died. *A. flavus* was cultured from 4 patients and *A. fumigatus* from 3 patients.

Discussion

Detection and identification of fungi by culture or PCR is important in determining the appropriate antifungal therapy. A successful outcome depends on early diagnosis, treatment, and recovery from neutropenia. Definitive proof of invasive aspergillosis is provided by culture or histological examination, although in thrombocytopenic patients, obtaining tissue biopsy specimens is not feasible and blood cultures for *Aspergillus* are often negative. The PCR assay seems to be specific [15] and the sensitivity was 19.4 times higher than culture [16]. Real-time PCR has successfully been applied for the diagnosis of fungal infections that are hard to detect by culture and has the added benefit of minimizing false positive results due to amplicon contamination resulting from tube opening post-PCR [12,17-19].

Invasive aspergillosis has two peaks of occurrence: the first is before engraftment during neutropenia, and the second is during the post engraftment period when corticosteroid is used or graft-versus-host disease occurs. We evaluated the recipients for 100 days after transplantation to cover these intervals, and the mean time of invasive aspergillosis clinical manifestations was 43 days. There was no correlation between the burden of DNA and the severity of infection. Some patients had small DNA burdens even in the presence of clinical and/or radiological signs and even just before death.

Kami *et al.* [12] reported a sensitivity of 79%, for PCR for the diagnosis of invasive pulmonary aspergillosis, which is higher than that of galactomanan detection. The sensitivity of the method used in the present study was found to be 86.7%, which is higher than that in the previously mentioned study [12] but less than the 91.6% detected in another study [20]. This result might be due to examining more samples from each recipient or to the type of population in this study. In the present study, 12 patients with no clinical signs and no positive culture from clinical samples had a single blood sample PCR result that was positive (of several that were tested negative) and so these positive PCR results were considered to be false positive. In the future, the performance characteristics of the assay may be improved by incorporating a requirement for multiple PCR-positive serial specimens before a PCR-based diagnosis of aspergillosis is made.

The incidence rate of invasive aspergillosis may vary considerably, from 7.3% in HLA-identical siblings [19] to 27% in alternative HSCT recipients [21]. In our study, the incidence rate of infection was 15.8%, and four patients were infected with both invasive aspergillosis and cytomegalovirus. The rate of infection depends on the management of BMT procedures and the sterility of the wards, recovery from neutropenia, and other infections. In this study, infections were detected in lungs (in computer tomography, the typical halo signs or air crescents were not visible), sinopulmonary sites, and liver (disseminated infection or liver abscess).

Early detection of infection has a great impact on clinical outcomes of many infectious diseases. Mikulska *et al.* [22] reported two patients with invasive aspergillosis (clinical signs) at the time of transplantation. In the present study, three patients had *Aspergillus* DNA in their blood samples prior to transplantation with no clinical criteria; only one patient survived. Other studies reported that molecular methods can help detect the fungal infections before clinical findings [23,24]. Our data show that fungal DNA can be identified prior to clinical manifestations in patients with hematological disorders, who received multiple chemotherapy and steroid treatments, and who were neutropenic at different stages of their sicknesses.

Taking into account the above-mentioned findings, it is important that recipients be checked for

Table 1. Characteristics of patients with invasive aspergillosis

No.	Age	Sex	Type transplant	Site of infection	Primary disease	Culture	Diagnostic criteria	GVHD	No. of positive samples (n)	First Pos. PCR result[1] (day)	Onset of clinical signs[2] (day)	Outcome
1	7	F	Allogenic	Liver	Cooley`s anemia	----	C/ R	-	6	+15	+35	Deceased
2	42	M	Autologous	Sinus	AML	*A. flavus*	C/R/Mb	+	7	+27	+52	Deceased
3	30	M	Allogenic	Lung	AML	*A. fumigatus*	C /Mb	+	5	-6	-1	Survived
4	6	F	Autologous	Lung/ Brain	t. major	---	C/ R	+	7	+30	+45	Survived
5	25	F	Allogenic	Lung	CML	---	C / R	-	13	-6	+3	Deceased
6	49	M	Allogenic	Lung/ Sinus	AML	*A. fumigatus*	C/R/Mb	+	6	-6	+7	Deceased
7	16	M	Allogenic	Lung	t. major	*A. flavus*	C/R/Mb	+	7	+11	+30	Deceased
8	13	F	Allogenic	No localized	AML	---	C	+	5	+14	+15	Deceased
9	8	F	Autologous	Lung	CML	*A. fumigatus*	C/R/Mb	+	8	+20	+48	Survived
10	32	M	Allogenic	Lung/ Sinus	Cooley`s anemia	*A. flavus*	C/R/Mb	-	4	+7	+21	Deceased
11	41	F	Autologous	No localized	t. major	---	C	-	11	+12	+35	Deceased
12	17	M	Allogenic	No localized	AML	----	C	+	8	+7	+21	Deceased
13	23	M	Allogenic	Lung	t. major	*A. flavus*	C/R/Mb	+	7	+21	+49	Survived

No.: number; F: Female; M: Male; AML: acute myelocytic leukemia; CML: chronic myelocytic leukemia; t. major: thalassemia major; C: clinical; R: radiological; Mb: microbiological; GVHD: Graft Versus Host Disease; Pos: positive

[1] Positive numbers indicate days post transplantation and negative numbers denote days prior to transplantation

[2] Positive numbers indicate days after first PCR-positive result and negative numbers denote days before first PCR-positive result

any fungal infections before transplantation. It is possible that the two patients who were infected before transplantation and who subsequently died would have survived after transplantation had the *Aspergillus* infection been detected earlier. In patients with graft-versus-host disease treated with high doses of corticosteroids, disabled leukocyte trafficking and aspergillosis are fatal. The average death rate is approximately 50% in leukemic patients and 90% in allogeneic BMTRs [25]. In the current study, 69.2% of the invasive aspergillosis infected recipients died.

We observed in this study that the PCR results became negative during antifungal treatment and the subsequent recovery of neutrophils. Empirical treatment with antifungals is initiated as a common practice in patients with hematological malignancy and fever who are unresponsive to antibacterial agents. In the present study, the PCR results in two recipients were negative but the patients were responsive to the antifungal agents. This might have been due to fungal species not detectable by the PCR assay but also could have been due to the receipt of empirical therapy prior to sampling. Therefore, to promote the diagnosis of invasive aspergillosis, it may be more effective to collect blood samples for PCR testing prior to the initiation of empiric antifungal therapy.

Conclusion

Better diagnosis and potentially improved treatment of patients with invasive aspergillosis can be achieved through the use of specific real-time PCR assays direct from patient blood samples. Since the PCR method is both relatively noninvasive and rapid, it could be helpful in the early detection of *Aspergillus* species and the quantification of fungal loads in order to monitor antifungal treatment. *Aspergillus* infection was detected in the blood of the BMT candidates in the present study. Therefore, closer observation of patients who are neutropenic and receiving immunosuppressive drugs is necessary to enhance their chances for survival and lower both the cost and length of their hospitalization.

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