Detection of Invasive Aspergillosis in Bone Marrow Transplant Recipients Using Real-Time PCR

Mojtaba Nabili[1,2], Tahereh Shokohi[1], Ghasem Janbabaie[3], Mohammad Bagher Hashemi-Soteh[4], Kamran Ali-Moghaddam[5], Seyed Reza Aghili[1]

[1]Department of Parasitology and Mycology, Invasive Fungi Research Center, [3]Departments of Internal Medicine, [4]Biochemistry, Biophysics & Genetics, Molecular and Cell Biology Research Centre, Sari Medical School, Mazandaran University of Medical Sciences, Sari, [2]Social Security Organization, Golestan, [5]Department of Internal Medicine, Hematology-Oncology Research Center and Stem Cell Transplantation (HORCSCT; formerly HORCBMT), Tehran University of Medical Sciences, Tehran, Iran

ABSTRACT

Objective: The invasive aspergillosis (IA) is a serious opportunistic infection caused by various species of *Aspergillus* in immunocompromised individuals. Basically, rapid and early diagnosis prevents IA progression. In this study we performed a Real Time PCR/ Fluorescence Resonance Energy Transfer (FRET) for diagnosis of IA in hematologic malignancies and bone marrow transplant recipients. **Materials and Methods:** Sixty two patients with hematologic malignancies and marrow transplant recipients were evaluated for IA in Sari and Tehran from 2009 to 2010. The primer and hybridization probe were designed to amplify the specific sequence of 18S rRNA genes using Light Cycler system and FRET. Galactomannan (GM) assay was performed on serums which obtained from selected patients using the Platelia *Aspergillus* kit. **Results:** According to the criteria defined by the European Organization for Research and Treatment of Cancer and Mycoses Study Group (EORTC/MSG) for IA, 18 (29%) patients out of 62 patients were stratified into probable and possible groups. The female-to-male ratio was 1:2; the mean age of the patients was 36 years. The most common malignancies in these patients were acute lymphoblastic leukemia (38.9%). The minimum detection limit was 10 conidia (10^1 CFU/ml) equivalents (100 fg) per PCR reaction. GM assay was positive in 20.9% and real-time PCR probe set assay were positive in 17.7% patients who had clinical signs and host factor according to the mentioned criteria. **Conclusion:** Using the Real-Time PCR/FRET assay in whole blood specimens seems to be a promising method for diagnosis of IA, especially when used in combination with the GM detection test.

Key words: Hematological malignancy, Invasive aspergillosis, Real-Time PCR

INTRODUCTION

IA is a serious opportunistic infection caused by different species of *Aspergillus*. It often occurs in people with impaired immunity, particularly bone marrow and other organs transplant recipients, those receiving chemotherapy for treating hematologic malignancies, neutropenic patients, end stage AIDS patients, and patients with chronic granulomatous disease.[1,2] Incidence of invasive aspergillosis oscillates between 1-15%. In case of delayed diagnosis, the mortality rate reaches 90%.[3] The risk of *Aspergillus* infection has increased due to increased usage of anti-cancer treatments and deficiencies in immune system. *A. fumigatus* is the most common species which causes aspergillosis; however, other species such as *A. flavus*, *A. terreus* and *A. niger* are also fairly common agents of *Aspergillus* infection.[4] Absence of early diagnosis, quick dissemination and delayed treatment of infection have led to frequent recurrence, increased health costs and ultimately death of the patients.[5,6] A standard cost of treatment with voriconazole is more than 30000$.[7] Conventional methods such as culture and histopathology have limited sensitivity and specificity.[8] Histological examination of tissue samples are often obtained during open lung biopsy or transbronchial lung biopsy. Unfortunately, severe thrombocytopenia is commonly present in these patients.[9] Blood cultures are rarely positive for patients with invasive aspergillosis.[8] For these reasons, methods other than direct examination of the tissue and culture have been developed,

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Address for correspondence:
Prof. Tahereh Shokohi, E-mail: shokohi.tahereh@gmail.com

including GM and β-glucan antigen detection in serum or other body fluids, as well as molecular techniques. However, these antigen assay yields to a number of false positive results, due to cross reaction between *Aspergillus* spp and other fungi.[10,11] Real-time PCR offers several advantages over conventional methods. While, real-time PCR testing has demonstrated equivalent sensitivity and specificity to the conventional PCR testing,[12] the turnaround time eliminates the necessity to perform post amplification processing and detection. Besides, by combining target amplification and detection in a single, closed-reaction vessel, real-time PCR testing reduces the possibility of environmental contamination with amplified nucleic acids.[13] This assay permits highly reproducible detection and quantification of fungal burden which is also important in monitoring the effectiveness of treatment.

The real time PCR (FRET) method for *Aspergillus* DNA detection in clinical samples is used in several studies and its advantage is proven through comparing with other methods.[7,14-16]

Another advantage of the real-time PCR is that it allows identification of *Aspergillus* at the species level. In present study, we set up Real time quantitative PCR assay to detect *Aspergillus* species DNA by using Fluorescence Resonance Energy Transfer (FRET).

MATERIALS AND METHODS

A cross-sectional descriptive study was designed. The aim of this study was to determine IA in patients with hematologic malignancies presenting at the oncology department of Imam Khomeini hospital which is affiliated to Mazandaran University of Medical Sciences in Sari and hematology-oncology and stem cell transplantation research centre which is affiliated to Tehran University of Medical Sciences in Tehran from September 2009 to September 2010. This research was approved by the Ethics Committee of Mazandaran University of Medical Sciences and informed consent was obtained from all patients.

Inclusion and exclusion criteria

Patients with hematologic malignancies, bone marrow (BMT) transplant recipients, including those with recent history of neutropenia (less than 500/mm^3) for >10days; individuals with persistent fever refractory to broad-spectrum antibiotics for more than 96 hours; those with fever higher than 38°C or lower than 36°C; and patients with long-term use of corticosteroids and immunosuppressant (3 weeks during the past 60 days) were included in this study.[17,18] And patients receiving β-lactam group antibiotics such as Piperacillin–Tazobactam, patients with infections caused by *Bifidobacterium*, *Staphylococcus* and *Pseudomonas* were excluded due to cross-reactivity with GM antigens and making false positive results.

The demographic and clinical data collected from the patients included

Age, sex, date of sampling, brief descriptions of the patient's signs like high fever, persistent or recurrent sign of acute respiratory failure, the findings of CT scan, bronchoscopy findings, sinus infection, central nervous system infection, blood cell counts such as WBC count and the presence of neutropenia, history of chemotherapy, number of chemotherapy courses, history of radiotherapy and number of radiotherapy courses, type of malignancy, anti-fungal treatment, and time of beginning antifungal treatment.

The definitions of invasive fungal infection are classified into "proven," "probable," and "possible" in immunocompromised patients with cancer and in hematopoietic stem cell transplant recipients. To define as a proven invasive fungal infection it is required that a fungus be detected by histological analysis or in the culture of a specimen of tissue taken from the infected site of the disease. To consider as probable and possible invasive fungal infection 3 factors must be present; host factors, clinical signs and symptoms consistent with the disease entity, and mycological evidence including; direct microscopic analysis, culture and serologic test such as GM. The gold standard methods for diagnosis of IA are histopathology and culture of the tissue samples. Since, Biopsies can be dangerous and cannot be performed on cancer patients who have thrombocytopenia due to cytotoxic chemotherapy regimens, to define the sensitivity and specificity of the Real Time PCR method, the EORTC criteria was used.

Patient management

IA was classified according to EORTC/MSG definition.[17] The results of PCR were not applied in clinical decision making purposes and antifungal prescriptions. Oral fluconazole was used for prophylaxis in BMT patients. Empirical antifungal therapy with Amphotericin B was started after 96 hours of the onset of fever in case of antibiotic resistant fever, as recommended for neutropenic patients. Voriconazole used in amphotericin B resistant cases as substitute. In case of suspicion to pulmonary IA, chest radiography and/or, chest CT scan were performed. Blood sampling for fungal culture was done on all patients at least 3 samples per week.

The whole blood with anticoagulant was kept at -20°C until DNA extraction. The blood samples without anticoagulant were centrifuged at 3000 g for 5 minutes and the serums were kept at -20°C in to sterile tubes until determination of GM antigen.

Sample preparation

A clinical isolate of *A. fumigatus* CBS (603.31), *A.niger* CBS (513.88), *A.terreus* CBS (116.46), *A. flavus* CBS (120.49) and *C.albicans* CBS (562) were grown on Sabouraud-dextrose agar for 96 hours at 35°C. Fungal suspension was adjusted in sterile normal saline (0.5 × MacFarland standards) to a concentration of 1×10^6–5×10^6 cells ml^{-1}.[19] For sensitivity and specificity testing, serial dilutions (10^6-10^1 cells) were prepared. Five milliliter of blood samples from healthy volunteers were spiked with serial dilutions of *Aspergillus spp* and *C.albicans* to test the sensitivity and specificity of the assay.[20,21]

DNA extraction from whole blood specimen

DNA was extracted from whole blood as described by Loffler *et al.*[22] Briefly, hypotonic lysis of erythrocyte with Red Cell Lysis Buffer (RCLB) (10mMTris [PH = 7.6], 5 mM $MgCl_2$. 10mMNaCl) was performed for 10 minutes in 37°C. The pellet was obtained by centrifugation at 5000 g for 10 minutes.[22] The pellets were vortexed using glass beads (425-600µm, Sigma-Aldrich Corp, St. Louis, MO USA) for 3 minutes and the supernatant were treated with 180 U lyticase (Sigma-Aldrich Corp, St. Louis, MO USA) at 37°C for 30 minutes.[19] Two hundred micro liter of the treated supernatant was extracted using the QIAmp DNA blood mini kit by following manufacturer's instruction. To monitor any contamination in the DNA extractions, saline or DNA from healthy controls were included. Positive controls were prepared in each extraction to verify extraction efficiency.

Real Time PCR assays

The primers which bind to the conserved regions of the fungal 18S rRNA gene were applied.[19,20] Two different of the probe were hybridized to an internal species-specific sequence of 18S RNA gene of *Aspergillus*, with suitable fluorphore as shown in Table 1.

Table 1: Primers and probes

Primers:
ASP-F(5′-ATTGGAGGGCAAGTCTGGTG)
ASP-R(5′-CCGATCCCTTAGTCGGCATAG-3′)
Probes:
LC640Red 5′-TGA GGT TCC CCA GAA GGA AAG GTC CAG C 3′PHO
5′-GTT CCC CCC ACA GCC AGT GAA GGC -3′ fluorescein

The Real Time PCR amplification and quantification were carried out on Light Cycler System (Roche Diagnostics, Mannheim, Germany). The detection system was based on FRET which is performed with two hybridization probes. One of them is located at end of 5' and is labeled with the fluorphore Light Cycler Red 640 and the other probe which is located at the end of 3' is labeled with fluorescein. In this system, fluorescein (donor) is excited by light source of the Light Cycler machine. The excitation energy is transferred to the Light Cycler Red 640 fluorphore (acceptor) and emission of the acceptor fluorescence is measured in annealing step. Primer and probe were designed based on previous studies[1,2,14,15,23,24] and in order to assess the validity of the primer and the probe sequences, the BLAST software at NCBI database were used and were synthesized by MWG Operon. [Table 1] PCR was performed in a final volume of 20 µl (17. 4 µl of the Master Mix, 0.6 µl of HS prime Taq DNA polymerase enzyme and 2 µl of DNA template) with 8 minutes at 95°C, followed by 45 cycles of denaturation 5 s at 95°C, annealing (15 s at 62°C) and extension (25 s at 72°C), with a temperature transition rate (TTR) of 20°C s^{-1}. The PCR was followed by melting temperature analysis cycle compromising 90°C for 20 s (TTR of 20°C s^{-1}), 45°C for 20 s (TTR of 20°C s^{-1}) and 90°C for zero s (TTR of 0.2°C s^{-1}) to check the specificity of the PCR product. And for the ending stage, a cooling rate of 40°C to 30 s for one cycle was defined. The reactions could be almost investigated during the final 45 minutes. To verify the real time PCR results, the products were visualized after gel electrophoresis on 2% agarose gel and ethidium bromide staining. Quantification was performed by determination of the PCR cycle numbers for which the exponential of real time amplification curve had it crossing point (CT- value). To avoid any contamination, PCR assay was performed in a laminar-air-flow bio hazard cabinet and in a separated room, liquated reagent, positive displacement pipettes, and aerosol resistant tips. Each PCR and Real Time PCR included a negative control consisting of water without template DNA to monitor possible contamination.

ELISA

At this point, GM antigen levels in serum samples taken from the patients were measured using ELISA kit (Pletelia™ *Aspergillus* EIA French Company Bio-Rad with the Lot No; 62769). According to kit instructions GM Assays were classified as positive when an optical density ratio of ELISA ≥ O.5 was observed in at least 2 samples. Results are expressed as "galactomannan index" (GMI), by comparison to the "cutoff". GMIs of 0.5 or higher are regarded as positive.[25]

RESULTS

Sensitivity

All of the serial dilutions of the conidia of *A.fumigatus* were successfully amplified with increased fluorescence during the reproduction signal probe by Real Time PCR method. Minimum amount of DNA, 10 conidia or cell (10^1 CFU/ml) equivalents (100 fg) was detected in each PCR reaction, which indicated a high sensitivity of this method [Figure 1].

Specificity

PCR reactions with DNA of *C.albicans*, bacteria and human genomic DNA were negative which indicated that the specificity was 100%. Specific melting temperature (Tm) was determined after amplification of *Aspergillus* species DNA. Melting analysis of *A.niger, A.flavus, A.terreus* and *A. fumigatus* only revealed a specific melting peak signal for each species that were 70°C, 68°C, 71°C, and 69°C, respectively [Figure 2].

Reliability

Amplification of the DNA from the same extraction of the serially dilutions of *A. fumigatus* conidia and DNAs from the different extraction of different serial dilutions of *A. fumigatus* were repeated 5 times to exclude inter-assay and intra – assay variation, respectively.

Patients' demographic and clinical data

From 2009 to 2010, 62 patients who had met our inclusion criteria were enrolled. Their age ranged from 2 to 74 years with the mean age of 32.17 years. Forty cases (64.5%) were male and (35.5%) were female with female to male ratio of about 1:2. Nineteen cases (30.6%) were diagnosed as acute lymphoblastic leukemia (ALL), (22.6%) acute myeloid leukemia (AML), and (17.7%) Hodgkin's lymphoma and other patients had lymphoma, multiple myeloma, Fanconi anemia (FA), Thalassemia, Hemophagocytic lymphohistiocytosis (HLH) and chronic lymphocytic leukemia that received chemotherapy.

Mortality rate in this study was 33.9%. The highest mortality rate was observed in patients with ALL (31.2%), and followed by AML and non-Hodgkin's lymphoma (25%) and Hodgkin's lymphoma (12.5%). Clinically, fever not responding to empirical antibiotics was the most common clinical signs (83.9%), followed by lethargy and weakness (62.9%), cough (22.6%), and up and down fever (14.5%). None of the patients had a positive blood culture for *Aspergillus* species.

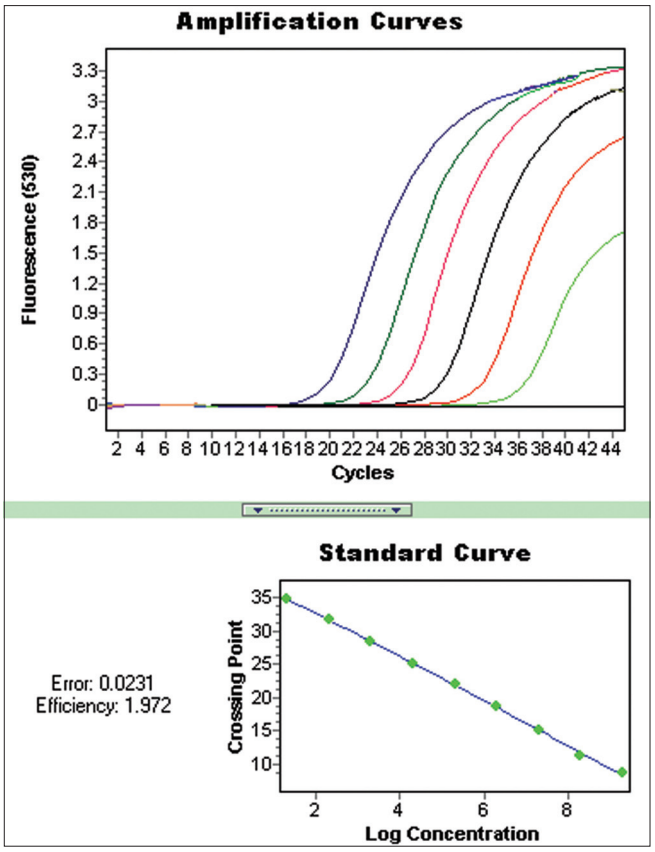

Figure 1: Quantitative amplification curve of *Aspergillus fumigatus* DNA dilution (10^1 to 10^6 copies/well of the target) by using Real-time PCR/FRET probe (upper) and Standard curve report of serially diluted *Aspergillus fumigatus* DNA (10^1 to 10^6) (lower)

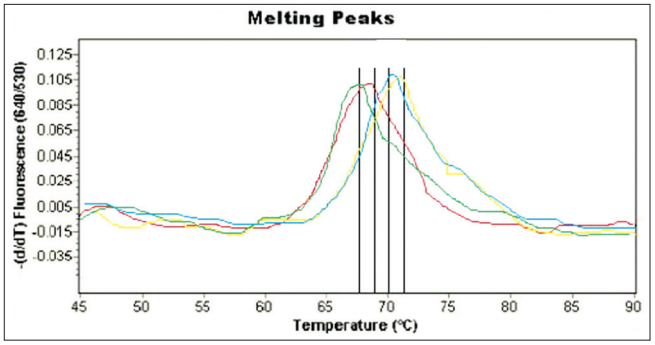

Figure 2: Specific melting temperature for *A.niger* (70°C), *A.flavus* (68°C), *A.terreus* (71°C) and *A.fumigatus* (69°C) are demonstrated

In this study, 56 (90.3%) patients out of 62 patients had neutropenic episodes. Most of the patients (96.8%) were under chemotherapy. In the study of sinus CT scans (8.1%) showed bilateral mucosal thickening of maxillary, etmoidal, and sphenoidal sinuses. The brain CT scan of 1 case showed brain atrophy and a pituitary hypertrophy. The chest CT scans of (4.8%) revealed diffuse infiltration. In none of these patients, the major signs for acute IA in sever neutropenic patients in CT scan, including halo sign and air crescent were evident [Table 2].

Real Time PCR results with clinical samples

A. fumigatus DNA was successfully amplified with the conventional thermo cycler and by Light Cycler System. All serially diluted samples showed a single band of 500 bp (amplicon) by gel electrophoresis; the band represented the fungus-specific amplicon [Figure 3]. Among hundred whole blood samples collected from 62 patients who met the clinical signs and host factor criteria; blood samples (19.3%) were positive in Real Time PCR/probe set assay. Compared to EORTC criteria sensitivity, specificity of the Real Time PCR to detect *Aspergillus* DNA in IA patients were 41%, 86%, respectively.

Melting Curve Analysis on the basis of melting temperature (Tm), (58.3%) *A.fumigatus*, (16.6%) *A.terreus*, (8.3 %) *A.niger* and (8.3%) *A.flavus* were identified.

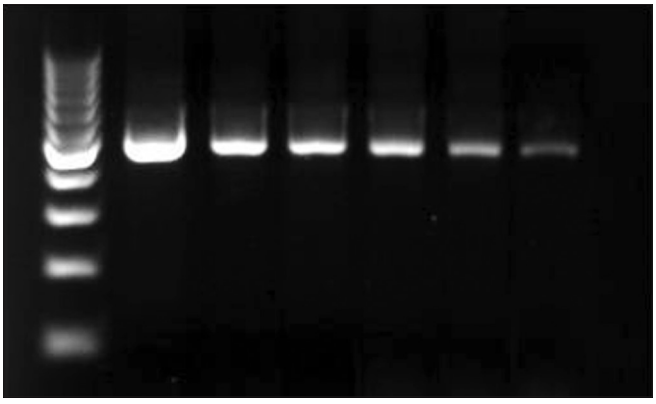

Figure 3: Agarose gel electrophoresis of serially diluted *A. fumigatus* conidia (10^6 to 10^1 CFU, corresponding to 10 ng to 100 fg of DNA) showing a single, specific band at 500 bp. Lanes: 1, 100-bp ladder; 2, 10^6 CFU ; 3, 10^5 CFU (1ng); 4, 10^4 CFU (100 pg); 5, 10^3 CFU (10 pg); 6, 10^2 CFU (1 pg); 7, 10^1 CFU (100 fg); 8, negative control (double-distilled H2O)

Serum Aspergillus GM test

The GM test of 100 serum samples of 62 patients, (with cut off ≥ 0.5) revealed positive result in (21%) who exhibited clinical signs and host factors of IA. Compared to EORTC criteria, the sensitivity, specificity of ELISA to detect *Aspergillus* GM in IA patients were 61%, 90%, respectively. Patients with invasive fungal infection are now classified according to the European Organization for Research and Treatment of Cancer/Mycosis (EORTC/MSG). These definitions allow patients' fungal infection to be categorized into proven, probable or possible groups. According to these criteria, (29%) were defined as IA which was stratified into probable: (28%) and possible: (72%) groups. In this study, autopsy and biopsy were not performed; therefore, we were not able to report any cases of definite IA (proven IA). The mortality rates among probable and possible IA were 55.6%.

DISCUSSION

To the best of our knowledge, there have been a few studies on the occurrence of invasive aspergillosis in immunocompromised patient in Iran.[26,27] The incidence and mortality rates of IA may vary considerably based on immune statues and the management of the patients and the environmental infection control of the ward and hospitals. In a retrospective cohort study of patients admitted between 1999 and 2003 to 18 hematology wards in Italy, to evaluate the incidence an outcome of invasive fungal infections (IFI). 11,802 patients with hematologic malignancies participated. There were 538 proven or probable IFI (4.6%); 373 (69%) occurred in patients with acute myeloid leukemia. Over half (346/538) were caused by molds (2.9%), in most cases *Aspergillus* spp. (310/346).

Table 2: Demographic characteristics and clinical data of eleven PCR positive patients with probable or possible IA

Patient no	Age, sex (yr)	WBC No	Primary disease or risk factor	Clinical symptom	CT	Agent	GM assay	IA classification	Outcome*
1	2/M	12400	HLH, ** BMT	High F, LRT	PI, SM, B	A.fumigatus	+	Probable	Death
2	58/F	300	ALL	High F, C, D	-	A.fumigatus	_	Possible	Death
3	45/F	500	L	F, C	-	A. flavus	_	Possible	Survival
4	29/F	800	HL** BMT	F, WL, LRT	SME	A. terreus	_	Possible	Survival
5	9/F	700	FA,** BMT	F, WL,	-	A. niger	+	Probable	Death
6	45/M	200	HL,** BMT	F, WL,	-	A.fumigatus	_		Survival
7	24/M	400	ALL, ** BMT	F, WL,	-	A.fumigatus	+	Probable	Survival
8	6/M	100	AML,** BMT	F, WL,	-	A.fumigatus	_		Survival
9	31/M	500	NHL,** BMT	F, WL,	-	A. terreus	_		Survival
10	19/M	100	ALL	F, WL,	-	A.fumigatus	_		Survival
11	28/M	100	HL** BMT	F, WL,	-	A.fumigatus			Survival

*After 6-10 months after diagnosis; **Candidate for BMT; ALL: Acute lymphoblastic leukemia, AML: Acute myeloid leukemia, HLH: Hemophagocytic lymphohistiocytosis, HL: Hodgkin's lymphoma, NHL: Non-Hodgkin lymphoma, L: Lymphoma, FA: Fanconi anemia, PI: Pulmonary infiltration, SM: Wall thickness of the maxillary sinus, SME: Wall thickness of the maxillary and Ethmoid sinus, B: Brain atrophy, D: Dyspnea, F: Fever, LRT: Lower respiratory tract, WL: Weakness and lethargy, C: Cough, Amp B: Amphotericin B

The mortality rates were for aspergillosis (42%). It seems that mortality rate for aspergillosis has dropped from 60-70% to approximately 40% which similar to our study.[28]

In another retrospective cohort study which was performed using the Kids Inpatient database in the United States during 2000, the highest incidence of IA was seen in children who had undergone allogeneic bone marrow transplantation (4.5%) and those with acute myelogenous leukemia (4%). Due to impact of IA on increases in mortality, length of hospital stay, and the burden of cost in the hospital setting emphasizes the need for improved mean of diagnosis, prevention, and treatment of IA in immunocompromised children.[29]

In an epidemiological surveillance network study by Cornet and *et al.* in 18 teaching hospitals in Paris, 621 cases (115 proven, 506 probable) of IA were analyzed. No seasonal variation was found. Hematological disorders (73%) including stem-cell transplantation (36%), solid-organ transplantations (10%) and AIDS (9%) were the main underlying conditions. The crude mortality was 63%. Incidence of IA was 8% in acute myelocytic leukemia and 6.3% in acute lymphocytic leukemia. Incidence was 12.8% following allogeneic stem-cell transplantation and 1.1% following autologous stem-cell transplantation.[30] In a prospective study (2005-2007) with 424 case-patients included, 15% had proven IA, 78% hematological conditions, and 92.9% had lung involvement. Acute leukemia (34.6%) and allogeneic stem cell transplantation (21.4%) were major host factors which this finding is similar to our study.[31] In the present study, 33.9% of the patients died despite antifungal therapy. It seems that this high mortality rate is due to delayed in diagnosis. In a systematic review, among 1941 patients with IA presented in 50 studies, the mortality rate despite antifungal treatment was 58%.[3,32] Shoham *et al*.,[33] reported the mortality rate as 95%. In the study done by Badiee *et al*.,[26] out of 14 patients who were positive for aspergillosis by PCR-ELISA molecular method, 5 patients (35.5%) died despite antifungal therapy.

Molecular diagnostic techniques such as detection of *Aspergillus* DNA in whole blood using real-time PCR are feasible approaches that may produce rapid diagnosis. It may be possible to identify infected patients at an early stage as we have demonstrated in this study.[34] In detecting the specificity of the Light Cycler PCR test, DNAs from bacterial pathogens and human genomic DNA were used. The entire PCRs with the bacterial pathogens and human genomic DNA were negative demonstrating a specificity of 100% for hybridization probes, alike to the results found by Skladny *et al*.[32] and Ramirez *et al*.[14] Sensitivity testing indicated a high sensitivity to detect 10 conidia or cell (10^1 CFU/ml) equivalents 100 fg of whole blood, which is equivalent to the limits of detection reported previously using real-time PCR and labeled oligonucleotide probes.[14,15]

In the study by Loffler *et al*. who had prepared *Aspergillus* conidia in dilutions of 10^0 to 10^4, they were able to determine an amount of 5 CFU/ml in each blood milliliter[24] In the study by Faber and colleagues, who had distinguished the *Aspergillus* species by using three different probes, they were able to determine the presence of at least 1-5 *Aspergillus* conidia in one milliliter of blood.[15] Badiee and *et al*. reported that sensitivity, specificity, negative, and positive predictive values of the PCR method compared to routine methods were 86.6%, 82%, 96.5% and 52%.[27] Several studies have also shown high sensitivity and specificity in the diagnosis of invasive aspergillosis with Real Time PCR.[5,15,23,24,35-41] It seems that the differences in the above mentioned sensitivity values depend on certain factors such as different DNA extraction methods, different primers and probes, thermal cyclers and implementation of PCR techniques. These molecular tests are capable to recognize 10-100 fg or 1-10 copies of the genome of *Aspergillus* DNA in blood, serum or probably BAL samples.In this study, *Aspergillus* growth of all performed blood cultures was negative. It is most important that blood cultures are rarely positive in patients with proven aspergillosis, and for this reason, it was not possible to compare the PCR-positive results for *Aspergillus* with blood cultures.[14]

Melting curve analysis after PCR amplification of *Aspergillus* species DNA, permits more discrimination between different species of *Aspergillus* based on the specific Tm pattern. Considering the increasing inherent resistance of some *Aspergillus* species other than *fumigatus* to conventional antifungal drugs, using this diagnostic modality in establishing rapid and accurate diagnosis of invasive aspergillosis improves patients' survival and prevents the expensive and potentially toxic antifungal treatments.

In the present study based on the melting curve analysis 4 species of *A. fumigatus*, *A.terreus, A. flavus* and *A.niger* were detected. The temperature range observed in the present study for *Aspergillus* species varied from 68.68ºC to 71.43ºC. In the study by Ramirez *et al*.[14] who studied 127 patients based on a Tm probe, they were able to differentiate 5 species of *A. fumigatus*, *A.terreus, A. flavus*, *A.niger* and *A. nidulans*.[14]

Faber and colleagues[15] showed that the Tm for each species is very useful for distinguishing the species from each

other. In this study, 4 species of *A. fumigatus*, *A. terreus*, *A.flavus*, and *A.niger* with temperature range of 68.1ºC to 72.9ºC were detected.[15] This difference in temperature ranges in different studies depends on several factors such as different extraction methods, different implementation of PCR techniques, and different components of master mixes like the amount of salt and ions and different types of the probes.

In the present study, in order to identify the DNA of different species of *Aspergillus* in the blood, Real Time PCR/FRET technique was used which has great advantages over other real-time methods such as SYBER green and TaqMan. One of the major disadvantages of SYBER green method is that the SYBER green color non- specifically binds to all double-stranded DNAs such as primer dimers and other non specific bands. Therefore, the fluorescent signals derived from the reaction, which is proportional to the amount of double-stranded DNA and the amount of fluorescence, increases resulting in decreased specificity and increased false positives. Using this method *Aspergillus* species and even fungal species cannot be distinguished from each other and only the presence of the fungi can be detected. In TaqMan method, probes are hydrolyzed and destroyed; analysis of the melting curve after replication is not possible. However, this method can differentiate the species of *Aspergillus* only if a specific probe is designed for each species, which will be very expensive.

But FRET method, which uses two fluorescent labeled probes, is specifically bound to the desired DNA. These probes are designed in a way that in case of even one different nucleotide, it does not bind to the DNA. As in this method the probes are not hydrolyzed, the analysis of the melting curve after replication is possible. The melting temperature of DNA, which depends on the DNA structure and factors such as length and number of nucleotides, probe concentration, salt concentration and GC content, can be a specific parameter to differentiate *Aspergillus* species from each other.

In addition to the fact that the real-time PCR assay (FERT) has the ability to diagnose, it can simultaneously quantify the fungal DNA load in a variety of clinical specimens. The clinical usefulness s of this method can include determining disease severity, monitoring response to treatment, estimating the advancement of disease.

Nevertheless, lack of standardization of the procedure is a major reason why the EORTC/MSG has not yet included PCR in its recently published list of criteria for the diagnosis of IA. There are only a few standardized assays that are commercially accessible. This particular challenge will be addressed by the Working Group 'EAPCRI' (European *Aspergillus* PCR Initiative) under the support of ISHAM. Twenty-four centers have started to organize a European standard for *Aspergillus* PCR. The main goal of these projects is to establish a standard for PCR that can be included into the next review of the EORTC/MSG definitions for IA.[42] Further prospective studies are required to evaluate the potential profits of early therapy based on Real Time PCR in patients at high risk for IA infections.

CONCLUSION

The Real Time PCR/FRET method is an appropriate diagnostic tool for detection of *Aspergillus* species DNA in the blood. Although the sensitivity of this test is shown to be relatively lower than GM, its specificity is almost identical to GM test. Therefore, Real Time PCR/FRET method in combination with GM test is a suitable method in detecting IA in patients with hematologic malignancies and bone marrow transplant recipients. Both tests must be used simultaneously in screening of the high risk patients to confirm the diagnosis of IA.

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