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Detection of *Aspergillus flavus* and *A. fumigatus* in Bronchoalveolar Lavage Specimens of Hematopoietic Stem Cell Transplants and Hematological Malignancies Patients by Real-Time Polymerase Chain Reaction, Nested PCR and Mycological Assays

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Background: Pulmonary aspergillosis (PA) is one of the most serious complications in immunocompromised patients, in particular among hematopoietic stem cell transplants (HSCT) and patients with hematological malignancies.
Objectives: The current study aimed to evaluate the incidence of PA and utility of molecular methods in HSCT and patients with hematological malignancies, four methods including direct examination, culture, nested polymerase chain reaction (PCR) and real-time PCR were performed on bronchoalveolar lavage (BAL) specimens in Tehran, Iran.
Patients and Methods: During 16 months, 46 BAL specimens were obtained from individuals with allogeneic HSCT (n = 18) and patients with hematological malignancies (n = 28). Direct wet mounts with 20% potassium hydroxide (KOH) and culture on mycological media were performed. The molecular detection of *Aspergillus fumigatus* and *A. flavus* was done by amplifying the conserved sequences of internal transcribed spacer 1 (ITS1) ribosomal DNA by nested-PCR and the β-tubulin gene by TaqMan real-time PCR.
Results: Seven (15.2%) out of 46 specimens were positive in direct examination and showed branched septate hyphae; 11 (23.9%) had positive culture including eight (72.7%) *A. flavus* and three (27.3%) *A. fumigatus*; 22 (47.8%) had positive nested-PCR and eight (17.4%) had positive real-time PCR. The incidence of invasive pulmonary aspergillosis (IPA) in these patients included proven IPA in 1 (2.2%), probable IPA in 10 (21.7%), possible IPA in 19 (41.3%) and not IPA in 16 cases (34.8%).
Conclusions: The incidence of IPA in allogeneic HSCT and patients with hematological malignancies was relatively high and *A. flavus* was the most common cause of PA. As molecular methods had higher sensitivity, it may be useful as screening methods in HSCT and patients with hematological malignancies, or to determine when empirical antifungal therapy can be withheld.

*Keywords:*Bronchoalveolar Lavage; Invasive Pulmonary Aspergillosis; *Aspergillus flavus*; *Aspergillus fumigatus*; Hematopoietic Stem Cell Transplantation; Hematological Malignancies

1. Background

Invasive mycoses represent a major cause of morbidity and mortality in patients with malignancy or undergoing hematopoietic stem cell transplantation (HSCT) (1, 2). Patients with hematologic malignancies are currently at higher risk of invasive fungal infections (IFI) caused by molds than yeasts, and the incidence of IFI is the highest among patients with acute myeloid leukemia. *Aspergillus* spp. are still the most common pathogens, followed by *Candida* spp. (3). Invasive aspergillosis (IA) occurs more often in patients with acute leukemia than in patients with chronic leukemia, lymphomas or multiple myeloma (4). In most reports, prognosis of the established aspergillosis is dismal; therefore, its prevention is of utmost importance. A meta-analysis of 50 studies on aspergillosis revealed mortality rates of approximately 60% in patients with acute leukemia and lymphoma and up to 90% in allogeneic stem cell recipients (5). The diagnosis of IA remains a major challenge. Definitive diagnosis of IA requires histopathological evidences of deep-tissue invasion or a positive culture from normally sterile sites (6).

To diagnose the invasive pulmonary aspergillosis (IPA) in high-risk patients, bronchoalveolar lavage (BAL) specimen is a useful tool. Early and precise diagnosis of IPA is important to start antifungal treatment in time and reduce the unnecessary use of toxic antifungal agents.

Although traditional approaches such as direct microscopic examination, histopathological evaluation and cultivation are still the gold standard, the diagnosis of IPA is generally difficult because of their inadequate sensitivity and specificity (7). Several molecular diagnostic methods are described to diagnose aspergillosis. However, they have only been used in experimental studies and not standardized yet.

2. Objectives

The current study aimed to evaluate the incidence of IPA in HSCT and hematological malignancies patients by using BAL specimens, and also the diagnostic potential of nested polymerase chain reaction (PCR) and real-time PCR were compared with conventional methods.

3. Patients and Methods

During a 16 month period (June 2009 to October 2010), 46 consecutive BAL fluid specimens were obtained from patients with hematological malignancies and HSCT, at Shariati Hospital and Medical Mycology Laboratory in the School of Public Health, Tehran, Iran. A portion of BAL specimens (4-7 mL) were obtained by specialist physicians under standardized techniques (8), and then collected in sterile vessels free of preservatives, and transferred to the laboratory within one hour. BAL specimens were centrifuged at 3,000 grpm for 20 minutes. The pellet was vortexed vigorously and resuspended in a small volume of supernatant, with the final volume of 400 to 600 microlitter. Aliquots of the specimens were kept at -20°C for future DNA extraction.

3.1. Culture

Seventy-five-microliter aliquots of the specimens were planted on Sabouraud glucose agar (4%) (Difco, USA) and Brain Heart Infusion agar (Difco, USA) plates equally (total 150 μL); then were incubated for three to seven days at 30°C.

3.2. Direct Examination

A wet mount was prepared with 150 μL of the sediment with a drop of 20% potassium hydroxide (KOH), and examined by 100× and 400× magnification of microscope.

3.3. DNA Extraction

For cell lysis of BAL fluid, each specimen was subjected to four times freezing in liquid nitrogen and thawing in boiling water, and then was crushed with a conical grinder for one minute. For sticky and viscous specimens, 2% dithiothreitol (Wako, Japan) was added, then sonicated for two to five minutes (9). Finally, DNA was extracted from each BAL specimen by phenol-chloroform method as described by Makimura et al. with a little modification (DNA of four specimens were lost) (10). The homogenized BAL sediment was added to an equal volume of the lysis buffer (100 mM Tris-HCl, pH = 8, 10 mM EDTA, 0.1% w/v SDS, 100 mM NaCl, 2% v/v Triton 100X) and an equal volume of phenol chloroform isoamyl alcohol (25: 24: 1), and was centrifuged at 5'000 g for 10 minutes. Chloroform was added to the supernatant and was centrifuged at 5'000 g for five minutes. Sodium acetate (3 M; 0.1 volume) and 2-propanol (equal volume) were added to the supernatant and kept at -20°C for 10 minutes, and then was centrifuged at 12'000 g for 12 minutes. The precipitated DNA was washed with 300 μL of 70% ethanol and re-suspended in 30 μL of ultrapure water and was kept at -20°C.

Furthermore, the analytical specificity of the PCR was assessed by testing the following fourteen genomic DNA of standard fungal strains, which were provided by Teikyo University Institute of Medical Mycology (TIMM), Tokyo, Japan: *A. flavus* (JCM 2061), *A. fumigatus* (ATCC 26430, TIMM 3968), *A. niger* (TIMM 0113), *Candida tropicalis* (ATCC 750), *C. albicans* (TIMM 3313), *C. glabrata* (ATCC 90030), *C. parapsilosis* (ATCC 22019), *Penicillium expansum* (TIMM 1293), *P. notatum* (TIMM 0883), *P. marneffei* (CBS 334.59), *Fusarium solani* (TSY 0403), *Trichosporon asahii* (CBS 2479) and *Sporobolomyces koalae* (JCM 18063).

3.4. Nested PCR

All the primers used for the nested PCR had been designed by Sugita et al. (11). The PCR mixture for contained 12.5 μL 2× premix (Ampliqon, Denmark), 0.5 μL (30 pmol) of each primer, and 2 μL of the DNA template solution, and enough distilled water up to 25 μL. The mixture was heated to 95°C for five minutes followed by 30 cycles of 94°C for 45 seconds; 60°C for one minute; and 72°C for one minute and a final step at 72°C for seven minutes. For the species specific primer sets of *A. flavus* and *A. fumigatus*, 1 μL of the first PCR product (diluted 1/100) was used as a template. Each mixture was heated to 95°C for five minutes and PCR was performed in cycles of 94°C for 50 seconds; 58°C for 40 seconds and 72°C for 45 seconds for 25 cycles and a final extention at 72°C for eight minutes. The reactions were run with a thermal cycler (Takara PCR Thermal Cycler Dice™ mini, TP100. Japan).

3.5. Real-time PCR

Real-time PCR was performed with a 7500 Fast Real-Time PCR System (Applied Biosystems) based on TaqMan chemistry. The real-time PCR primers and probes were designed based on multiple alignments of various sequences of partial β-tubulin gene by Zarrinfar et al. as described previously (12). All of the real-time PCR primers and probes were obtained from Applied Biosystems (USA). Reactions were performed using Eagle Taq Mastermix with ROX (Roche, Basel, Switzerland) according to the manufacturer's recommended protocol. Each reaction mixture contained 2 μL of template DNA solution, 0.2 μL (10.4 μM) of probes A. fumi-p, 0.16 μL (12.9 μM) of probe *A. flavus*-p, 0.4 μL (10 μM) of primer A. fumi-f, 0.4 μL

(10 μM) of primer A. fumi-r, 0.4 μL (10 μM) of primer *A. flavus*-f, 0.4 μL (10 μM) of primer *A. flavus*-r, 10 μL of master mix and enough distilled water up to the final volume of 20 μL. Following an initial denaturation step at 95°C for 10 minutes, PCR amplification was performed for 45 cycles consisting of 95°C for three seconds and 60°C for 30 seconds DNA extracted from *A. fumigatus* (TIMM 3968) and *A. flavus* (TIMM 2912) were used as positive and three tubes containing water instead of DNA were used as negative controls in each run.

A. fumigatus and *A. flavus* PCR products were cloned using a TA cloning kit and pCR 2.1 vector (Invitrogen Corp). The PCR 2.1 plasmid containing the DNA target was sequenced to confirm the insertion of a single copy of amplicon. The quantitation, accuracy, and precision of the real-time PCR assay were validated via preparation of serial dilutions of the plasmid containing the cloned target. The DNA concentration was determined according to the absorbance at 260 nm and molecular weight of the plasmid. In addition, the plasmid was used as the positive control in all reaction runs. Quantitative results were expressed by determination of the threshold cycle (Ct) of detection, or crossing point (Cp), which marked the cycle at which fluorescence of the specimen became significantly different from the baseline signal. The analysis of the results for a positive specimen was designated when at least one replicate had a Ct value of ≤ 38 cycles. The efficacy of the DNA isolation system in releasing *Aspergillus* DNA from the fungal cells for PCR were evaluated by analyzing serial dilutions of *A. fumigatus* conidia in seven concentrations ($10^6, 10^5, 10^4, 10^3, 10^2, 10^1$ and 10^0 conidia/mL saline).

4. Results

Forty-six BAL specimens were obtained from allogeneic HSCT (n = 18) and patients with hematological malignancies (n = 28), 70% being men (n = 32) with an average age of 38 and 30% being women (n = 14) with an average age of 35 years. Among the patients with hematological malignancies, six were ALL (acute lymphoblastic leukemia); 11 AML (acute myeloid leukemia); five had CLL (chronic lymphocytic leukemia); one had NHL (non-Hodgkin lymphoma) and also five had Lymphoma (Table 1).

According to the EORTC/MSG 2008 criteria (13), a diagnosis of proven IA can be established by the culture from a sterile tissue biopsy specimen, or the needle aspiration, or the microscopic detection of branched septate hyphae in such specimens with histopathological evidence of the associated tissue damage. Isolation of *Aspergillus* spp. from respiratory specimen or sinus aspirate is regarded as evidence for probable infection in a high-risk patient with relevant clinical and radiological findings. The radiological findings include typical pulmonary high resolution computed tomographic findings, such as the halo sign, air-crescent sign, or a cavity with a consolidated area, also findings in other tissues suggestive of fungal infection. If the radiological and mycological evidence for IA are not obtained but include the host factor and clinical criteria consistent with the infection, the diagnosis can only be classified as possible. Therefore, based on this classification, 2.2% (n = 1) were classified as proven IPA, 21.7% (n = 10) as probable IPA, 41.3% (n = 19) as possible IPA, and 34.8% (n = 16) as not IPA (Table 2).

Table 1. Demographics of the Enrolled Patients With Different Types of Hematological Malignancy [a]

Hematological Malignancy of Patients	Gender, Male/Female	Mean Age Range	BAL Specimens, No.
ALL	6/0	30.7	6
AML	8/3	30.9	11
CLL	4/1	58.6	5
NHL	0/1	35	1
Lymphoma	2/2	49.25	5

[a] Abbreviations: BAL, bronchoalveolar lavage fluid; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; NHL, non-Hodgkin lymphoma.

Table 2. Molecular Diagnosis Results and Classification of Patients With Hematological Malignancies and HSCT [a]

Underlying Disease	Proven IPA Cases, No.	Probable IPA Cases, No.	Possible IPA Cases, No.	Positive Nested PCR Results for *A. flavus*, No. [b]	Positive Nested PCR results for *A. fumigatus* No. [b]	Positive Real-Time PCR results for *A. flavus* No. [b]	Positive Real-Time PCR results for *A. fumigatus* No. [b]
ALL	0	1	5	3	1	0	1
AML	0	2	9	5	3	2	1
CLL	0	1	4	1	1	0	1
NHL	0	0	1	1	1	0	0
Lymphoma	0	2	3	2	0	0	0
HSCT	1	4	6	9	3	3	1

[a] Abbreviations: IPA, invasive pulmonary aspergillosis; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; NHL, non-Hodgkin lymphoma; HSCT, hematopoietic stem cell transplants.
[b] Some of BAL specimens had positive results in nested PCR for *A. flavus* and *A. fumigatus*, and in real-time PCR for *A. flavus* and *A. fumigatus*, together.

4.1. Direct Microscopy and Culture

Branched septated hyphae were observed in wet mount preparitons of seven (15.2%) BAL specimens, and one tissue biopsy specimen in the direct microscopic examination. Eleven (23.9%) specimens were positive in the culture including eight (72.7%) *A. flavus* and three (27.3%) *A. fumigatus*. The tissue specimen had positive culture by *A. flavus*.

4.2. Nested PCR

Of the 46 specimens tested, 22 (47.8%) were positive by the nested PCR including 13 *A. flavus* and one *A. fumigates* specimens, and eight specimens were positive for both *A. flavus* and *A. fumigatus*. The proven IPA specimen had positive nested PCR for *A. flavus*. Among the 10 probable IPA specimens, two (20%) showed positive results for *A. flavus* and three (30%) for both *A. flavus* and *A. fumigatus*. Among the 19 specimens with possible IPA, five (26%) showed positive results of the nested PCR for *A. flavus*, 1 (5%) for *A. fumigatus* and three (16%) for both *A. flavus* and *A. fumigatus*. Of the 16 specimens with not IPA, five (31%) had positive nested PCR for *A. flavus* and two (12.5%) were positive for both *A. flavus* and *A. fumigatus*.

4.3. Real-Time PCR

Eight specimens showed positive results by real-time PCR, including five for *A. flavus* and three for *A. fumigatus*. One of the specimens showed positive results for both *A. flavus* and *A. fumigatus*. Between the 10 specimens with probable IPA, two (20%) were positive for *A. fumigatus*. Of the, two (10.5%) out of 19 specimens with possible IPA had positive real-time PCR results, which one of them was positive for *A. flavus* and the other one for both *A. flavus* and *A. fumigatus*. Two (12.5%) out of the 16 specimens without IPA showed positive real-time PCR results for *A. flavus* and one (6.25%) showed positive real-time PCR result for *A. fumigatus*.

In the current study, the lower limit of detection (LoD) of used TaqMan real-time PCR method was 35 copies per assays for *A. fumigatus* and 40 copies per assays for *A. flavus*. Specificity assays did not show cross-reaction with DNA of the other fungal standard strains. In addition, the lowest detectable number of conidia in 1 mL saline was 10^2, which would be detectable by the molecular methods.

5. Discussion

Several approaches have been described for early diagnosis of aspergillosis in different patients. As a reliable and rapid diagnosis would improve the survival rate in high-risk patients especially among HSCT and patients with hematological malignancies, the current study evaluated routine laboratory methods, nested PCR and real-time PCR to diagnose PA due to *A. fumigatus* and *A. flavus*. The assay was carefully validated and applied to an analysis of BAL specimens.

To define IPA, the study used the diagnostic criteria described by the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases, Mycoses Study Group (EORTC/MSG) (13). *Aspergillus* spp. infection is one of the main causes of death in HSCT (14) and patients with hematological malignancies (15). On time diagnosis and treatment of PA in such patients is essential and decreases mortality rate. However, conventional definitive diagnostic methods are difficult and time consuming. Although conventional mycological and histopathological methods are still useful for a definite diagnosis of IPA, new non-invasive diagnostic methods including molecular biomarkers are now available (16). These new diagnostic methods facilitate an early diagnosis of invasive fungal disease and allow for utilization of a pre-emptive treatment approach, which may ultimately lead to improved treatment outcomes in HSCT and patients with hematological malignancies.

Although microscopic examination and cultivation of clinical specimens for PA diagnosis are still gold-standard methods; in general, they do not have enough sensitivity and specificity. Furthermore these methods show positive results only in the end stages of infection where an increased fungal burden exists. On the other hand, it is rather difficult to obtain deep biopsy specimens. Furthermore, fungal culture is often confounded by antifungal treatment, since the initiation of empirical treatment is a common practice in HSCT and patients with hematological malignancies. The molecular diagnostic methods are commonly used to diagnose PA. Although there are many published articles on the application of PCR to detect *Aspergillus* DNA, to date, a standard commercially developed molecular diagnostic test is not available.

Real-time PCR assay is widely used to detect fungal pathogens in the molecular studies, and considerably rapid and highly sensitive results are obtained in pediatric patients (7). According to the definition provided by EORTC/MSG (13), the IPA incidence of patients examined in the current study was 28% and 21% in HSCT and patients with hematological malignancies, respectively. While in the other reported studies (3, 7, 17), the incidence of IPA was lower than that of the current study. In the current study, 7 (15.2%) specimens were positive in direct examination while the positive results of culture (23.9%), real-time PCR (17.4%) and nested PCR (47.8%) were higher than it. Only two specimens had positive results by all the four methods; however, two other specimens showed positive results in direct examination, culture and nested PCR together.

The reasons why there were mismatches between the various methods is not quite clear. Potential explanation includes the possibility of the empirical therapies in these patients (18), lower sensitivity of traditional methods and specimen contamination (7). Moreover, isolation of the fungus from non-sterile specimens such as BAL specimen may also reflect colonization of the airway in-

stead of invasive infection (19). In the present study, unlike most reports that show *A. fumigatus* as a common cause of PA, in this study, *A. flavus* was the most frequent species isolated by culture and PCR while *A. fumigatus* was the second etiologic agent (20). These findings may be consistent with the increase of non-*fumigatus Aspergillus* spp. and or the difference of geographical locations. Other studies conducted in Iran have shown that *A. flavus* is the most frequent species isolated from patients and air (12, 21-23).

In the 16 specimens with not IPA, none of them had a positive direct examination and culture but seven and three of the specimens had positive nested PCR and positive real-time PCR, respectively. This may be due contamination and or a hidden suppressed immune system in the patients (24). However, in immunocompromised patients with characteristic clinical presentation, observation of *Aspergillus* in culture or PCR assay, even if obtained from sputum or BAL, has a high diagnostic value for IPA (25). Although this conclusion is based on a limited number of patients with hematological malignancies and HSCT for whom PA were suspected, the performance was promising. Despite this seemingly small number of subjects, the study reports a high number of proven/probable IPA cases in terms of evaluating diagnostic tests or surrogate parameter performance in BAL specimens for PA.

Although there was a weak correlation between the results of several methods that may not increase the diagnosis power, it is useful to make diagnostic decisions especially in patients with hematological malignancies and HSCT with clinical signs suspicion to PA. In conclusion, incidence of IPA in allogeneic HSCT and patients with hematological malignancies was relatively high and *A. flavus* was the most common cause of PA. As molecular methods had higher sensitivity, it may be useful as the screening methods in HSCT and patients with hematological malignancies, or to determine when empirical antifungal therapy can be withheld.

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Authors' Contributions

Hereby all the authors guarantee that this paper is an original work. It has not been submitted to any other journal. Specimens collection and performance of routine and molecular procedures: Hossein Zarrinfar; study concept and design: Hossein Mirhendi; data collection training and advising, and writing scientific report: Abdolmajid Fata; cooperation in molecular procedures: Hossein Khodadadi. Specimen collection training and advising, and routine diagnosis procedures: Parivash Kordbacheh.

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