Bone Marrow Transplantation (2009) 44, 595–599

www.nature.com/bmt

ORIGINAL ARTICLE

Impact of PCR-based diagnosis of invasive pulmonary aspergillosis on clinical outcome

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The mortality rate of 60–90% in invasive pulmonary aspergillosis (IPA) is partly explained by diagnostic delay due to the limitation of current diagnostic tests. We assessed the influence of *Aspergillus* species (ASP) DNA detection by PCR from bronchoalveolar lavage (BAL) fluid, a new tool for diagnosing IPA, on the outcome of this disease in immune-compromised patients. The study population comprised 107 consecutive patients with hematological malignancies from a single medical center with IPA diagnosed between 1998 and 2005. Clinical variables and mortality rates were compared between two groups diagnosed according to traditional criteria without and with PCR-based ASP DNA detection in BAL fluid. The overall mortality rate during the study period was 38.3%. The addition of PCR to the diagnostic criteria shifted 31 patients from possible to probable IPA. Patients diagnosed with probable IPA according to traditional microbiological methods had significantly higher mortality rates compared to their counterparts who had in addition a PCR-based diagnosis (80 vs 35.6%, $P = 0.003$). This study demonstrates that PCR-based ASP DNA detection for a diagnosis of IPA from BAL fluid has a significant effect on the outcome of patients with IPA, probably related to earlier diagnosis.
Bone Marrow Transplantation (2009) **44,** 595–599; doi:10.1038/bmt.2009.65; published online 23 March 2009
Keywords: *Aspergillus*; immune suppression; lung; mortality; PCR

Introduction

Invasive pulmonary aspergillosis (IPA) is a common, often life-threatening infection in immune-compromised patients, especially those with hematological malignancies (HM) and/or BMT recipients. Early (in-hospital) mortality rates of 60–90% from IPA are explained in part by diagnosis in advanced stages of the infection due to the limitations of current diagnostic tests.[1,2]

Early diagnosis of IPA and administration of antifungal treatment is expected to improve outcome, but this goal is difficult to achieve as cultivation of the causative agent *Aspergillus* species (ASP) from respiratory secretions has poor sensitivity.[3–7] Deep tissue biopsy specimens are difficult to obtain in this setup because many affected patients have poor respiratory status in addition to coagulation defects. PCR-based ASP DNA detection in bronchoalveolar lavage (BAL) fluid theoretically enables earlier and more frequent diagnosis of IPA.[8–11] The implication of this test on the outcome of affected patients had not been studied as yet.

This study was designed to assess the added value of this test by comparing the outcome of immune-compromised patients (with HM and BMT recipients) with IPA, diagnosed either with the traditional methods alone or with the traditional methods combined with PCR-based ASP DNA detection in BAL fluid.

Patients and methods

Patients

This retrospective study was undertaken at the Department of Hemato-Oncology and at the Division of Pulmonary Medicine at Rambam Health Care Campus, a 1000-bed primary and tertiary care, university-affiliated hospital over an 87-month period (1 May 1998 to 31 July 2005). The study population included 107 consecutive hospitalized patients with HM and BMT recipients who underwent bronchoscopy and BAL for the evaluation of pulmonary infiltrates, and who were diagnosed with IPA. BAL is part of the routine workup performed at Rambam for immune-compromised patients with pulmonary infiltrates.

The study population was divided into two groups. Group 'A' included 42 patients diagnosed with IPA from May 1998 to November 2002 using traditional microbiological techniques, whereas group 'B' included 65 patients

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Received 6 May 2008; revised 3 February 2009; accepted 6 February 2009; published online 23 March 2009

diagnosed with IPA from November 2002 to July 2005, when PCR-based ASP DNA detection in BAL fluid was added to the routine diagnostic evaluation performed on BAL specimens for these patients. IPA was diagnosed according to the European Organization for Research and Treatment of Cancer and Mycoses Study Group criteria 2002[12] and was categorized as possible, probable or proven.

- Possible IPA was defined as clinical and radiological findings highly suggestive of IPA in patients with the appropriate host factors without supportive laboratory evidence.
- Probable IPA was defined as clinical and radiological findings highly suggestive of IPA in patients with the appropriate host factors and detection of ASP (from BAL) by smear or culture, or positive galactomannan (GM) Ag in serum.
- Proven IPA was defined as clinical and radiological findings highly suggestive of IPA in patients with the appropriate host factors and histopathological evidence of tissue invasion and damage by ASP or isolation of ASP from lung biopsy.

Bronchoscopy
Bronchoscopies were performed by fiber-optic bronchoscope in the bronchoscopy suite or bedside in the hematology department with pulse-oximeter and cardiopulmonary monitoring. The procedure was performed following sedation (midazolam 5–10 mg, i.v.) and local anesthesia (lidocaine 2%) through the nose or oral cavity. The BAL specimens were sent to the microbiology, virology and cytology laboratories and were cultivated there for aerobic and anaerobic bacteria, viruses, fungi and mycobacteria.

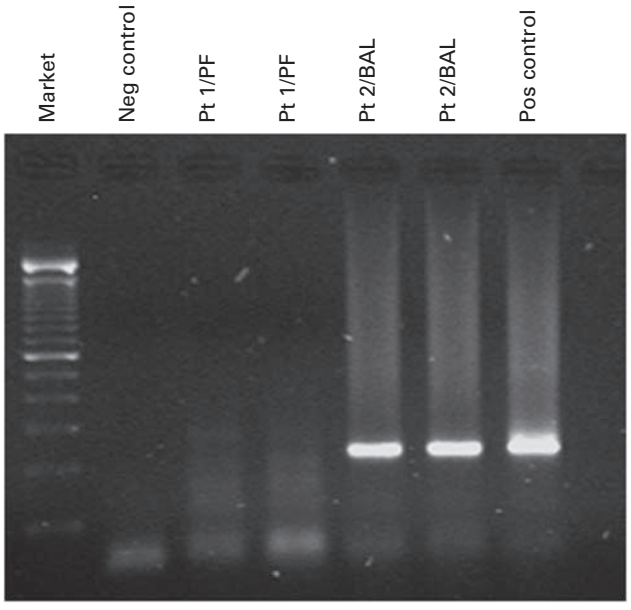

Figure 1 PCR-based assay for detection of aspergillus DNA. DNA was extracted from patient 1 (Pt1) and patient 2 (Pt2) pleural fluid (PF) and bronchoalveolar lavage (BAL). Negative and positive controls are shown as well.

Radiological evaluation
The chest CT studies were performed with 2-slice (CT Twin), 4-slice (Mx8000 Quad) or 16-slice (Mx8000IDT) scanners (Philips Medical Systems) as available at the time of the examination with scanning protocols directed by the attending radiologist. The review of the CT scans was done on the Easy-Vision PACS (Philips Medical Systems, Andover, MA, USA).

ASP DNA detection
A new method of detecting ASP DNA from BAL specimens by PCR test using an in-house developed protocol was introduced at our institution in November 2002. BAL samples were sent to the laboratory and processed immediately. Total DNA was prepared from 0.2 ml of BAL fluid using a QIAmp DNA mini kit (Qiagen, Hilden, Germany). Approximately 50–150 ng of total DNA was amplified using a two-step (nested) PCR assay. First-round PCR primers were forward AFU7S and reverse AFU7AS and the second-round PCR primers were forward AFU5S and reverse AFU8AS, which specifically amplify a highly conserved ASP-specific region of the 18S ribosomal RNA gene.[9] First-round PCR conditions were as follows: 2 min at 94 °C followed by 23 cycles of 40 s at 94 °C, 1 min at 65 °C and 1 min at 72 °C with a final step of 5 min at 72 °C. For the second-round (nested) PCR, 2 μl of the first-round PCR product were amplified as follows: 2 min at 94 °C followed by 35 cycles of 40 s at 94 °C, 1 min at 65 °C and 1 min at 72 °C, with a final step at 72 °C for 5 min. A 232 bp PCR fragment encoded by the human *β-globin* gene was amplified in parallel to control for the presence of DNA. PCR products were separated on 2% agarose gels, stained with ethidium bromide and visualized with UV light. The first PCR step results in amplification of a 404 bp fragment, and the second step amplifies an internal fragment of 220 bp. (Figure 1). To definitely establish the identity of the PCR product, we occasionally sequenced resulting amplicons. The PCR assay detection threshold is 100 fg/μl, and sensitivity, specificity, positive predictive and negative predictive values are 75, 100, 100 and 87%, respectively (in preparation).

Data collection
Demographic and clinical characteristics were retrieved from the computerized hospital system. The status of the HM was defined for each patient. For BMT recipients, time and type of transplantation performed was recorded. The respiratory status of the patients was defined as normal when no oxygen supplementation was needed and abnormal when a need for oxygen supplementation or respiratory support was present. Categorical variables, such as age, gender, type and status of HM, neutropenia, respiratory status, extrapulmonary organ dysfunction and outcome were compared between the groups.

Outcome
The end point of this study was the in-hospital mortality rate, which was compared between the two groups, A and B, and between subgroups with different diagnostic probabilities.

Statistics
Means and s.d. were used to express approximately normal distributed variables. The association between categorical variables and survival was assessed by the Fisher's exact test. A *P*-value <0.05 was considered as significant.

Results

The baseline characteristics of the whole study population are shown in Table 1. There were 75 men and 32 women with a mean age of 46.6 ± 15.2 years (range 17–77 years). A total of 65 (60.7%) patients were BMT recipients. Acute leukemia was the most common HM, present in 58 patients, with multiple myeloma in 15 patients and lymphoma in 21 patients. Thirty-nine patients had active hematological disease. Twenty-seven patients had acute respiratory failure requiring mechanical ventilation.

The baseline epidemiological and clinical characteristics comparison between the two groups is shown in Table 2. There was no statistically significant difference in any of the demographic and clinical variables between the two groups. The time gap between onset of symptoms to bronchoscopy was similar for both groups—1.8 vs 2.0 days.

Radiological variables, such as administration of i.v. contrast and slice thickness, did not differ between the two groups. The radiological manifestations of IPA are presented in Table 3. Pulmonary nodules with and without halo sign were the most common radiographic findings in both groups. Air-crescent sign and cavitary nodules were more prevalent in patients from group 'A' than from group 'B' but the difference was not statistically significant.

Galactomannan Ag detection was introduced to our laboratory at the end of the year 2002, and at the beginning was not routinely performed. Altogether, the test was performed for 46 patients from group B (46 of 65), was positive in 16 and negative in 30. When available, GM Ag test result was included in the microbiological criteria for categorization of the probability of IPA.

Table 1 Patient characteristics

Number of patients	107
Age, years (range)	46.6 ± 15.2 (17–77)
Gender	
Male	75 (70%)
Female	32 (30%)
Hematological disorders	
Acute leukemia	58
Chronic leukemia	13
Lymphoma	21
Multiple myeloma	15
BMT	65 (60%)
Autologous	14 (13%)
Allogeneic	51 (47%)
Hematological status	
Active disease	39 (36.4%)
Respiratory failure	27 (25.3%)

The in-hospital mortality rate of the entire cohort was 38.3%. Overall, the mortality rates did not differ significantly between the two groups (47.6 vs 32.3%, $P=0.387$). Stratification of the study population using the diagnostic criteria as published by the European Organization for Research and Treatment of Cancer and Mycoses Study Group[12] revealed 15 and 28 probable cases in the two groups, as well as 27 and 37 possible cases. There was no single case of definite diagnosis in any of the groups. The mortality rates among patients with probable and possible diagnoses of IPA did not differ between the two groups

Table 2 Baseline characteristics of the two study groups

	A, 1998–2002	*B, 2002–2005*	P-*value (Fisher's)*
Age	45.195 ± 15.93	47.7 ± 14.6	NS
Gender (% male)	30 (71.4)	45 (69.2)	NS
Hematological disorder			
Acute leukemia	22	36	
Chronic leukemia	7	6	
Multiple myeloma	5	10	
Lymphoma	8	13	
Active hematological disease	15 (35.7%)	24 (36.9%)	NS
BMT	26	39	NS
Autologous	6	8	NS
Allogeneic	20	31	NS
Time from BMT <1 m	15 (38.4%)	23 (35.30%)	NS
Extrapulmonary organ failure	19 (46.1%)	29 (45)	NS
Mechanical ventilation	11 (26.1%)	16 (24.6%)	NS

Table 3 Radiological characteristics

	Subgroup A (42)	*Subgroup B (65)*
Nodules, *n* (%)	13 (31)	34 (52)
Cavitations, *n* (%)	8 (19)	5 (8)
Wedge-shaped infiltrates, *n* (%)	7 (17)	5 (8)
Nonspecific infiltrates, *n* (%)	14 (33)	21(32)

Table 4 Mortality data on subgroups of patients with different diagnostic criteria

	A (n = *42*)	*B* (n = *65*)	P-*value*
(A) In-hospital mortality rates of two groups at all levels of certainty of diagnosis, according to standard criteria			
All	20/42 (47.6%)	21/65 (32.3%)	NS
Probable	12/15 (80%)	15/28 (53.6%)	NS
Possible	8/27 (29.6%)	6/37 (16.2%)	NS
(B) In-hospital mortality of two groups at all levels of certainty of diagnosis, when adding PCR+ in BAL as microbiological evidence for probable diagnosis			
All	20/42 (47.6%)	21/65 (32.3%)	NS
Probable	12/15 (80%)	21/59 (35.6%)	0.003
Possible	8/27 (29.6%)	0/6 (0%)	NS

(Table 4A). Repeated statistical analysis, adding positive aspergillus DNA in BAL detected by PCR as a supportive laboratory evidence to group B (which shifted 31 possible cases to probable ones) demonstrated that the mortality rate of patients with a probable diagnosis using the PCR result was significantly lower compared to the probable cases by the traditional definition (35.6 vs 80%, $P=0.003$), with a relative risk reduction of 56% (Table 4B).

Discussion

The mortality rate from IPA in immune-compromised patients, especially those with HM, is exceedingly high. Early identification and initiation of antifungal treatment is expected to improve the survival of these patients.[3] The current approach in which clinical probability, fungal culture and cytology examination serve as guidelines for therapy is not satisfactory.[4,5] For this purpose, more rapid confirmation of aspergillus infection with non-culture-based testing may be more useful. Detection of ASP DNA in BAL fluid theoretically enables early diagnosis and early guided optimal therapy that is expected eventually to improve the outcome of IPA in high-risk patients.[6–11] In our hands, the turnaround time for PCR-based detection of aspergillus DNA from BAL sampling until delivery of the results is approximately 1–2 working days, with the molecular diagnostic procedure lasting for about 6 h.

This study evaluated the contribution of ASP DNA detection to the diagnostic workup of immune-compromised patients with IPA by comparing the mortality rates during two different time periods, with and without the addition of ASP PCR testing. In-hospital mortality was chosen as the outcome variable, despite the fact that nowadays the disease, especially in allogeneic BMT recipients, can present in the community long after transplantation, because in our hospital the standard of care was to admit patients suspected of having IPA, and treat them in hospital for this diagnosis, even a long time after the BMT. Besides, it was documented by Upton *et al*.[13] who compared the outcome of patients with IPA between two time periods that although overall the outcome was better in the recent period, the mortality rate persistently and equally increased with time during 1 year of follow-up in the two study periods. Another point in favor of this decision is that there was no statistically significant difference in the number of allogeneic BMT recipients between the two study groups.

We found that the mortality rate in patients with probable IPA diagnosed with the addition of ASP DNA detection was much lower (35.6 vs 80%, $P=0.003$), compared to the probable cases diagnosed traditionally in the first period. Although the proportion of probable cases increased in the second period over possible ones, the reduced mortality was apparent in the second group as a whole, compared to the first one (32.3 vs 47.6%), although it did not reach statistical significance. This difference in mortality might reach statistical significance in a larger group of patients.

Regardless of the mode of diagnosis, the mortality rates from IPA in our institution are lower when compared to reports from other sites,[1,2] probably reflecting the effect of experienced dedicated staff in our tertiary BMT center with a high incidence of IPA and early usage of novel antifungal medications, such as voriconazole[14] and caspofungin.[15] In other words, relying on fungal culture might lead to a delay in the onset of antifungal therapy, probably because fungal culture tends to become positive in subjects with advanced, sometimes incurable, disease whereas PCR testing enables detection of IPA in an earlier stage.

Our study population includes patients with nonspecific pulmonary infiltrates, as specified by the original disease-defining criteria,[12] a criterion that is not present anymore in the recently revised definitions of invasive fungal disease.[16] However, in any patient with nonspecific pulmonary infiltrates, there was at least one more clinical criterion present (cough, chest pain, hemoptysis, dyspnea, pleural rub). Moreover, 13 of 21 patients with nonspecific pulmonary infiltrates in group B were shifted from the possible to the probable category based on the ASP/PCR results performed on BAL specimens. Thus it is likely that most of the possible cases were indeed cases of IPA, despite the inclusion of patients with nonspecific infiltrates.

Despite excellent sensitivity and specificity, ASP DNA detection in BAL has not yet been standardized or externally validated. Standardized, commercial PCR diagnostic assay are not available in Israel nor has it been incorporated into the standard diagnostic criteria of IPA. In our opinion, a sensitivity of 75–100% and a specificity of 80–93% for PCR, as published in the literature, compared to a sensitivity of 30–50% for ASP culture from BAL specimens, justify incorporation of this test into the diagnostic workup of high-risk patients with suspected IPA. Clearly, multicenter studies comparing different PCR assays as well as large clinical trials are mandatory before inclusion of the PCR assay into official diagnostic criteria.

Our data are consistent with previous studies in suggesting that PCR performed on BAL has high positive and negative predictive values. The high negative predictive value of the PCR test might help clinicians to withhold antifungal therapy in high-risk patients with a negative PCR from BAL fluid, because of a low probability for fungal infection,[9,10] whereas positive PCR results in a high-risk population justify antifungal therapy.[11] However, before incorporating the PCR assay into clinical official diagnostic criteria, large, multicenter clinical trials that compare different PCR assays are mandatory.

In summary, using PCR testing for ASP species in BAL for the diagnosis of IPA improved the outcome of immune-compromised patients with IPA. As this test can be performed easily on BAL fluid that is commonly sampled from these patients, it facilitates rapid confirmation and prompt institution of antifungal therapy, or alternately, rules out IPA without a need for invasive procedures. We believe that this test should be included in the diagnostic criteria of IPA.

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