Diagnosis of Invasive Pulmonary Aspergillosis Using Polymerase Chain Reaction-Based Detection of Aspergillus in BAL*

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Study objective: To assess the value of Aspergillus polymerase chain reaction (PCR) test performed on the BAL in diagnosing invasive pulmonary aspergillosis (IPA).

Design: Between January 1996 and 1997, we prospectively followed up 249 cancer patients with pulmonary infiltrates suggestive of pneumonia. Bronchoscopy with fungal stains, cultures, and PCR was performed on all patients. PCR was used for the detection of Aspergillus mitochondrial and alkaline protease gene DNA. The PCR products were visualized either directly on polyacrylamide gel or after Southern transfer and probing with specific probes for mitochondrial and alkaline protease DNA.

Results: The 249 patients consisted of 10 patients with proven IPA (tissue invasion), 22 patients with probable IPA (microbiologic culture), 18 patients with possible IPA (consistent clinical and radiologic findings), and 199 control patients with no evidence of IPA. PCR positivity was strongly associated with all forms of IPA (p < 0.002). The sensitivity, specificity, positive predictive value, and negative predictive value of PCR were 80%, 93%, 38%, and 99%, respectively, for proven IPA, and 64%, 93%, 52%, and 96%, respectively, for probable IPA. Southern blotting analysis did not improve the diagnostic yield of the PCR test.

Conclusion: PCR performed on BAL is associated with high specificity and negative predictive value for IPA. The low positive predictive value could be related to the transient colonizing presence of aspergilli in the respiratory tract. The sensitivity correlates with the certainty of the diagnosis based on tissue invasion.

Key words: alkaline protease gene; aspergillosis; fungal pneumonia; polymerase chain reaction; mitochondrial DNA; molecular diagnostics

Abbreviations: bp = base-pair; CI = confidence interval; IPA = invasive pulmonary aspergillosis; PCR = polymerase chain reaction; SDS = sodium dodecyl sulfate

The mortality of patients with invasive pulmonary aspergillosis (IPA) remains unduly high despite antifungal therapy in immunocompromised patients, particularly leukemia and bone marrow transplant recipients. A definitive diagnosis is established by the demonstration of deep tissue invasion through histopathologic specimen, which is often contraindicated in the clinical setting of bone marrow aplasia and severe thrombocytopenia. This often requires delay in establishing the diagnosis, as well as the indiscriminate heavy use of empiric nephrotoxic antifungal therapy. Noninvasive methods and early diagnosis could result in early treatment and improved outcome. Occasional clinical or radiologic findings, such as pleuritic friction rub or the halo or crescent signs demonstrated on CT, are highly specific for this infection, but are not very sensitive. Using different sets of primer genes, polymerase chain reaction (PCR) has been suggested in several studies to be useful in establishing the diagnosis of IPA in high-risk patients. However, most of the studies were retrospective in nature. In addition, they involved a small number of patients. This study was conducted on a large cohort of cancer patients with pneumonia who were prospectively

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evaluated in order to determine the diagnostic usefulness of PCR performed on BAL in the diagnosis of IPA.

**Materials and Methods**

**Patient Enrollment**

Between February 1996 and October 1997, 249 cancer patients with chest radiographic findings suggestive of pneumonia were prospectively evaluated. All patients underwent bronchoscopy, and the BAL fluid was sent for microscopy, cytology, and bacterial, viral, and fungal cultures. Information was obtained on all patients as to their age, gender, race, underlying diagnosis, and history of bone marrow transplant. In addition, further information pertaining to clinical manifestations of the infection, such as fever, cough, hemoptysis, dyspnea, pleuritic chest pain, sinus symptoms, and prior lung disease, was obtained. Clinical signs of the infection, particularly pleuritic friction rub, rales, or rhonchi, were assessed. In addition, the risk factors for the infection, such as use of steroids, duration of neutropenia, and prior chemotherapy were also determined. Information was also obtained on the radiologic and laboratory findings, such as CT of the chest, as well as the chest radiograph and sinus radiographs. Histopathologic findings obtained on the BAL, transbronchial, fine-needle, or open-lung biopsy, and autopsy if available were also documented. The treatment that the patient received, in terms of antibiotics and antifungal therapy, was also determined on all patients. Patients were followed up prospectively until death or discharge.

**BAL**

The main indication for BAL was the development of new pulmonary radiographic infiltrates. Fiberoptic bronchoscopy was performed using a conventional technique.

Because immunocompromised patients frequently have some degree of thrombocytopenia, the oral route was preferred due to the lower risk of bleeding when compared with the nasal route. Because immunocompromised patients frequently have some degree of thrombocytopenia, the oral route was preferred due to the lower risk of bleeding when compared with the nasal route.

The fiberoptic bronchoscope was wedged into a subsegmental bronchus, and four to six 20-mL aliquots of sterile normal saline solution were instilled and retrieved. The BAL fluid was then sent for cytologic and microbiologic examinations: Gram stain and hemosiderin staining.

**PCR Assay and DNA Isolation**

Fungal DNA was extracted from BAL using a standard protocol. Briefly, 1 mL of BAL sample was centrifuged in microcentrifuge at 12,000 revolutions per minute for 10 min, and the pellet was dissolved in 600 µL of lysing buffer (sodium acetate with sodium dodecyl sulfate [SDS]) and vortexed briefly. The mixture was digested using 5 mL of proteinase kinase and incubated for 1 h at 55°C. After digestion, the mixture was phenol/chloroform extracted and DNA was precipitated in alcohol and resuspended in Tris/ethylenediamine tetra-acetic acid buffer.

**Southern Blot Analysis**

To ensure specificity, the PCR products were transferred onto nylon membranes and hybridized with an oligonucleotide labeled at the 5' end with 32P and complementary to the amplified mitochondrial DNA (5'-GGTTGATGTAATAGT-3'). Membranes were prehybridized for 2 h with Hybridiz (Oncor; Gaithersburg, MD) [50% formamide, 60 mM sodium chloride/sodium citrate, 10% dextran sulfate, 1% SDS, sheared DNA, and modified Denhardt’s solution], and then hybridized overnight with the probe. Washing was first carried out at 4°C with 6 x sodium chloride/sodium citrate for 20 min, followed by two 20-min washes at 54°C in 3 mol/L tetramethylammonium chloride, 50 mM Tris pH 8.0, 2 mM ethylenediamine tetra-acetic acid pH 8.0, and 0.1% SDS. The filters were exposed to x-ray film for overnight and 3 days at 70°C.

**Figure 1.** Representative example of the detection of the Aspergillus alkaline protease gene amplification in samples in lanes 3, 7, 8 and 10. Lane C, positive control; N, negative control.

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Definitions

All patients included in this study were classified according to the European Organization for Research and Treatment of Cancer and Mycoses Study Group criteria\textsuperscript{14} and categorized into one of four groups: (1) possible IPA, (2) probable IPA, (3) proven IPA, (4) no IPA, or control group (Table 1).

Possible IPA was defined clinical and radiologic findings highly suggestive of IPA in the absence of histopathologic and microbiologic evidence of this infection. The clinical and radiologic findings that were considered to be highly suggestive of IPA were derived from a study\textsuperscript{15} performed at our institution, whereby patients with definite IPA were matched with control patients with pneumonia from other causes. By logistic multivariate analysis, independent predictive factors of IPA were underlying leukemia, prolonged neutropenia (> 7 days), pleuritic chest pain or friction rub, nodular lesions, and cavitations on chest radiograph or CT scan suggestive of IPA.\textsuperscript{15} The presence of four of the criteria listed above in patients with pneumonia was considered to be consistent with possible IPA. Probable IPA was defined as positive culture for Aspergillus species from a respiratory specimen (endotracheal aspirate sputum or BAL) with clinical and radiologic findings suggestive of IPA as defined above. Proven IPA was defined as histopathologic evidence of tissue invasion and damage by Aspergillus species with clinical and radiologic findings suggestive of IPA as defined above.

Statistical Analysis

The significance of the differences between the study groups was determined with the use of the Fisher’s Exact Test or \( \chi^2 \) test for categorical variables, and Student’s \( t \) test or Mann Whitney test for continuous variables. All \( p \) values were based on two-tailed test of significance with a level of significance at \( p < 0.05 \). Sensitivity, specificity, negative predictive value, and positive predictive value of PCR on BAL were determined with 95% confidence interval (CI) for proven, probable, or possible IPA, respectively compared with control cohort cancer patients included in this study. Statistical analyses were performed using software (SPSS version 8.0 for Windows; SPSS; Chicago, IL).

Results

Characteristics of Patients

Most (165 patients, 66\%) of the 249 cancer patients with pneumonia who were included in this study had hematologic malignancy (leukemia, lymphoma or myeloma). The mean age of the cohort of patients included in this study was 50 years (range, 15 to 86 years). Most (174 patients, 70\%) received chemotherapy within 6 months prior to the onset of the pneumonia. Only 36\% received high-dose steroids within 6 months of onset of the pneumonitis; 39\% were neutropenic (< 500 neutrophils per microliter) at the onset of the evaluation.

There were 50 patients with IPA (22 probable, 18 possible, and 10 proven) with a mean age of 52 years (range, 15 to 82 years). Most had hematologic malignancies (40 patients, 80\%), cough (35 patients, 70\%) and received chemotherapy during the 6 months prior to the onset of pulmonary problems (37 patients, 74\%). Twenty-three patients (46\%) had received high-dose steroids during the prior 6 months, and 24 patients (48\%) were neutropenic.

None of the control patients had radiologic manifestations suggestive of IPA, such as halo or crescent sign or nodular and cavitary lesions consistent with IPA. Table 2 compares between all cases of aspergillosis (50 cases) and control (199 cases). Patients with or suspected of having aspergillosis had leukemia (\( p < 0.001 \)), pleuritic chest pain (\( p < 0.001 \)), and friction rub (\( p = 0.03 \)). They also tended to have cough (\( p = 0.08 \)) and to have neutropenia for longer duration (\( p = 0.07 \)). Two factors that remained significant by multiple logistic regression analysis in describing patients with aspergillosis were leukemia (\( p < 0.001 \)) and pleuritic chest pain (\( p = 0.001 \)). Patients with possible IPA were more likely to have an underlying leukemia and neutropenia (\( p < 0.05 \)).

Table 1—Definition of IPA Subgroups and the Frequency of PCR Positivity

<table>
<thead>
<tr>
<th>IPA Subgroups</th>
<th>Definition</th>
<th>Patients With Positive PCR Finding, No. (%)</th>
<th>Cases, No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Possible IPA</td>
<td>Clinical and radiologic findings suggestive of IPA</td>
<td>18, 6 (33)</td>
<td></td>
</tr>
<tr>
<td>Probable IPA</td>
<td>Clinical and radiologic findings suggestive of IPA with positive culture finding for Aspergillus from respiratory specimen</td>
<td>22, 14 (64)</td>
<td></td>
</tr>
<tr>
<td>Proven IPA</td>
<td>Clinical and radiologic findings suggestive of IPA with histopathologic evidence of lung tissue invasion</td>
<td>10, 8 (80)</td>
<td></td>
</tr>
</tbody>
</table>

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**Table 2—Characteristics of Patients***

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Aspergillosis</th>
<th>Control†</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (range), yr</td>
<td>52 (15–82)</td>
<td>50 (18–86)</td>
<td>0.40</td>
</tr>
<tr>
<td>Male/female sex, No.</td>
<td>26/24</td>
<td>131/168</td>
<td>0.07</td>
</tr>
<tr>
<td>Underlying disease</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Leukemia</td>
<td>36 (72)</td>
<td>64 (32)</td>
<td></td>
</tr>
<tr>
<td>Other cancers†</td>
<td>14 (28)</td>
<td>135 (68)</td>
<td></td>
</tr>
<tr>
<td>Friction rub</td>
<td>3 (6)</td>
<td>1 (1)</td>
<td>0.03</td>
</tr>
<tr>
<td>Sinus symptoms present</td>
<td>6 (12)</td>
<td>16 (8)</td>
<td>0.40</td>
</tr>
<tr>
<td>Cough</td>
<td>35 (70)</td>
<td>112 (56)</td>
<td>0.08</td>
</tr>
<tr>
<td>Hemoptysis</td>
<td>9 (18)</td>
<td>33 (17)</td>
<td>0.81</td>
</tr>
<tr>
<td>Pleuritic chest pain</td>
<td>15 (30)</td>
<td>20 (10)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Chemotherapy‡</td>
<td>37 (74)</td>
<td>137 (69)</td>
<td>0.48</td>
</tr>
<tr>
<td>Neutropenia‡</td>
<td>24 (48)</td>
<td>72 (36)</td>
<td>0.13</td>
</tr>
<tr>
<td>Duration of neutropenia, mean (range)</td>
<td>22 (1–90)</td>
<td>14 (1–91)</td>
<td>0.07</td>
</tr>
<tr>
<td>Steroids‡</td>
<td>23 (46)</td>
<td>66 (33)</td>
<td>0.09</td>
</tr>
<tr>
<td>Duration of steroids, mean (range)</td>
<td>28 (5–60)</td>
<td>24 (1–99)</td>
<td>0.48</td>
</tr>
</tbody>
</table>

*Data are presented as No. (%) unless otherwise indicated.†Includes lymphoma, myeloma, and solid tumors.‡Neutropenia is < 500 neutrophils/µL.¶Six were confirmed by autopsy.¶¶Fifteen were confirmed by autopsy and 1 by transbronchial biopsy.

Patients with probable or proven IPA did not differ from control patients in their clinical characteristics.

**PCR Test on BAL**

There is a strong association between positive PCR test on BAL and all forms of IPA (p < 0.002). The association was strongest with proven IPA. Most of the patients with proven IPA (80%) had positive BAL PCR test results (Table 1), while only 7% of the control patients had a positive test result (p < 0.001). Similarly, 64% of patients with probable IPA had a positive PCR test, compared with 7% in the control group (p < 0.001). The sensitivity of the BAL PCR test for all forms of IPA was 56%; specificity was 93%, positive predictive value was 68%, and negative predictive value was 89%. The sensitivity of the test was correlated with the certainty of the diagnosis based on the histopathologic demonstration of tissue invasion, as demonstrated in the proven cases. The sensitivity for possible IPA was only 33% (95% CI, 13 to 59%). It increased to 64% (95% CI, 41 to 83%) for probable IPA, and was as high as 80% (95% CI, 44 to 97%) for proven IPA (Table 3). The specificity was 93% for all subgroups of IPA. This test was also associated with a high negative predictive value, ranging from 94% (95% CI, 90 to 97%) to 99% (95% CI, 96 to 100%) for possible IPA and proven IPA, respectively. However, the BAL PCR test was associated with a low positive predictive value, ranging from 32% (95% CI, 13 to 57%) to 52% (95% CI, 32 to 71%) for various forms of IPA.

After visualizing the PCR directly, Southern blot transfer, and probing with specific probes, the two sets of primers were performed on 68 specimens; the two sets of primers had negative PCR test results and negative Southern blot test results; 13 specimens had positive PCR and Southern blot test results. Hence, the Southern blotting analysis did not improve the diagnostic yield of the PCR test in the 68 specimens examined.

**Discussion**

This prospective clinical trial shows that the PCR test using two sets of primers (alkaline protease and mitochondrial DNA) performed on BAL is highly useful in ruling out IPA in cancer patients presenting with pulmonary infiltrates and, therefore, is associated with a high negative predictive value. This test is also sensitive and highly specific for proven IPA in cancer patients.

Several studies4–9 have suggested that PCR (using different genes) performed on BAL could be useful in the diagnosis of IPA. However, these studies included a very small number of patients with IPA (Table 4), ranging from 3 to 21 patients for all cases (including those with possible IPA) and 3 to 7 patients with proven or probable IPA. In addition, most of these studies were retrospective in nature,4–8 with the exception of one prospective study that included only three cases of probable IPA.9 This current study represents the largest prospective trial, including a large cohort of 249 cancer patients with pulmonary infiltrates that included 50 patients with IPA, of whom 32 patients had proven or probable IPA.

The fact that patients with possible IPA were more likely to have an underlying leukemia and neutropenia is related to the definition used of possible IPA, which included leukemia and neutropenia. Also, the need for a diagnostic test is demonstrated in our
study by the lack of differences of clinical characteristics between patients with probable or proven IPA and the control patients.

Our data are consistent with previous studies in suggesting that PCR performed on BAL would have a high negative predictive value. The negative predictive value in five of the six previous studies was 100% (Table 4).4–7,9 The negative predictive value in the sixth study by Verweij et al8 was 73%. This high negative predictive value is consistent with the fact that PCR is such a sensitive marker for any colonizing or infecting presence of aspergilli in the respiratory tract. Therefore, a negative PCR test result in a patient with a suspected infection highly suggests that the patient does not have IPA and most likely does not have organism colonization.

The PCR using the two genes is also specific for IPA in cancer patients with pulmonary infiltrates. Therefore, cancer patients with a clinical picture compatible with invasive aspergillosis, particularly those with prolonged neutropenia, hematologic malignancy, and prior use of steroids who have pneumonia and a positive PCR test result on BAL, most likely have IPA. The specificity of the test in previous studies4–9 has ranged from 75 to 94.4%. Hence, the test done on BAL is useful in confirming the diagnosis in situations where IPA is considered in the differential diagnosis of cancer patients with pneumonia.

The sensitivity of the test varied from 33% (95% CI, 13 to 59%) for possible cases to 80% (95% CI, 44 to 97%) for proven cases. Hence, the sensitivity correlated with the certainty of the diagnosis based on the histopathologic demonstration of tissue invasion. Recently, Reichenberger et al16 studied the diagnostic yield of bronchoscopy, over a 10-year period, using conventional and culture methods in diagnosing invasive pulmonary aspergillosis. Of the 23 patients with proven aspergillosis demonstrated by histopathologic evidence of tissue invasion, only 7 patients (30%) had positive evidence of aspergillosis based on BAL cultures or cytology examination. Therefore, the PCR test using the two primer genes described in this study increased the sensitivity of BAL in proven IPA from 30% (based on conventional fungal cultures and microscopy) to 80%.

The low positive predictive value of the PCR on BAL (ranging from 32% for possible IPA to 52% for probable IPA) is a function of the fact that the Aspergillus hyphae are often present in the air and could transiently colonize the respiratory tract of noninfected individuals. The PCR performed on unprotected BAL could not, therefore, differentiate between the infecting vs the colonizing hyphae in the oropharyngeal and bronchoalveolar space of various cancer patients. Bart-Delabesse et al17 demonstrated that 25% of BAL samples from healthy individuals are positive for the PCR test used to detect aspergilli. The positive predictive value of the PCR test is improved if it is done on serum or whole blood. Several studies7,18–23 have reported a positive predictive value of 95 to 100% if the test is done on serum or whole blood.

More recently, sandwich enzyme-linked immunosorbent assay for the detection of galactomannan was performed on the blood of patients with suspected IPA, showing that this test is associated with high sensitivity, specificity, and predictive value, even higher than PCR test itself.20,24,25 The use of Southern blot analysis did not improve the diagnostic yield of the PCR test in our patient population. However, testing the blood for either PCR or enzyme-linked immunosorbent assay galactomannan may help confirm the diagnosis and differentiate between colonization and infection in patients with positive PCR findings on BAL.20,24 In addition, one of the limitations of our study is that only a small number of the control patients had biopsies or autopsies were done.

**CONCLUSION**

In conclusion, Aspergillus PCR test using alkaline protease gene and mitochondrial primers done on
BAL is associated with high negative predictive value for IPA in cancer patients with pneumonia. Hence, a negative test result on BAL is useful in ruling out IPA. In addition, the test is sensitive and highly specific. However, the test is associated with a low positive predictive value. If IPA is considered in the differential diagnosis along with other infectious etiologies and bronchoscopy is undertaken, performing Aspergillus PCR on the BAL is useful in ruling out IPA. A positive test result on BAL needs to be further verified by a positive PCR or galactomannan enzyme-linked immunosorbent assay done on the blood.

References