Polymerase Chain Reaction Amplification of Asp f1 and Alkaline Protease Genes from Fungus Balls: Clinical Application in Pulmonary Aspergillosis

Tomoyuki Urata, Makoto Kobayashi, Jun Imamura, Yuji Tanaka, Hidenori Muneishi, Yoshihito Iwahara, Yoshiki Uemura, Hirokuni Taguchi and Isao Miyoshi

Asp f1 (18 kDa) and alkaline protease (33 kDa) are the 2 major antigens which are derived from Aspergillus (A.) fumigatus and have been implicated as possible virulence factors in the pathogenesis of Aspergillus-induced diseases. We attempted to detect fragments of genes encoding both proteins from fungus balls obtained at surgery or autopsy by polymerase chain reaction (PCR) amplification and then used PCR to test clinical samples. Frozen-stored fungus ball samples from a patient with acute myeloid leukemia complicated by Aspergillus pneumonia and from a patient with pulmonary aspergilloma were studied. We successfully amplified a 315 bp PCR product, the target sequence for Asp f1, and a 747 bp PCR product as a target sequence for alkaline protease (ALP) in both cases. In addition, 13 clinical samples including sputum specimens from patients with pulmonary aspergillosis were also examined. PCR analysis for the Asp f1 (ALP) gene in clinical samples showed positive results in 5/10 (6/10) patients with pulmonary aspergilloma and in 3/3 (1/3) patients with invasive pulmonary aspergillosis. Culture data on A. fumigatus revealed positive results in 3/9 patients with pulmonary aspergilloma and in 2/3 patients with invasive pulmonary aspergillosis. This method can be used to recognize the involvement of A. fumigatus in various clinical settings where conventional culture results are not readily available.

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Key words: Aspergillus fumigatus, polymerase chain reaction (PCR) diagnosis, virulence factor, respiratory secretion

Introduction

Aspergillus (A.) fumigatus is thought to be the most pathogenic Aspergillus species for humans. This fungus is found ubiquitously and is capable of causing a wide spectrum of human diseases including allergic and invasive aspergillosis (1).

Asp f1 and alkaline protease (ALP) are the 2 major allergens or antigens derived from A. fumigatus and they have been implicated as possible virulence factors in the pathogenesis of Aspergillus-induced diseases (2-4). Asp f1 has been reported to be an 18 kDa IgE binding protein and a member of the mitogillin family of cytotoxins, which promotes colonization and infection by A. fumigatus over other Aspergillus species (2, 4).

In contrast, ALP, which is a serine protease of the subtilisin family, has a molecular weight of 32-33 kDa (5, 6). This enzyme is responsible for most, if not all, detectable extracellular elastinolytic activity, and is also capable of destructing lung tissue and it facilitates tissue invasion by the fungus (5-7). To date, DNA sequences of both genes have already been elucidated by gene cloning (2, 3, 8). In this study, we focused on these 2 molecules and attempted to detect fragments of genes encoding both proteins from fungus balls obtained at surgery or autopsy by polymerase chain reaction (PCR) amplification. Then, we also examined the clinical samples from patients with pulmonary aspergillosis by PCR in order to develop more sensitive methods compared to the conventional culture or immunodiagnosis.

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Materials and Methods

Fungus balls

Two frozen-stored fungus ball samples derived from a patient with acute myeloid leukemia complicated by Aspergillus pneumonia and from a patient with pulmonary aspergilloma were studied (Table 1). The diagnosis of A. fumigatus infection was made based on the histopathological studies at autopsy or on surgically removed specimens reviewed by the pathologists. Chest radiographs of these patients are shown in Fig. 1. Chest radiograph of patient 1 showed multiple nodular shadows in the bilateral lung fields which progressively worsened over a 2-week period until he died. Chest radiograph of patient 2 shows a small nodule located in his left lower lobe. A chest computed tomographic scan taken before left lower lobectomy revealed a thin layer of air space surrounding the nodule.

Fungus balls were removed at autopsy or surgery and stored at -70°C until used. These samples were vortexed with glass beads and treated with extraction buffer containing 1% SDS and 100 µg/ml proteinase K (Sigma, St. Louis, MO, USA) according to the method described by Tang et al (7). Then, DNA was

<table>
<thead>
<tr>
<th>Samples</th>
<th>Patients</th>
<th>Age (yrs)/ Sex</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>P*</td>
<td>Sputum isolate</td>
<td>MC 62/M</td>
<td>Aspergillus pneumonia</td>
</tr>
<tr>
<td>1</td>
<td>Frozen stored fungus ball obtained at autopsy</td>
<td>KT 50/M</td>
<td>Aspergillus pneumonia acute myeloid leukemia</td>
</tr>
<tr>
<td>2</td>
<td>Frozen stored fungus ball obtained at surgery</td>
<td>CT 55/M</td>
<td>Pulmonary aspergilloma</td>
</tr>
</tbody>
</table>

P*: positive control.

Figure 1. (A) Chest radiograph of patient 1 showing multiple nodular shadows in the bilateral lung fields which progressively evolved over a 2-week period until he died. (B) Chest radiograph of patient 2 showing a small nodule in the left lower lobe (arrow).
extracted with phenol/chloroform. PCR amplification was carried out with each set of corresponding primer pairs. PCR products were analyzed on 2% agarose gels, stained with ethidium bromide, and visualized by UV transillumination.

**Fungal strains**

Clinical sputum isolate from a patient with invasive pulmonary aspergillosis was used as the source of positive control DNA. *A. flavus* (IFM 46870), *A. terreus* (IFM 46871), *A. nidulans* (IFM 41395), and *A. niger* (IFO 4414) were kindly supplied by Prof. Kazuko Nishimura (Research Center for Pathogenic Fungi and Microbial Toxices, Chiba University). DNA was extracted from these strains as described above. DNA extracted from a clinically isolated strain of *Candida albicans* and human leucocytes were used as the negative controls.

**PCR primers**

For the detection of the *Asp f1* gene, we used a set of primer pairs which is predicted to generate a 315 bp amplified product. The PCR primers for *Asp f1* were as follows: The upper primer was 5' TGG-ACA-TGC-ATC-AAC-CAA 3' (ASP1) which is a modification of that used by Reddy et al (9), and the lower primer was 5' GTC-AAA-CTT-ATA-GTC-GTG 3' (ASP2) for the amplification of the 315 bp gene fragment.

For the detection of the *ALP* gene, we used a single primer pair which is predicted to generate a 747 bp amplified product (10). The upper primer was 5' AGC-ACC-GAC-TAC-ATC-TAC 3' (ALP1) and the lower primer was 5' GAG-ATG-GTG-TTG-GTG-GC 3' (ALP2).

**Figure 2.** PCR analysis of amplified products from fungus balls. Lanes M, DNA size marker; P, positive control; 1, Case 1; 2, Case 2; N, no template. Upper panel indicates ethidium bromide-stained 2% agarose gels, and the lower panel indicates corresponding Southern blot hybridization by the chemiluminescence method. (A) PCR amplification of the *Asp f1* gene with a primer pair, ASP1 and ASP2. The digoxigenin-labeled *Asp f1*-specific internal oligonucleotide was used as the probe. (B) PCR amplification of the *ALP* gene with the ALP1 and ALP2 primer pair. Digoxigenin-labeled PCR probe (747 bp) was used for hybridization.
PCR conditions

For amplification of the Asp f I gene, PCR reactions were performed in a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT, USA) for 45 cycles in 100 μl volume containing 10 mM TRIS-Cl at pH 8.3, 50 mM KCl, and 1.5 mM MgCl₂ with 100 pmol of both primers, and 2.5 U TaKaRa Taq DNA polymerase (Takara Shuzo, Tokyo) with denaturation at 94°C for 1 minute, annealing at 45°C for 1 minute, and extension at 72°C for 3 minutes.

For amplification of the ALP gene, the thermal cycling conditions used were 42 cycles at 94°C for 30 seconds, 63°C for 45 seconds, and 72°C for 2 minutes. As a precaution to prevent template contamination, preparation of DNA samples, preparation of reaction solutions, and PCR amplification were performed in different rooms using a safety cabinet or clean bench and aerosol resistant pipette tips (Molecular Bio-products, Inc, San Diego, CA, USA).

Gel electrophoresis and Southern blot hybridization

PCR products (10 μl) were electrophoresed in 2% agarose, stained with ethidium bromide (0.5 μg/ml), and visualized with UV light. The DNA was transferred from the gels by Southern transfer onto nylon membranes (Hybond N, Amersham International, Buckinghamshire, UK). The membranes were hybridized with 5' end-digoxigenin-labeled internal oligonucleotide in the earlier part of this study. However, digoxigenin-labeled PCR probes were alternatively used in the later study, because the probe synthesis is more simple. Briefly, a digoxigenin labeling mixture (Boehringer-Mannheim, GmBH, Germany) was used in place of dNTP in the PCR reaction amplifying the respective target gene from *A. fumigatus* DNA under the same thermal cycling conditions, as for the synthesis of digoxigenin incorporated PCR probe (11).

Subsequently, non-radioisotopic chemiluminescence detection was performed using CSPD™ (Tropix, Bedford, MA).
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USA) as a substrate according to the manufacturer’s instructions (12).

**Clinical samples**

Further, 12 sputum samples from patients with pulmonary aspergilloma (n=10), a patient with invasive pulmonary aspergillosis (n=1), and a patient with eosinophilic pneumonia (n=1) were tested. In addition, a bronchial aspirate from a patient with culture proven invasive pulmonary aspergillosis (n=1), bronchoalveolar lavage fluid (BALF) from a patient with surgically confirmed invasive pulmonary aspergillosis (n=1), and pleural effusion from a patient with probable invasive aspergillosis (n=1) were also examined.

Pulmonary aspergilloma was diagnosed from the characteristic radiological appearance of the presence of a radio-opaque mass in the lung cavity with an air-crescent sign (13, 14). Two patients with invasive pulmonary aspergillosis were pathologically confirmed by autopsy (15, 16), as was another case with invasive pulmonary aspergillosis, who underwent left upper surgical lobectomy for life-threatening hemoptysis (16). A patient with probable invasive pulmonary aspergillosis was assumed based on the co-existence of invasion to the pleural space and the parietal pericardium, which had never been implicated by the involvement of the underlying disease (16). A patient with eosinophilic pneumonia was initially thought to have allergic bronchopulmonary aspergillosis, but was found to have normal levels of serum IgE and negative specific IgE values for *A. fumigatus* afterwards (17).

All of the clinical samples were added to an equal volume of extraction buffer. After incubation at 65°C for 3 hours, the mixture was extracted according to a modification of the method described by Wakefield et al (18).

**Results**

**PCR detection of Asp fI and ALP genes in fungus balls**

Agarose gel electrophoresis and Southern blot hybridizations are shown in parallel (Fig. 2). The 315 bp amplified gene fragment of *Asp fI* (Fig. 2A) and the 747 bp amplified gene fragment of *ALP* were both generated by PCR (Fig. 2B). These results demonstrated that both fungus balls resulted from an *A. fumigatus* infection as shown below.

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**Figure 4. Sensitivity of the PCR.** (A) PCR amplification of the *Asp fI* gene with the ASP1 and ASP2 primer pair. (B) PCR amplification of the *ALP* gene with the ALP1 and ALP2 primer pair. Upper panel shows the ethidium-bromide stained gel, and lower panel represents the corresponding Southern blot hybridization.
Specificity of the PCR detection of A. fumigatus DNA

Specificity studies were conducted as follows. DNA (100 ng) from other members of the Aspergillus species, Candida albicans, and human leukocytes were used as targets. Ethidium bromide-stained gels and Southern blot hybridizations are shown in Fig. 3.

Noted that a 315 bp gene fragment was amplified from A. fumigatus DNA while the 315 bp product was amplified from neither fungal strain sample nor human DNA (Fig. 3A). After Southern blot hybridization, a 315 bp amplified product could be detected solely from A. fumigatus DNA, representing the detection of the Aspfl gene as shown in Fig. 3A.

In contrast, the use of PCR for the detection of the ALP gene, amplification products could be detected both from A. fumigatus and A. flavus DNAs after ethidium bromide and Southern blot analysis. In the ethidium bromide-stained gel, the size of the predicted amplification product from A. fumigatus was 747 bp, and that from A. flavus was 690 bp. In Southern blot analysis, the hybridization signal was intense for A. fumigatus, but it was faint for A. flavus, probably because the digoxigenin-incorporated PCR amplified fragments of A. fumigatus (747 bp) were used to probe the Southern blots (Fig. 3B).

Sensitivity of PCR detection of A. fumigatus DNA

Sensitivity studies were carried out by using serially diluted DNA extracted from A. fumigatus as the target. As the result, 10 pg of A. fumigatus DNA could be detected by PCR for the Asp fl gene after Southern blot analysis (Fig. 4A). In contrast, 100 pg of A. fumigatus DNA could be detected by PCR for the ALP gene after Southern blot analysis (Fig. 4B). Based on this study, amplification of the Asp fl gene has an advantage over that of the ALP gene in terms of sensitivity.

PCR detection of the Asp fl gene in clinical samples

A set of PCR primer pairs which is predicted to generate a 315 bp product was chosen for the PCR detection of the Asp fl gene from clinical samples including sputum. An amplification product corresponding to the size of the fragment (315 bp) from A. fumigatus could be detected in 5 out of the first 12 samples (Fig. 5).
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**PCR detection of the ALP gene in the clinical samples**

PCR was carried out using the same DNA samples as used for the PCR of the *Asp f I* gene. An amplification product corresponding to the size of a fragment (747 bp) from *A. fumigatus* could be detected in 6 out of the first 12 consecutive samples (Fig. 6).

**Summary of patient characteristics and PCR results**

The PCR results, culture data on *A. fumigatus*, and patient profiles are summarized in Table 2. The first consecutive sample (patient) number, from 1 to 12, coincides with the lane number as shown in Figs. 5 and 6. Three additional samples whose patient numbers were from 13 to 16 were also examined and are included in the list.

PCR for the *Asp f I* gene revealed that 5 of 10 patients with pulmonary aspergilloma and 3 of 3 patients with invasive pulmonary aspergillosis gave positive results. In contrast, PCR for the *ALP* gene showed that 6 of 10 patients with pulmonary aspergilloma, and 1 of 3 patients with invasive pulmonary aspergillosis gave positive results. Another sample of pleural effusion from a patient with probable invasive aspergillosis was only PCR positive for the *ALP* gene. The culture data on *A. fumigatus* showed that 3 of 9 patients with pulmonary aspergilloma, and 2 of 3 patients with invasive pulmonary aspergillosis gave positive results.

Consequently, the overall results indicated that the positive rate for PCR analysis of the *Asp f I* gene was 8/13, for the *ALP* gene was 7/13, and that for the culture data was 5/12, in pulmonary aspergillosis.

**Discussion**

To date, several PCR-based amplification methods for the detection of *A. fumigatus* from clinical samples have been reported (19–21). Most methods utilize 18S ribosomal RNA as the target sequence. Therefore, some of these methods allow detection of *Penicillium* species together with *Aspergillus* species. In contrast, 2 studies chose other molecules as target sequences. One selected *Asp f I* (9), and the other *ALP* (10), both of which are thought to be possible virulence factors in the pathogenesis of pulmonary aspergillosis.

*Asp f I* is exclusively produced in association with germination and the fungal growth of *A. fumigatus* and *A. restrictus*, and causes human antibody responses in patients with various forms of pulmonary aspergillosis. In fact, the highest levels of IgG and IgE antibodies against *Asp f I* are found in sera from...
Table 2. Clinical Characteristics of the Patients and the PCR Results

<table>
<thead>
<tr>
<th>Patients No.</th>
<th>Age/Sex (yrs)</th>
<th>Diagnosis</th>
<th>Clinical samples</th>
<th>Culture</th>
<th>PCR Asp f I</th>
<th>PCR ALP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>KM</td>
<td>71/F Pulmonary aspergilloma</td>
<td>Sputum</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>MI</td>
<td>61/F Pulmonary aspergilloma</td>
<td>Sputum</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>HH</td>
<td>73/F Pulmonary aspergilloma</td>
<td>Sputum</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>MM</td>
<td>58/M Pulmonary aspergilloma</td>
<td>Sputum</td>
<td>–</td>
<td>ND*</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>KM</td>
<td>65/M Invasive pulmonary aspergilosis</td>
<td>Bronchial aspirates</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>ST</td>
<td>49/M Pulmonary aspergilloma</td>
<td>Sputum</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>MM</td>
<td>69/M Pulmonary aspergilloma</td>
<td>Sputum</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>MM</td>
<td>75/M Pulmonary aspergilloma</td>
<td>Sputum</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>HK</td>
<td>43/M Pulmonary aspergilloma</td>
<td>Sputum</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
<td>MU</td>
<td>64/M Invasive pulmonary aspergilosis</td>
<td>Sputum</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>11</td>
<td>SM</td>
<td>59/F Eosinophilic pneumonia</td>
<td>Sputum</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>12</td>
<td>IO</td>
<td>69/M Probable invasive pulmonary aspergilosis</td>
<td>Pleural effusion</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>TK</td>
<td>66/M Pulmonary aspergilloma</td>
<td>Sputum</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>YK</td>
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<td>Sputum</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>KS</td>
<td>46/M Invasive pulmonary aspergilosis</td>
<td>BALF**</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

ND*: not done, BALF**: bronchoalveolar lavage fluid.

patients with allergic bronchopulmonary aspergillosis and pulmonary aspergilloma, while *A. restrictus* is unable to cause human diseases because of its inability to grow at 37°C (2, 4). Reddy et al amplified the *Asp f I* gene by PCR and successfully detected *Asp f I* DNA from urine samples from patients with invasive aspergillosis (9). In our study, we chose the 315 bp nucleotide sequence of the *Asp f I* gene as the target, based on the results of gene cloning (3, 4).

In contrast, *ALP* is an elastinolytic protease specifically derived from *A. fumigatus* and *A. flavus*, both of which can cause human infection. Tang et al reported the successful PCR amplification of a 747 bp sequence of the *ALP* gene from BALF in immunosuppressed and non-immunosuppressed patients (10).

Amplified *Asp f I* and *ALP* gene fragments were detected in frozen stored fungus balls from patients with pulmonary aspergillosis by PCR. Thus, this combined PCR approach allows us to discriminate *A. fumigatus* from other members of the *Aspergillus* species.

We used PCR to examine clinical samples including sputum specimens. We analyzed respiratory secretions (sputum, bronchial aspirate, and BALF), by PCR for the *Asp f I* gene, and found that 5 of 10 patients with pulmonary aspergilloma and 3 of 3 patients with invasive pulmonary aspergillosis gave positive results. Conversely, PCR for the *ALP* gene showed 6 of 10 patients with pulmonary aspergilloma, and 1 of 3 patients with invasive pulmonary aspergillosis gave positive results. The overall results indicated the positive rate for PCR for the *Asp f I* gene was 8/13, for the *ALP* gene was 7/13, and for the culture data was 5/12, in pulmonary aspergillosis. In this sense, this study does not conclusively demonstrate the superiority of our PCR method.
In order to validate whether our approach is more sensitive than conventional culture, a prospective study including larger numbers of samples is clearly required.

In our series, there appears to be a lower incidence of PCR positivity in patients with pulmonary aspergillosis. This may be due to the fact that aspergilloma may be caused by other members of Aspergillus species and that there is a lower shedding rate of fungal DNA into the sputum without the invasive process. Because the germination of spores and fungal growth is closely associated with the active expression of the Asp fI gene, it is less likely to occur in this situation (4). We presume the respiratory secretions from invasive pulmonary aspergillosis, if obtainable, should be good materials for PCR diagnosis.

The discrepancy of the PCR results between the Asp fI and ALP genes in some of the samples seems to be explained by the time lag of the secretion of these proteins by A. fumigatus, but this point needs to be confirmed with a larger number of samples (22). Additionally, there is a concern that the size of the PCR product from the ALP gene is rather longer to be amplified easily from materials than that of the Asp fI gene, depending on the quality of the samples. In fact, PCR analysis of the ALP gene showed a lower positive rate (1/3) in samples from confirmed invasive pulmonary aspergillosis in comparison with PCR analysis of the Asp fI gene (3/3). The present view is that PCR for the Asp fI gene is better than PCR for the ALP gene. However, further investigations including the selection of the more optimal target sequences for the ALP gene are required.

In conclusion, because more than 95% of the invasive pulmonary aspergillosis in humans are reported to be caused by A. fumigatus (1), the Asp fI gene in conjunction with ALP gene appears to be a promising candidate for the target of PCR diagnosis with regards to respiratory secretions, particularly in clinical settings where conventional culture results are not readily available.

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References


