Identification of fungal DNA in BALF from patients with home-related hypersensitivity pneumonitis

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KEYWORDS
Hypersensitivity pneumonitis; Trichosporon; Fusarium; Fungal DNA; Bronchoalveolar lavage fluid

Summary
Background: In Japan, a major type of home-related hypersensitivity pneumonitis (HP) is summer-type HP, which is caused by Trichosporon asahii (T. asahii) or Trichosporon mucoides. Some patients with home-related HP test negative for antibodies against Trichosporon; yet, a causative mold antigen cannot be identified.
Methods: We analyzed 19 patients with home-related HP, 8 healthy volunteers, and 35 patients with other diseases. We extracted DNA from cell pellets of bronchoalveolar lavage fluid (BALF), amplified the DNA by PCR using Trichosporon-specific primers or other fungus-specific primers, and cloned as well as sequenced the PCR amplicon. Other primers used were specific for Acremonium chrysogenum, Aspergillus fumigatus, Aspergillus niger, Fusarium napiforme, Humicola fuscoatra, Penicillium corylophilum, and Pezizula domiciliana.
Results: We detected Trichosporon DNA (n = 17) and F. napiforme DNA (n = 2) by PCR in 19 patients with home-related HP; however, these species were not identified in healthy volunteers. After sequencing of the PCR amplicon for Trichosporon species, we identified T. asahii (n = 11), Trichosporon japonicum (n = 1), and Cryptococcus uzbekistanensis (n = 4).
Conclusion: We could detect fungal DNA in BALF cell pellets from patients with home-related HP. These data suggest that this method might be useful to detect antigens responsible for home-related HP.
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Introduction

Hypersensitivity pneumonitis (HP) is an immunological lung disease induced by repeated inhalation of a variety of causative antigens. Thus, many individuals may be at risk for exposure in their occupational, domestic, and recreational environments. Jacobs and colleagues reported that interstitial lung disease is associated with the presence of fungi in individual homes, which is the cause of home-related HP. Selman and colleagues also described clinical features of fungi-related HP, and most of these were occupational and domestic cases. In Japan, a major type of home-related HP is summer-type HP, which is caused by Trichosporon asahii (T. asahii) or Trichosporon mucoides. Because some patients with home-related HP test negative for antibodies against Trichosporon, other fungi might be causative for the disease. However, little is known about home-related HP other than summer-type HP, regarding the etiological and diagnostic aspects.

Identification of the causative antigen of HP is important for a definitive diagnosis and appropriate treatment. First, we used a cultivation test to detect the presumptive microorganisms in samples from the patients’ environment, and then we diagnosed the disease. A causative agent was identified if the immunological examinations are positive as well as the inhalation provocation tests. However, a complete implementation of such procedures is quite challenging. The early growing microorganisms that have no pathogenicity often cover culture medium, which disturbs the growth of causative microorganism in a conventional culture method.

Recently, new molecular biological methods to detect pathogenic microorganisms have been developed. We previously demonstrated the application of the ribosomal DNA intergenic spacer (IGS) region sequence of Trichosporon by using samples obtained from patients with deep-seated trichosporonosis and from the houses of patients with summer-type HP. Although there are few reports about direct detection of fungal DNA in bronchoalveolar lavage fluid (BALF), we examined fungal DNA in BALF cell pellets from the patients with home-related HP to detect the causative antigen to support the diagnosis.

Methods

Subjects

Patients with home-related HP (n = 19), healthy volunteers (n = 8) as normal subjects, and patients with other diseases (n = 35) were included from January 2000 to December 2009. Acute HP and chronic HP were diagnosed according to the criteria of Ando and Yoshizawa, respectively. We defined home-related HP as clinical improvement after withdrawal from the domestic environment fulfilling the above criteria. This study conformed to the Declaration of Helsinki and was approved by the institutional review board in Tokyo Medical and Dental University (approval number: 534). Informed written consent was obtained for each subject.

Immunological examinations

To examine specific antibodies against Trichosporon, an ELISA was performed, as described previously. Serum was diluted at 1:400, and then 100 µl of the diluted samples were tested. The results were expressed as the absorbance values (O.D. at 490 nm and 620 nm). The cut-off value was determined as mean +2 S.D. of normal controls.

DNA extraction from BALF

We performed bronchoalveolar lavage (BAL) as previously described. BAL was performed using three 50-ml aliquots of sterile 0.9% saline. The cellular composition of the BAL was determined by using a cytoplasm smear with Wright stain and counting of 200 cells.

For extraction of genomic DNA, we suspended the cell pellets from 1 ml of BALF in 150 µl of lysis buffer (100 mM Tris—HCl, pH 7.5, 0.5% sodium w/v, 10 mM EDTA) and 350 µl TE buffer (10 mM Tris—HCl, 1 mM EDTA, pH 8.0). After vortex mixing for 5 s, this mixture was subjected to three cycles of freezing in liquid nitrogen and thawing at 100 °C for 15 min for mechanical lysis. Then, 200 µl of phenol—chloroform—isooamyl alcohol (25:24:1, vol/vol/vol) was added and vortexed for 30 s. This mixture was centrifuged at 14,000 rpm for 5 min, and the supernatant was transferred to a new tube. Subsequently, 200 µl of chloroform—isooamyl alcohol (24:1, vol/vol) was added and vortexed for 30 s. This mixture was centrifuged at 14,000 rpm for 5 min, and the supernatant was transferred. To precipitate DNA, 1/30 vol. 3 M sodium acetate, 1/100 vol. Ethachinimine (Nippon Gene, Toyama, Japan), and 2.5 vol. 100% ethanol were added to the supernatant. The sample was centrifuged at 14,000 rpm for 20 min, and the pellet was washed with 70% ethanol, dried, and resuspended in 30 µl TE.

PCR and sequencing

DNA amplification was performed using primers targeting the IGS 1 region of Trichosporon species, which is located between the 26S and 5S ribosomal DNA. PCR was also carried out using various fungus-specific primers. The species of fungus were selected by referring to the previous case reports about home-related HP. The sequence of these primers is listed in Table 1. Extracted DNA (1 µl) from each sample was added to 49 µl of the PCR master mixture, which consisted of 5 µl of 10× PCR buffer (Takara Inc., Shiga, Japan), 4 µl of 200 µM deoxynucleoside triphosphates (an equimolar mixture of dATP, dCTP, dGTP, and dTTP; Takara Inc., Japan), 50 pM of each primer, and 1.25 U of Takara Ex Taq polymerase (Takara Inc., Japan). PCR was performed in a Thermocycler (model 9700; Applied Biosystems, Foster City, USA) with an initial 1-min denaturation at 94 °C followed by 30 cycles that consisted of 30 s at 94 °C, 30 s at 59 °C, 1 min at 72 °C, and a final 10-min extension at 72 °C. The product from the first reaction underwent a second run with the same conditions as the first. Only primers targeting the IGS 1 region of Trichosporon species were used for both the first and the second run.
run. PCR products were electrophoresed in 1.5% agarose gels and visualized by staining with ethidium bromide. Then, positive PCR products by using primers targeting the IGS 1 region of *Trichosporon* species were cloned by using a TA Cloning Kit (Invitrogen Corp., Carlsbad, USA) and sequenced with an ABI 310 DNA-sequencing apparatus with the BigDye Terminator Cycle Sequencing Ready Reaction kit version 3.1 (Perkin-Elmer Applied Biosystems), according to the manufacturer’s instructions.

### Statistical analysis

Data were analyzed using GraphPad Prism version 5.2 (GraphPad Software, Inc., USA). Comparisons between two groups were analyzed using the Mann—Whitney test, and comparisons among more than three groups were analyzed using the Kruskal—Wallis test. When appropriate, a non-parametric post hoc multiple comparisons test, Dunn’s test, was used to evaluate differences between the groups. We used \( \chi^2 \) tests for categorical variables. A \( p \)-value less than 0.05 was considered to be significant.

### Results

#### Characteristics and BALF profiles of patients

We obtained BALF samples from 62 subjects including the following: 19 patients with home-related HP, of whom 10 were acute-type and 9 were chronic-type; 8 normal controls; 17 patients with collagen vascular disease associated interstitial pneumonia (CVD-IP); 10 patients with sarcoidosis; and 8 patients with other lung diseases (Table 2). CVD-IP included rheumatoid arthritis \((n = 7)\), dermatomyositis \((n = 5)\), systemic sclerosis \((n = 3)\), and Sjögren syndrome \((n = 2)\). Other lung diseases included chronic bird-related HP \((n = 2)\), idiopathic pulmonary fibrosis \((n = 1)\), chronic eosinophilic pneumonia \((n = 1)\), lung cancer \((n = 1)\), pulmonary thromboembolism \((n = 1)\), radiation pneumonitis \((n = 1)\), and Alport syndrome \((n = 1)\). Most patients with home-related HP had wooden houses. There was no difference in the age of house among all groups.

In BALF profiles, no difference was detected in the fluid recovery percentage among all groups (Table 3).
number of total cells was significantly increased in patients with acute home-related HP and CVD-IP compared with normal controls (94.4 \times 10^4/ml vs. 15.6 \times 10^4/ml, p < 0.01 and 126.0 \times 10^4/ml vs. 15.6 \times 10^4/ml, p < 0.001). The macrophage percentage was significantly lower in patients with acute home-related HP and CVD-IP than normal controls (15.1% vs. 86.2%, p < 0.05 and 33.5% vs. 86.2%, p < 0.05). The lymphocyte percentage was significantly higher in patients with acute home-related HP than in patients with chronic home-related HP, CVD-IP, other lung diseases, and normal controls (81.1% vs. 14.9%, p < 0.01; 81.1% vs. 9.8%, p < 0.01; 81.1% vs. 6.0%, p < 0.001; and 81.1% vs. 12.7%, p < 0.01).

Table 3  Bronchoalveolar lavage fluid profiles.

<table>
<thead>
<tr>
<th></th>
<th>Acute home-related HP</th>
<th>Chronic home-related HP</th>
<th>Normal</th>
<th>CVD-IP</th>
<th>Sarcoïdosis</th>
<th>Other lung diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery, %</td>
<td>62.3</td>
<td>62.0</td>
<td>71.3</td>
<td>58.0</td>
<td>64.0</td>
<td>56.7</td>
</tr>
<tr>
<td>Median</td>
<td>49.3–70.0</td>
<td>24.7–80.7</td>
<td>56.0–74.7</td>
<td>36.7–71.3</td>
<td>44.0–77.3</td>
<td>48.7–67.3</td>
</tr>
<tr>
<td>Range</td>
<td>94.4</td>
<td>36.5</td>
<td>15.6</td>
<td>126.0</td>
<td>40.0</td>
<td>44.2</td>
</tr>
<tr>
<td>Macrophages, %</td>
<td>7.7–180.0</td>
<td>20.0–94.5</td>
<td>8.8–25.2</td>
<td>21.5–919.0</td>
<td>23.7–80.1</td>
<td>17.0–75.0</td>
</tr>
<tr>
<td>Median</td>
<td>15.1</td>
<td>74.3</td>
<td>86.2</td>
<td>33.5</td>
<td>57.3</td>
<td>71.1</td>
</tr>
<tr>
<td>Range</td>
<td>0.4–49.5</td>
<td>16.9–88.8</td>
<td>62.3–97.6</td>
<td>1.9–89.3</td>
<td>16.0–97.0</td>
<td>34.1–94.2</td>
</tr>
<tr>
<td>Lymphocytes, %</td>
<td>81.1</td>
<td>14.9</td>
<td>12.7</td>
<td>9.8</td>
<td>42.3</td>
<td>6.0</td>
</tr>
<tr>
<td>Median</td>
<td>45.9–99.0</td>
<td>0.0–81.0</td>
<td>2.4–37.1</td>
<td>0.0–63.4</td>
<td>2.4–84.0</td>
<td>2.2–46.0</td>
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<td>Neutrophils, %</td>
<td>0.9</td>
<td>5.2</td>
<td>2.1</td>
<td>10.0</td>
<td>0.0</td>
<td>4.1</td>
</tr>
<tr>
<td>Median</td>
<td>0.0–5.0</td>
<td>0.6–39.0</td>
<td>1–32.1</td>
<td>0.6–88.5</td>
<td>0.0–0.6</td>
<td>1.0–8.8</td>
</tr>
<tr>
<td>Eosinophils, %</td>
<td>1.0</td>
<td>0.9</td>
<td>1.2</td>
<td>5.0</td>
<td>0.0</td>
<td>4.7</td>
</tr>
<tr>
<td>Median</td>
<td>0.0–6.5</td>
<td>0.0–11.2</td>
<td>0.0–61.9</td>
<td>0.0–15.3</td>
<td>0.0–0.9</td>
<td>0.0–61.9</td>
</tr>
<tr>
<td>Range</td>
<td>0.0–61.9</td>
<td>0.0–15.3</td>
<td>0.0–0.9</td>
<td>0.0–61.9</td>
<td>0.0–0.9</td>
<td>0.0–61.9</td>
</tr>
</tbody>
</table>

PCR and sequencing

The results of the PCR analysis revealed that *Trichosporon* species were detected in the BALF from 9 out of 10 patients (90%) with acute home-related HP, from 8 out of 9 (88.9%) patients with chronic home-related HP, and from 3 out of 17 (17.6%) patients with CVD-IP but not in the BALF from patients with other lung diseases and normal subjects (Fig. 1). *Fusarium napiforme* was detected in 2 out of 10 patients with acute home-related HP. None of the other fungal DNA were detected by PCR in any of the study subjects.

The clone sequences of the PCR products amplified with primers targeting the IGS 1 region of *Trichosporon* species were determined. *T. asahii* was detected in 7 patients with acute home-related HP, in 4 patients with chronic home-related HP, and in 3 patients with CVD-IP (Table 4). *Cryptococcus uzbekistanesis* (*C. uzbekistanensis*) was detected in 1 patient with acute home-related HP and in 3 patients with chronic home-related HP. *Trichosporon japonicum* was detected in one patient with chronic home-related HP.

Correlation of *T. asahii* DNA from BALF with specific antibody from sera

The results of the relationship between *T. asahii* DNA from BALF and specific antibodies from sera of patients with HP are summarized in Table 4 and Fig. 2. All patients with acute home-related HP and 5 out of 9 patients with chronic home-related HP were positive for antibodies against *T. asahii*. Among the 4 patients with chronic home-related HP in which antibodies against *T. asahii* were not detected, 2 patients had *T. asahii* DNA in their BALF (cases 11 and 14), and 2 patients had other fungal DNA (*C. uzbekistanensis* and *T. japonicum*) in their BALF (cases 15 and 16).

Discussion

The present study demonstrates that fungal DNA is present in BALF of patients with home-related HP, and this method would benefit in the diagnosis of this disease.

We showed that *T. asahii* was the most frequent species among the detected fungi in the BALF of patients with...
These fungi, as well as *F. napiforme* has been reported to be a causative antigen of *Sporotrichum species* are known to be phylogenetically close to *C. uzbekistanensis* sequenced in several cases. CVD-IP in this study (Fig. 1) even though these patients were negative for antibodies against home-related HP.22 In this report, they analyzed neither the DNA nor any other fungi, and subjects were limited to acute summer-related HP. Therefore, we expanded the subjects to include other fungi, and subjects were limited to acute summer-related HP (Table 4 and Fig. 1). This result is consistent with previous reports that revealed that the major causative antigen of home-related HP was *T. asahii*.19,20 Due to 60% of the houses being wooden21 and a rainy month every summer in Japan, it is easy for *Trichosporon* to grow since it prefers hot and humid conditions.

Only one report has demonstrated that *Trichosporon* DNA was observed in the BALF of patients with summer-type HP.22 In this report, they analyzed neither the species nor the genus *Trichosporon* by sequencing, nor any other fungi, and subjects were limited to acute summer-type HP. Therefore, we expanded the subjects to include patients that were negative for *T. asahii* antibody and patients with chronic home-related HP.

The other fungi besides *T. asahii* that could be detected in the BALF of patients with home-related HP in the present study included *T. japonicum, C. uzbekistanensis*, and *F. napiforme*. Although we performed a PCR using *Trichosporon*-specific primers, *C. uzbekistanensis* DNA was sequenced in several cases. *C. uzbekistanensis* and *Trichosporon* species are known to be phylogenetically close to each other. Moreover, among detected fungus species, *F. napiforme* has been reported to be a causative antigen of HP.15 These fungi, as well as *T. asahii*, are capable of being the antigens responsible for home-related HP.

*T. asahii* DNA was detected in the BALF in 3 patients with CVD-IP in this study (Fig. 1) even though these patients were negative for antibodies against *T. asahii*. We had initially considered the possibility of the contamination during bronchoscopy or assay procedures. However, this consideration was rejected because no fungal DNA was detected in the BALF of patients with sarcoidosis, patients with other lung diseases, or normal subjects. Although the pathogenicity of *T. asahii* in the BALF of CVD-IP patients is unclear, it is speculated that home-related HP is complicated in these CVD-IP patients or that *T. asahii* might be involved in the development of CVD-IP.

For diagnosis of home-related HP, clinical features, radiological findings, laboratory data, and environmental condition should be comprehensively evaluated according to the criteria. Both detection of specific DNA in BALF and positive results of specific antibodies in the sera might be not index of the diagnosis of home-related HP, but the index of the exposure to the agents. However, the items of diagnostic criteria include immunologic examination such as specific antibody and lymphocyte proliferation to detect responsible antigen of the disease. Therefore, both examinations are thought to be important to diagnose of home-related HP. In this study, positive results of *T. asahii* DNA in BALF did not completely correlate with positive results of specific antibodies in the sera of patients with chronic home-related HP (Fig. 2B) even though there was a fairly good correspondence between the two analyses in patients with acute-type HP (Fig. 2A). This disassociation may be related to the sensitivity of each examination. Detection of fungal DNA in BALF partly depends on the quality of the samples during preservation. The sensitivity of specific antibody tests depends on the time
when the blood sample was collected, the amount of exposure to the antigen, and the antibody production ability of the host. Therefore, sensitivity is often insufficient. Indeed, some patients with home-related HP showed negative data by only one examination in our study. To make diagnostic sensitivity of home-related HP higher, it appears to be important to combine both tests.

The limitation of our study is that the number of samples that were in good condition was small and that patients were included retrospectively. Further investigation of a larger number of samples in home-related HP and other diseases would be required.

In conclusion, we were able to detect fungal DNA in the BALF from patients with home-related HP. This method might be especially useful for patients with home-related HP with a negative antibody test to help identify a causative antigen. Further prospective studies are warranted to determine the clinical usefulness of this method.

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Conflict of interest

All authors have no potential for conflict of interest.

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