ABSTRACT

Background: Humidifier lung (HL) is a hypersensitivity pneumonitis resulting from exposure to humidifiers, with fungi from the humidifier as one of the etiologic agents. However, identification of the fungal species responsible for each case can be challenging because of difficulties in culturing fungi, their accurate identification, and interpreting the results of specific serum IgG testing.

Objective: To clarify the best way to determine the causative fungal species of each HL case.

Methods: We report 2 cases with HL in which rare fungi were identified as causative agents. In addition, we searched MEDLINE for previous publications on HL caused by fungi and performed a literature review focusing on clinical testing for the determination of causal fungal species.

Results: In our 2 cases, we identified \textit{Fusarium oxysporum} species complex, \textit{Purpureocillium lilacinum}, \textit{Acremonium sclerotigenum/egyptiacum} as the causative fungal species, based on findings that these could be cultured from humidifier water (HW) and precipitins against these fungi were also positive. The literature review identified 31 HL cases in which the causative fungal species had been documented. In more than half of the cases (17 of 31) there was a concordance between the fungal species cultured from HW and the presence of specific IgG in the blood.

Conclusion: We recommend performing culture of fungi from HW and specific serum IgG testing for the accurate determination of the causative fungal species in HL, and concordance between them serves as a rationale for the determination of causative fungal species.

Keywords: Humidifier lung; Air conditioner; Hypersensitivity pneumonitis; Fungi; Mycoses

INTRODUCTION

Hypersensitivity pneumonitis (HP) is an immune-mediated respiratory disease caused by repeated inhalation of antigens from environmental sources, including birds, fungi, and bacteria [1]. Various types of equipment in human habitations used to heat, humidify or
Humidifier lung caused by fungi

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Conflict of Interest
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Cool air are recognized as one of the sources of such antigens from fungi and bacteria. Humidifiers are common domestic appliances that may cause HP, and the term “humidifier lung (HL)” is used for the HP associated with humidifier use [2, 3]. The first HP cases caused by humidifiers were reported by Banaszak et al. in 1970 [4], which was a small outbreak that occurred in office workers exposed to Thermophilic actinomyces contaminating the air-circulation system supplying their office. After this first report, additional reports of outbreaks of HL in work places caused by office forced-air heating or air-conditioning systems have also been documented [3, 5]. However, not only large air-handling units in work places or industrial settings, but also home humidifiers can also cause HL [2, 6, 7]. Because home humidifiers have been becoming more affordable and available in recent years, the number of cases with HL has been increasing worldwide [7-9]. However, because of the difficulty in accurately diagnosing HL and lack of appropriate diagnostic procedures, HL may be underdiagnosed especially when it occurs in isolated cases after repeated exposure to a home humidifier [10].

Accurate identification of potential causative agents in HP plays an important role for antigen avoidance, better disease prognosis, and understanding the pathogenesis of HL [11, 12]. In general, a detailed patient history, testing for serum IgG against suspected antigens, and specific inhalational challenge (SIC) with suspected causative agents have been recommended to identify the agents inciting HP [12, 13]. Regarding HL, fungal antigens, nontuberculous mycobacteria and endotoxin have all been identified as causative agents [13]. However, there are difficulties in accurately identifying fungal species associated with HL. The reason for this is related to the fact that too many fungal species that do not contribute to HL pathogenesis are also cultured from HW, and that the detection of specific IgG antibodies is only possible for some particular fungal species. Additionally, it is difficult to identify fungal species only from their morphologic features. Thus, the determination of the actual causative fungal species in each individual HL case is challenging.

The first aim of this study is to report our 2 cases with HL in which rare fungi were identified as causative antigens. The second aim is to review the literature on HL caused by fungi, focusing on clinical testing for the identification of the causative species. We finally propose procedures for the diagnosis of HL and the identification of causative fungal species.

MATERIALS AND METHODS

Case reports and literature review
We report 2 cases with HL treated at the Sagamihara National Hospital between 2017 and 2018. Informed consent has been obtained from both patients.

We searched PubMed [National Library of Medicine, Bethesda, MD; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi] for cases of HL related to fungi. We selected published articles in PubMed described in English or in Japanese on 13th July 2021. The keywords used for searching were as follows: (hypersensitivity pneumonitis OR extrinsic alveolar) AND (humidifier OR air system OR air conditioner OR hot tub) AND (hypersensitivity pneumonitis OR extrinsic allergic alveolitis) AND (fungi OR mold OR thermophilic actinomycete). This search yielded 133 articles published between January 1970 and July 2021 (Fig. 1). After reviewing titles and abstracts, we excluded 38 publications of HL not caused by fungi (28 nontuberculous mycobacteria, 6 bacteria, 4 endotoxins), 25 publications not
written in either English or Japanese, 11 publications not included cases with HL, and one publication not on humans. Although *Thermophilic actinomyces* is currently classified as a bacterium, patients with HL caused by this agent were not excluded because this organism is historically classified as a species of fungus, and there are many publications describing HL caused by it. After full text screening of the remaining 58 publications, we excluded 33 (original articles, review articles) that did not include clinical information on the cases and one that could not be accessed [14]. Thus, the remaining 24 publications were finally included in this literature review [3-8, 15-35].

By this means, we collected clinical information as follows: age and sex of the cases, the types of humidifier that caused HL, the results of SIC, the causative fungal species for each case as documented by the authors, the results of fungal culture from humidifier water (HW), the results of the precipitin/serum IgG testing, and the results of genetic analysis for the identification of fungi.

Fig. 1. Flow-chart for the inclusion of articles in the literature review.
Specific inhalational challenge
We performed SIC with their own home ultrasonic humidifiers after obtaining written informed consent from the patients. The ultrasonic humidifiers used in the patients’ homes were run at the bedside in a private room in our hospital, and vital signs were observed every hour. The results of the SIC were interpreted according to the criteria of Ohtani et al. [36].

Culture test
We took samples from the HW and cultured them at 25°C and 37°C for about 7 days on potato dextrose agar and Dichloran 18%-Glycerol agar medium. The growing colonies were visually determined and the number of colonies was counted.

Preparation of crude fungal antigen extracts
Fungal colonies isolated from the above cultures were further cultured in Czapek-Dox liquid medium with shaking for about 2 months at 25°C. The culture supernatant was filtered and centrifuged to remove bacterial cells, and was then dialyzed, concentrated, and freeze-dried to obtain crude fungal antigen extract as a powder.

Precipitin reaction
The Ouchterlony gel double immunodiffusion method was performed for detection of precipitin reactions between 5-fold concentrated patient serum and various antigen extracts, including 20-fold concentrated HW and crude fungal extract (1 mg/mL) [37]. Five-fold concentrated patient serum was obtained by lyophilizing 2 mL of serum and redissolving in 400 µL of distilled water. Twenty-fold concentrated HW was obtained by lyophilizing 20 mL of HW and redissolving in 1 mL of Tris-Buffered Saline. The reactions were classified into strong positive, weak positive, and negative according to the criteria of Kurup and Fink [38].

Genetic identification of fungal species
We identified the fungal species that yielded positive results in the precipitin assay. We made sequencing of the 28S ribosomal DNA along with a BLAST search in GenBank for the identification.

RESULTS

Case reports
A 60-year-old man (case 1) and 73-year-old man (case 2) with fever and dyspnea were admitted to our hospital (Table 1). Although their symptoms seemed to have improved after a week’s antibiotic treatment, symptoms recurred 5 hours (case 1) or 8 hours (case 2) after returning home. The patients were readmitted to hospital with fever, dyspnea, and elevated white blood cell and serum C-reactive protein level. After detailed history-taking, they were suspected to be suffering from HL caused by their home ultrasonic humidifiers, and their symptoms improved without any antibiotic treatment. SICs were performed by running the problematic humidifiers in a private hospital room, which triggered symptoms in both cases. The patients were discharged and returned home after the humidifiers had been removed. There were no repeated exacerbations.

Identification of causative fungal species
Precipitin reactions against HW were performed for cases 1 and 2, and were positive in both. Two and 3 different species of fungi were cultured from HW at 25°C from the home of case 1...
and 2, respectively, as identified by genetic analysis. Crude antigen extracts of these fungi were prepared and precipitin reactions against these extracts were also examined. In case 1, a positive precipitin reaction was observed for an extract of *Fusarium oxysporum* species complex, although precipitins to all the 28 fungal species in the screening panels for HP and ABPM (allergic bronchopulmonary mycosis) were negative. In case 2, a positive reaction to *Purpureocillium*

### Table 1. Characteristics of our 2 cases with humidifier lung caused by fungi

| Characteristic         | Case 1                                | Case 2                                |
|------------------------|----------------------------------------|----------------------------------------|
| Age (yr)/sex           | 60/M                                   | 73/M                                   |
| Symptoms               | Fever, dyspnea                         | Fever, dry cough, dyspnea              |
| Past history           | Colon polyp                            | COPD                                   |
| Smoking (pack-year)    | 12                                     | 45                                     |
| Vital signs            |                                        |                                        |
| Body temperature (°C)  | 38.8                                   | 38.8                                   |
| Blood pressure (mmHg)  | 103/62                                 | 150/84                                 |
| Heart rate (beats/min) | 98                                     | 80                                     |
| SpO₂ (room air) (%)    | 98                                     | 92                                     |
| Laboratory data‡       |                                        |                                        |
| WBC (cells/mL)/neutrophils (%) | 13,000/86               | 12,990/79                             |
| CRP (mg/dL)            | 5.5                                    | 21.01                                  |
| LDH (IU/L)             | 171                                    | 231                                    |
| KL-6 (U/mL)            | 287                                    | 212                                    |
| Arterial blood gas     |                                        |                                        |
| pH                     | 7.432                                  | 7.414                                  |
| PaO₂ (Torr)            | 110                                    | 71.7                                   |
| PaCO₂ (Torr)           | 39.2                                   | 41.2                                   |
| HCO₃⁻ (mmol/L)         | 25.1                                   | 26.4                                   |
| HRCT findings          | Pale, ground-glass opacities in the bilateral upper lobes | Patchy, ground-glass opacities in bilateral lobes emphysema |
| Bronchoscopy           |                                        |                                        |
| CD4/8 ratio            | 1.16                                   | NI                                     |
| Lymphocytes (%)        | 29                                     | NI                                     |
| Histological findings  | No significant findings                 | NI                                     |
| Humidifier type        | UH                                     | UH                                     |
| Duration of humidifier usage in the same season | About 2 months | About 6 months |
| Specific inhalation challenge | Positive                             | Positive                             |
| Diagnosis according to the ATS/JRS/ALAT Clinical Practice (2020) | High-confidence HP | Moderate-confidence HP |
| Diagnosis according to the CHEST Guideline and Expert Panel Report (2021) | HP | HP |
| Fungi cultured from HW | *Fusarium oxysporum* species complex † | *Purpureocillium* lilacinum †        |
|                        | Black colony (not identified)           | Acremonium sclerotigenum/egyptiacum † |
|                        |                                        | Black colony (not identified)          |
| Serum IgG testing (precipitins) |                        |                                        |
| To fungi in the screening panel † | Negative for all             | Strong positive only to *Aureobasidium pullulans* |
| To HW                   | Strong positive                       | Strong positive                        |
| To fungi cultured from HW | Weak positive to *Fusarium oxysporum* species complex | Strong positive to *Purpureocillium lilacinum* |
|                         | Negative to black colony              | Weak positive to *Acremonium sclerotigenum/egyptiacum* |
|                         |                                        | Negative to black colony               |
| Our diagnosis regarding causative fungi for HL | *Fusarium oxysporum* species complex | *Purpureocillium lilacinum* |
|                         | Acremonium sclerotigenum/egyptiacum   |                                        |

Informed consent has been obtained from both cases.

COPD, chronic obstructive pulmonary disease; WBC, white blood cell; CRP, C-reactive protein; LDH, lactate dehydrogenase; HRCT, high-resolution computed tomography; NI, no information; UH, ultrasonic humidifier; HW, humidifier water; HP, hypersensitivity pneumonitis.

† Consisting of 28 fungal species which are frequent causes of HP and ABPM. Including: *Penicillium glaucum*, *Cladosporium*, *Aureobasillium pullulans*, *Tricoderma viride*, *P. mix*, *Cepharosporium acerimonium*, *Aspergillus fumigatus*, *A. flavus*, *A. glaucus*, *A. nidulans*, *A. niger*, *A. restrictus*, *A. terreus*, *A. clvatus*, *Trichosporon asahi*. *Cryptococcus neoformans*, *Candia albicans*, *Mannan A*, *Pigeon serum*, *P. digitatum*, *T. cutaneum*, *A. versicolor*, *Mold Mix*, *P. notatum*, *Trichopyton Mix*, *Bjerkandera adusta*, *Schizopy commune*, *P. casei*, *P. globurum*, *P. luteum*, *Alternaria kikuchiana*, parakeet. ‡Identified using genetic testing by using ribosomal DNA. † Normal range: WBC 3,500–8,500 (cells/mL), CRP 0–0.4 (mg/dL), LDH 124–222 (IU/L), KL-6 0–499 (U/mL).
Humidifier lung caused by fungi

*lilacinum* and *Acremonium sclerotigenum/egyptiacum* was observed, although again, precipitins to all the items in the screening panels (except for *Aureobasidium pullulans*) were negative.

Cases with HL caused by fungi, as identified by the literature search

Table 2 shows 92 cases with HL caused by fungi, comprising 90 cases found in 24 publications in the literature and our 2 cases. Because detailed clinical information on type of causative fungi and clinical testing for the identification thereof for each case was lacking for 47 cases from 3 publications [3, 8, 17], they were excluded and the remaining 45 cases were finally included in the following detailed analysis.

### Table 2. Cases with humidifier lung caused by fungi (including Thermophilic actinomycetes) in the literature and the results of clinical tests for the identification of causative fungi

| Study                          | N  | Age/sex | Humidifier type | Specific inhalational challenge | Causative fungal species | Serum IgG testing† | Detection of causative fungi from HW | Genetic analysis |
|-------------------------------|----|---------|-----------------|--------------------------------|--------------------------|-------------------|-------------------------------------|-----------------|
| Banaszak et al. (1970) [4]    | 4  | 43/M    | CH²             | +                              | Thermophilic actinomycete | +                 | +                                   | +               |
|                               | 56/M | CH²   |                 |                               | + Thermophilic actinomycete | +                 | +                                   | +               |
|                               | 58/M | CH²   |                 |                               | + Thermophilic actinomycete | +                 | +                                   | +               |
|                               | 55/F | CH²   |                 |                               | + Thermophilic actinomycete | +                 | +                                   | +               |
| Fink et al. (1971) [20]       | 1  | 54/F    | FAS             | +                              | Thermophilic actinomycete | +                 | +                                   | +               |
| Fink et al. (1976) [5]        | 8  | 56/F    | FH¹             | +                              | + Thermoaetinomycetes candidus | +                 | +                                   | +               |
|                               | 55/F | FH¹   |                 | +                              | + Thermoaetinomycetes vulgaris | +                 | +                                   | +               |
|                               | 50/M | AC    |                 | +                              | + Thermoaetinomycetes vulgaris | +                 | +                                   | +               |
|                               | 39/F | FH¹   |                 | +                              | + ND                    | -                 | -                                   | -               |
|                               | 62/M | NI    |                 | NI                             | + Thermoaetinomycetes vulgaris | +                 | +                                   | +               |
|                               | 42/M | FH¹   |                 | NI                             | + Thermoaetinomycetes candidus | +                 | +                                   | +               |
|                               | 50/M | AC    |                 | NI                             | + Thermoaetinomycetes vulgaris | +                 | +                                   | +               |
|                               | 8/F  | NI     |                 | NI                             | + Mucor faeni           | +                 | +                                   | +               |
| Miller et al. (1976) [24]     | 3  | 9/F     | CH²             | +                              | ND                      | -                 | +                                   | -               |
|                               | 8/NI | CH²   |                 | -                              | ND                      | -                 | +                                   | -               |
|                               | 7/NI | CH²   |                 | NI                             | ND                      | -                 | +                                   | -               |
| Burke et al. (1977) [18]      | 2  | 46/M    | FAS             | +                              | Thermophilic actinomycetes | -                 | +                                   | +               |
|                               | 57/M | H     |                 | Thermophilic actinomycetes     | +                       | +                 | +                                   | +               |
| van Assendelft et al. (1979) [14] | 2  | 37/F    | CMH             | +                              | Thermoaetinomycetes vulgaris | +                 | +                                   | -               |
|                               | 53/F | AH    |                 | Aspergillus fumigatus          | +                       | +                 | +                                   | +               |
| Solley and Hyatt (1980) [11]  | 1  | 39/M    | FH               | +                              | Penicillium spp         | +                 | +                                   | +               |
| Ganier et al. (1980) [3]      | 27 | NI      | FAS             | +                              | Cephalosporium spp      | +                 | +                                   | +               |
| Patterson et al. (1981) [27]  | 3  | 36/F    | HH               | +                              | ND                      | -                 | +                                   | -               |
| Robertson et al. (1987) [28]  | 3  | 53/M    | OH               | +                              | ND                      | -                 | +                                   | -               |
|                               | 41/M | OH    |                 | +                              | ND                      | -                 | +                                   | -               |
|                               | 57/F | OH    |                 | ND                             | -                      | +                 | +                                   | -               |
| Baur et al. (1988) [17]       | 9  | NI      | HH, AC           | +                              | Mucor species, Aspergillus species, Cladosporium spp, Fusarium spp | +                 | +                                   | +               |
| Shiu et al. (1990) [30]       | 2  | 80/M    | UH               | +                              | ND                      | -                 | Ni                                  | -               |
|                               | 59/M | UH    |                 | +                              | ND                      | Ni                 | Ni                                  | Ni              |
| Gemma et al. (1991) [21]      | 2  | 64/M    | UH               | +                              | Aspergillus fumigatus   | +                 | -                                   | Ni              |
|                               | 64/M | UH    |                 | +                              | Cephalosporium acremonium | +                 | +                                   | +               |
| Volpe et al. (1991) [6]       | 1  | 29/F    | UH               | +                              | ND                      | Ni                 | Ni                                  | Ni              |

(continued to the next page)
Analysis on 45 cases with HL with detailed clinical information

The mean age of the patients was 49.4 years, and 67% were men. Among the 45 cases, 16 were caused by ultrasonic humidifiers (all home-type) and 14 by heated humidifiers. Three outbreaks of HL were included in these publications, consisting of 4 cases in an office with an air-conditioning humidifier, 3 cases associated with a contaminated home central humidifier attached to the heating system, and 3 cases in a printing factory caused by a contaminated cold water humidifier.

SIC with HW and precipitation of HW were the 2 most commonly documented exposure assessment tools for HL in the literature. Positive results in SIC were reported in 32 cases (71%). In most of these cases, environmental provocation was performed as the procedure for SIC, for example by the patient staying in the room (in the hospital or in their house) while the problematic humidifiers were running. SIC were carried out by inhalation of HW in a further 8 cases, but there were no reports of the use of a challenge chamber. Positive results in the precipitin reaction against HW were documented in 13 cases (28%) and in 77% (35 of 45 cases), a positive reaction was documented for either precipitin to HW or SIC with the humidifier.

Table 2. (Continued) Cases with humidifier lung caused by fungi (including Thermophilic actinomycete) in the literature and the results of clinical tests for the identification of causative fungi

| Study                        | N | Age/sex | Humidifier type | Specific inhalational challenge | Causative fungal species                      | Serum IgG testing† | Detection of causative fungi from HW | Genetic analysis |
|------------------------------|---|---------|-----------------|---------------------------------|-----------------------------------------------|-------------------|--------------------------------------|-----------------|
| Suda et al. (1995) [7]       | 5 | 64/M    | UH              | +                               | Candida sp                                    | +                 | NI                                   | +               |
| 61/M                        |   |         | UH              | +                               | Cepharosporium sp                             | -                 | NI                                   | -               |
| 64/M                        |   |         | UH              | +                               | ND                                            | -                 | NI                                   | +               |
| 63/M                        |   |         | UH              | NI                              | Cepharosporium sp                             | -                 | NI                                   | +               |
| 73/M                        |   |         | UH              | NI                              | ND                                            | -                 | NI                                   | +               |
| Nakaya et al. (1997) [25]    | 1 | 61/M    | UH              | +                               | ND                                            | NI                | -                                    | -               |
| Alvarez-Fernández et al. (1998) [15] | 1 | 34/M    | UH              | +                               | Rodotorula                                    | 1                 | +                                    | +               |
| Da Broi et al. (1999) [19]   | 1 | 65/M    | OWH             | +                               | Aspergillus fumigatus                         | 1                 | NI                                   | +               |
| Sakurai et al. (2001) [29]   | 1 | 62/M    | FH              | +                               | Cephalosporium acremonium                     | +                 | NI                                   | NI              |
| Yamamoto et al. (2002) [35]  | 1 | 65/M    | UH              | +                               | Debaryomyces                                  | +                 | +                                    | +               |
| Koschel et al. (2005) [6]    | 11| Mean age, 40 yr/7 men and 4 women | UMF             | +                               | ND                                            |                   |                                      |                 |
| Ando et al. (2017) [16]      | 1 | 32/F    | UH              | +                               | Candida guilliermondii                        | -                 | -                                    | +               |
| Tomoda et al. (2019) [32]    | 1 | 60/M    | UH              | +                               | Cephalosporium acremonium                     | +                 | NI                                   | NI              |
| Okabe et al. (2020) [26]     | 1 | 5/F     | HT              | NI                              | Tricosporon asahii                            | NI                | NI                                   | NI              |
| Our cases (2021)             | 2 | 61/M    | UH              | +                               | Fusarium oxysporum species complex            | +                 | +                                    | +               |
| 73/M                        |   |         | UH              | +                               | Purpureocillium lilacinum                     | +                 | +                                    | +               |

N, number of cases; NI, no information; "-", positive; "-", negative; CH, central humidifier; FAS, forced-air system; FAH, factory humidifier; HH, home humidifier; HR, humidifier reservoir; CMH, cooling-mist-humidifier; AH, atomizer of humidifier; OH, office humidifier; UH, ultrasonic humidifier; HT, hybrid type (steam + ultrasonic humidifier); OWH, oxygen water humidifier; UMF, ultrasonic misting fountains; AC, air conditioner; FH, fornace humidifier; ND, not determined.

Includes outbreak cases. †The results of precipitins unless otherwise noted. ‡Heated type humidifier. §Not included for the detailed analysis shown in Table 2 and Fig. 2 due to lack of information regarding identification of causative fungi. ‡Positive for enzyme-linked immunosorbent assay. ¶Positive for radioimmunoassay.
Analysis of 31 cases for which the causative fungal species was identified
Of the 45 cases, the fungal species that caused the disease was documented in 31 (68%). Fig. 2 shows the list of causative pathogens in descending order. Thermophilic actinomycetes was the most frequently documented (14 cases) [4, 5, 18, 20, 34], followed by Acremonium spp. (6 cases) [7, 21, 27, 29, 32], and Aspergillus fumigatus (3 cases) [19, 21, 34]. The clinical testing used for the identification of the fungal species was different in the different reports. Table 3 shows the rationale for the determination of causative fungal species in these cases. In 17 (54%), the rationales were detection of the fungi after culturing HW, accompanied by positivity of specific serum IgG to the same fungal species. In 12 cases (38%), only the results of serum antibody testing were documented and in another 2 cases, only the results of HW fungal culture were employed. There were no reported cases for which genetic analysis was used to identify fungal species, except for our 2 cases.

Fig. 2. Causative fungi for cases with humidifier lung in the literature by humidifier type. †Including fungi reported as Cephalosporium spp.

Table 3. Rationale for the identification of fungi causing humidifier lung: findings from 31 cases in which causative fungi were documented

| Rationale for the identification of causative fungi | No. of cases (n, % of total) |
|---------------------------------------------------|-------------------------------|
| Agreement between type of fungi cultured from humidifier water and specificity of serum IgG from the patient* | 17 (54) |
| Positive in precipitins | 15 (48) |
| Positive in other serum-specific IgG testing | 2 (6) |
| Positivity in specific IgG testing | 12 (38) |
| Positive in precipitins | 11 (35) |
| Positive in other serum-specific IgG testing | 1 (3) |
| Detection of the fungi by culturing humidifier water | 2 (6) |
| Total | 31 (100) |

*Serological IgG test includes precipitins, enzyme-linked immunosorbent assay, radioimmunoassay and complement fixation test.
DISCUSSION

In this study, we report 2 patients with HL caused by rare fungal species identified by performing fungal culture of HW, genetic analysis, and precipitin testing against the fungal extract prepared from the cultured fungi. Additionally, a literature review of HL caused by fungi indicated that precipitins to HW, or SIC by running the humidifier have been performed as exposure assessment tools for HL in most previous reports. Also, causative fungal species have been determined on the basis of concordant findings between fungal species cultured from HW and specific serum IgG testing. Based on these findings, we propose procedures for the diagnosis of HL and accurate determination of the causative fungi, as shown in Fig. 3. We recommend performing cultures of HW, followed by testing serum IgG specificity for these cultured fungi, for the accurate identification of the causative fungal species in HL. Furthermore, we consider that genetic analysis, in addition to morphological identification, is desirable for the accurate identification of the fungal species cultured from HW.

Causative fungi of HL

In the literature review, the most common HL causative fungi were *Thermophilic actinomycetes* with 14 cases. In addition, the majority of HL cases caused by *Thermophilic actinomycetes* (9 of 14) were associated with using heated humidifiers. The optimal growth temperature of *Thermophilic actinomycetes* is 50°C–52°C [39] and they can grow even at temperatures as high as 65°C [40]. They are also known as causative pathogens for other HP, such as farmer’s lung [41] and mushroom lung [42]. *Thermophilic actinomycetes* is currently classified as a bacterium, but we note that when culturing this pathogen, culture conditions, such as isolation medium, culture temperature, and culture periods, are different from those of other bacteria. When

![Diagram](https://apallergy.org)

**Fig. 3.** Proposal for procedures for the diagnosis of humidifier lung and the identification of causative fungi. Refers to the latest diagnostic criteria [1](AJRCCM 2020;202:e36-69). [2](Chest 2021;160:e97-156), performed by inhalation of nebulized humidifier water, by exposure in a challenge chamber, or environmental provocation.
HL is clinically suspected, it is desirable to culture HW at 50°C–52°C in Czapek-Dox medium according to the method of Hopwood and Ferguson [43].

In our patients, 3 causative fungi were identified, namely, *Fusarium oxysporum* species complex, *Purpureocillium lilacinum*, and *Acremonium sclerotigenum/egyptiacum*. Although *F. oxysporum* species complex, and *A. sclerotigenum/egyptiacum* have been reported as causative fungi for HL in the literature, *Purpureocillium lilacinum* (formerly *Paecilomyces lilacinus*) had not been reported before. *P. lilacinum* has been reported as causing infections in immune-compromised hosts [44] and has been isolated from a variety of environments like soil, rotting crops, and air-conditioning contamination in factories. Because *P. lilacinum* has also been reported as a fungus in water [45], we presume that it can grow under the environmental conditions in the humidifier.

**Problems with ultrasonic humidifiers for HL**

Both of our cases were caused by using ultrasonic humidifiers. In the literature review, the frequency of HL caused by ultrasonic humidifier was relatively high at 16 of 38 (42.1%), and all of these were home appliances. These findings suggest that ultrasonic humidifiers may be more likely to cause HL than other types of humidifiers. One possible reason for this may be related to the fact that water in the ultrasonic humidifier is not usually heated up to a fungicidal temperature, and fungi may easily grow in the HW. Another possible reason may be the small particle size (0.5–3 µm) produced by ultrasonic vibrations which is small enough to reach the peripheral airways and lung parenchyma [7]. Furthermore, the generator itself, jets, towers, and nozzles in ultrasonic humidifiers are generally difficult to clean [30].

**Clinical testing of exposure assessment in the diagnosis of HL**

Exposure identification constitutes one of the 3 primary domains of diagnostic criteria for HP according to the ATS/JRS/ATRA guideline [13]. Exposure assessment tools include history-taking, exposure questionnaires, SIC, serum IgG testing, lymphocyte proliferation testing, and others [46]. However, the clinical relevance of each assessment tool is quite heterogeneous and their clinical utility remains a matter of debate [12].

Among the 45 HL cases that we surveyed in the literature, positive results in SIC with the humidifier were documented in 32 (71%). This suggests the clinical importance of this test for the diagnosis of HL. Regarding the procedure for SIC, environmental provocation has been used more frequently than inhaling HW with nebulizers.

Positive results for specific IgG testing against HW were documented in 28% (13 of 45) of HL cases in the literature, less frequent than SIC. Precipitins were more frequently used as detection tools for serum IgG antibodies than immunoassays such as enzyme-linked immunosorbent assay. Positivity in this test can show the presence of specific IgG antibody to any component in the HW, but cannot determine the causal agents. The relatively lower frequency of cases who underwent IgG testing to HW may be explained by the fact that these tests, especially those against clinical samples like HW, cannot be performed in commercial facilities in most countries.

**Laboratory testing for the determination of the causative fungal species for HL**

Accurate determination of agents inducing HL may not be essential for the management of each case, but is important for the understanding of pathogenesis and future prevention of HL. Culture of HW is the gateway for the accurate identification of causal agents of HL. However, performing fungal culture tests and interpreting the results can be challenging.
One reason for this is related to the fact that too many fungal species are usually cultured from HW, including those that do not contribute to pathogenesis. Picking up all the colonies cultured from HW is technically challenging and sometimes difficult. Additionally, the fungal species in HW may change over time depending on the storage environment of the humidifier, and it may also be necessary to establish cultures using several different types of separation medium which are appropriate for the target species. In our literature review, causative fungi were cultured from HW in 19 of 45 cases. However, it should be noted that the fungal species cultured from HW are not always the genuine cause of HL, and that genuine causative fungi may not always be amenable to culture.

When fungi are cultured from HW, they are usually identified by morphological findings, including color and appearance of colonies and microscopic findings. However, recent developments in molecular biology have led to the use of genetic analysis for the identification of fungal species. Compared to identification methods that used only morphological findings, genetic identification is superior because it is objective and reproducible. Detailed classification based on nucleotide sequences may also be available now. In our patients, genetic testing was used to accurately identify the fungal species cultured from HW. To the best of our knowledge, these 2 patients are the first cases in the literature for whom the causative fungal species were identified by genetic testing.

Performing specific IgG testing on the same fungi as those cultured from HW can contribute to confirming the accurate determination of causative fungal species. Indeed, in 17 of 31 cases of HL (54%) in the literature, concordant findings between fungal species cultured from HW and those bound by specific IgG have been documented. However, attention is also required for the interpretation of positive results in IgG testing against specific crude fungal extracts, because fungal antigen extracts contain many cross-reactive antigenic proteins. Exposure and IgG reactions to one particular fungal species can result in positive findings in IgG to other fungal species [47]. In order to minimize the risk of overdiagnosis of causative fungi due to fungal cross-reactivity, we recommend performing precipitin to panels of fungal extracts including common fungal species causing HP, as well as the fungal species which have been cultured from HW. Specific and strong reactivity to the latter increases the possibility that this fungus is the genuine causative agent.

Proposal of procedures for the diagnosis of HL and the identification of causative fungal species

Fig. 3 shows proposed procedures for the diagnosis of HL and the accurate identification of the causative fungi, on the basis of findings from the literature review. As a prerequisite, the current diagnostic criteria of HP must be met for patients to be considered as possibly suffering from HL. This can be diagnosed when a patient has a clinical history compatible with HL and who tests positive either (1) possessing precipitins for HW (or other specific serum IgG), or (2) SIC with humidifier. Among the 45 HL cases in the literature, 35 (77%) met these diagnostic criteria.

To identify causative fungal species in HL, culture of the HW is the first step. Cultured fungi should be regarded as causative agents for HL only when specific serum IgG testing to the same fungi is positive at the same time. As mentioned above, in 17 of 31 cases of HL (54%) in the literature, causative fungi were determined on the basis of concordant findings between fungal species cultured from HW and those to which serum IgG was present. Furthermore, it is desirable to add genetic analysis as well as morphological identification in order to
identify the causative fungal species more accurately. Our proposed procedure is easy to use and may be valuable in real-life clinical settings because it clearly documents the sequence of necessary clinical tests for diagnosis of HL or the accurate determination of causative fungi, and how to evaluate the results of each test.

**Potential limitations of our proposed diagnostic procedure**
A potential limitation of our proposed procedure is related to publication bias for cases with HL in the literature. We generated this procedure on the basis of findings from analysis of cases in the literature. However, cases with HL in the literature might have been biased towards those with higher rates of positive results in specific IgG tests and SIC. Another problem of our proposed procedure is that it requires IgG testing against HW or fungi cultured from HW. Performing these tests would be difficult for some institutes because they require special techniques and trained laboratory staff and equipment.

In conclusion, we report 2 cases with HL caused by rare fungal species. Additionally, we propose a procedure for diagnosis of HL and identification of the causative fungal species. We believe that our proposed diagnostic procedure would contribute to more accurate diagnosis of HL, more appropriate identification of causative fungi and hence better disease prognosis for patients with HL.

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