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Hypersensitivity pneumonitis and antigen identification – An alternate approach

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ABSTRACT

Objectives: Identification of the causal antigen for patients with hypersensitivity pneumonitis (HP) is challenging in a standard clinical setting. The purpose of this pilot study was to determine whether it was possible to evaluate the home/workplace of patients, and identify the causal antigen.
Methods: Using a case-control study design we compared the presence of antibody to antigen collected in the environment of individuals with HP and controls consisting of family members/co-workers. Based on patient interviews, homes/workplaces were evaluated and suspected sources of antigen collected for use in immunoassays.
Results: Nineteen individuals with HP participated with 15 classified as having fibrotic disease. Up to 54 bulk samples were collected from each patient's environment, with multiple isolates (antigens) cultured from each. Of the seven individuals who tested positive to one or more environmental samples, three had a positive response to more than 1 antigen from the environmental sample (range 1–9). Twelve individuals tested positive to antigen(s) on a standard panel, with only one overlapping with the antigen from the home/workplace sample. A significant association existed between results of interviews/site evaluations, and ability to collect antigen eliciting a positive response ($p < 0.001$).
Conclusion: Antigen identification was successful for patients with 'active' disease. Antigens for which patients test positive on standard panels may not be present in their environment. One benefit to patient-centered testing is the ability to develop recommendations specific to their environment. As most individuals tested positive for >1 antigen, further investigation is warranted to determine the actual antigen responsible for disease.

1. Introduction

Hypersensitivity pneumonitis (HP) is a well characterized respiratory disease that is the result of an immune response to inhaled organic antigen (e.g. *Thermoactinomyces candidus*) or low molecular weight chemicals (e.g. isocyanates) [1–4]. If the antigen is identified and removal from exposure occurs prior to the initiation of fibrotic changes, it is possible for the disease to resolve. With repeated exposure, the disease may progress to end stage pulmonary fibrosis. HP has been described in workers in multiple occupations such as farmers, pigeon breeders, and metal machinists [2] and from environmental exposures related to hot tubs, humidifiers and water damaged homes [3]. Over 200 antigens have been identified as causal agents for HP [5]. Typically these antigens have an aerodynamic diameter of 1–3 microns, small enough to penetrate into the lower airways upon inhalation [1,2,5,6]. Prevalence has been reported to be as great as 5–15% among individuals who are known to have been exposed to antigens associated with the disease [7]. HP has been described in multiple countries [8–11], and a study in the United States showed a significant increasing trend in the overall age-adjusted mortality rates between the years of 1980 and 2002, with the highest mortality rate occurring in Wisconsin at 1.04 per million [12].

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Diagnosis is often difficult. Multiple diagnostic criteria have been proposed [13–15]. A common component of the proposed diagnostic criteria is the identification of the etiologic agent by history and/or detection of antibody. More recently, some centers have begun doing specific antigen challenge testing [16]. Although corticosteroids are used to treat HP, the most effective treatment is removal of the patient from exposure to the causal antigen; however, frequently the causal antigen cannot be identified nor confirmed [17]. Results for standard clinically available serum-HP screening antigen panel, which allows testing for IgG antibodies in the patient's serum against (up to) eight antigens or more extensive panels that include 12 antigens or up to six additional antigens for bird protein, are often negative. At present, clinicians do not have access to testing that allows them to individualize the panel to the actual exposure of their patients.

The purpose of this pilot study was to evaluate HP patients' home and work environments and to collect and isolate antigens from these environments important in the etiology of the patient's HP.

2. Methods

2.1. Experimental design

This investigation was approved by the Michigan State University Human Research Protection Program (Biomedical and Health IRB) and the University of Michigan Health System IRB. All study subjects and controls provided informed consent. This was a pilot 'proof-of-concept' study developed to determine whether it was possible to collect samples from a subject's home/workplace and test whether these samples contained antigens against which the patient sera contained antibodies. A case-control study design was developed to compare the presence of antibodies to the suspected antigen(s) causing the patient's HP with a control group consisting of co-workers and/or family members.

2.2. Subject identification

Subjects were identified from referrals to the University of Michigan (n = 17) or Michigan State University (n = 2). The diagnosis of HP was determined at the University of Michigan interstitial lung disease multi-disciplinary clinical-radiographic-pathologic conference following American Thoracic Society guidelines for the diagnosis of interstitial lung disease [18]. The two subjects from MSU met clinical criteria for HP [13]. Subjects were classified as 'definite' with diagnostic surgical lung biopsy or 'probable' with consistent radiographic and bronchoscopic evaluation. Further sub-classification included 'fibrotic' based on surgical lung biopsy or radiographic characteristics in those without biopsy, versus 'inflammatory,' the lack of fibrotic changes [17,19–21]. Each case was asked to identify one control, either a family member or co-worker, depending on the most likely suspected source of the exposure.

2.3. Environmental sampling, sample collection, and laboratory testing

Environmental sampling took place at each case's home and/or workplace based on patient interviews using a standardized questionnaire. The sampling protocol was standardized but remained flexible in order to maximize the possibility of collecting and subsequently identifying the offending antigen(s). If, based on the interview and site visit, it was suspected that the subject was likely no longer exposed to the offending antigen, or that it was not possible for sampling to occur at the likely source of the suspected offending antigen (i.e. a vacation home out of state), samples were still collected from the home/workplace, but a notation was made that samples 'were not able to be collected from the suspected source'. Because of budget constraints a hierarchical scheme of analysis was developed. All cases had their serum tested against the standard HP antigen panel, and against samples collected from their workplace/home. If the case's serum tested positive to antigen collected in the sample, determination of the specific antigen contained in that sample was performed. If we were unsuccessful in identifying the offending antigen(s) in the first set of samples, we re-visited the case's workplace and/or home to collect additional samples.

Only those cases who had antibodies either to the standard HP panel or to antigen collected from their workplace/homes had the sera of their matched control subject tested, the rationale being that if the case did not produce antibodies to antigens collected in their work/home environment, we would not have identified the offending antigen(s) for that particular HP case, and testing referent sera would be futile.

2.3.1. Environmental sampling – questionnaire

The 'work environment questionnaire' included: previous employment history, length of time at current job, job classification, task description, types of chemical materials utilized, type of ventilation, knowledge of other worker complaints, and the availability of industrial hygiene and medical monitoring results. Information captured by a 'housing questionnaire' included: length of time lived in the home, age of structure, type (i.e. ranch style, bi-level, etc.), construction (i.e. brick, wood, etc.) and history and location(s) of water damage/leaks, types of HVAC systems utilized (i.e. forced heat, air conditioner, etc.), use of a humidifier, type(s) of flooring, ventilation preferences, rural or urban environment, description of landscaping, hobbies performed at residence (or elsewhere), and number and type(s) of pets.

2.3.2. Environmental sample collection procedures

Sampling locations were determined based on responses to the questionnaires. Environmental sampling focused on the collection of contaminants (fungal, bacterial, or chemical) which had the potential to become airborne. Therefore, media with which to collect the samples varied based on contaminant/substrate type. Sampling consisted of wipe sampling from ledges, sampling of liquid materials from bulk fluid tanks/humidifier reservoirs, micro-vacuum sampling of carpeting or air vents, swab sampling of wallboard, or the collection of bulk samples from furnace filters and soil/debris. Each environmental sample was suspended in 2% tryptone-20% glycerol (designed to support viability of microbial contaminants) and then split into multiple aliquots. One aliquot was stored frozen at −20 °C for use in immunoassays and the remaining aliquots were stored frozen at −80 °C for subsequent culture for bacteria and fungi.

2.3.3. Blood collection procedures

Fifteen milliliters of blood were collected from all cases and controls by a trained phlebotomist. If blood had not already been collected for a standard HP antigen panel by the referring physician, collection was done at the same time.

2.3.4. Commercial laboratory testing

Clinical laboratories provided a standard HP panel which utilized immunodiffusion to measure IgG antibodies to multiple antigens. The standard HP panel was performed by multiple laboratories unless it had not been performed as part of the patient's clinical care and we ordered the test as part of our project.

2.3.5. Immunodiffusion

All cases had their serum evaluated for antibodies against antigens in the environmental samples collected in their home/workplace. The first step in the 'global' assessment of whether or not a case reacted to a particular environmental 'agent' (e.g. microbes from water in a humidifier reservoir), was to utilize an immunodiffusion technique [5] in which the case's serum was tested against each of the environmental samples. Samples were evaluated as collected and were also prepared by ultrasound dissolution [22]. These soluble materials were used as "antigen" in an immunodiffusion assay carried out on agar-coated microscope plates with patient serum from the corresponding case. Evidence of immunologic recognition between antigen in the environmental sample and immunoglobulins in the serum was noted by the presence of a precipitin band following staining [23]. When the case's serum reacted to one or more of the environmental samples, the serum of their designated control was tested against the same sample.

2.3.6. Isolation and testing of microorganisms from environmental samples

After initial serum precipitin testing, it was possible there were multiple samples which yielded positive results against the associated case's serum. The initial immunodiffusion assays tested the case's serum with a sample that potentially contained multiple antigens, many of which were predicted to be associated with specific microorganisms. To isolate individual bacteria and fungi, each sample that had tested positive was cultured on Sabouraud medium (saprophytic fungi) and trypticase soy agar (bacteria) at 20 °C, 37 °C, and 55 °C for up to 10 days. Plates were screened daily and individual colonies were selected and subcultured until pure cultures were achieved. Each pure culture was suspended in 2% tryptone-20% glycerol; one aliquot was archived at −80 °C for subsequent culture and a second was prepared as described above for environmental samples. The immunodiffusion assay was repeated with each pure culture against patient serum to identify reactivity against individual microorganisms. Environmental samples which did not react with patient serum, i.e., did not contain reactive antigens, were not subcultured.

Individual microorganisms for which the patient had a positive reaction by immunodiffusion assay were identified to at least the genus level. Bacteria were cultured and stained, and cell morphology and gram reaction, as well as colonial morphology recorded. For each bacterial sample, 2–3 colonies were suspended in 20 μl of 50 mM NaOH and the sample incubated at 95 °C for 10 min to lyse the bacterial cells. This cell lysate was used as template in a PCR reaction with universal 16s rDNA primers [24] and the resulting PCR product sequenced and compared to the bacterial 16s rDNA database at the Ribosomal Database Project [25,26] to identify each organism. Sequencing was performed at the Michigan State University Research Technology Support Facility.

Fungi were identified by morphologic characteristics of organisms grown on Sabouraud's medium and in slide culture, in consultation with Dr. A. Leonel Mendoza, a fungal expert at Michigan State University.

2.3.7. Immunoblot analysis

To prepare antigens used for immunoblot analysis, isolates of *Cladosporium* and *Penicillium* were grown on Sabouraud agar at 20 °C and *Thermoactinomyces vulgaris* was grown on tryptic soy agar at 55 °C. Fungal or bacterial colonies were scraped from the agar and dense suspensions made in 2% tryptone-20% glycerol. Aliquots of these suspensions were mixed with SDS-PAGE sample buffer and the mixture boiled for 10 min. Samples were separated by discontinuous SDS-PAGE on 12% acrylamide gels [27]. Gels were either stained with Coomassie blue to visualize antigens, or the proteins were electrophoretically transferred onto nitrocellulose membranes for immunoblot analysis [27]. Blots were incubated with patient sera diluted 1:200 in TBST (10 mM Tris–HCl, pH 8, 150 mM NaCl, 0.05% Tween 20) and developed with horseradish peroxidase-tagged protein A and NiCl-diaminobenzidine color development reagent (BioRad).

2.4. Data analysis

Sample size for this pilot study was limited based on the amount of funds available and the costs of laboratory testing. Each subject's data file was complete (no missing data). Fisher's Exact Test was used to determine the association between testing positive on a standard HP Panel Test and antigen collected during home/workplace environmental sampling. This same test was also used to determine the association between the 'suspected antigen source' identified during the home/workplace visit, and the ability to collect antigen testing positive with the subject's serum. Pearson's correlation coefficient was utilized to determine whether there was correlation between results from immunoblot, immunodiffusion, and standard HP Panel tests. All analyses were performed utilizing SAS© version 9.4.

3. Results

Nineteen individuals with 'definite' or 'probable' diagnoses of hypersensitivity pneumonitis were included in the study (Table 1). Of these, 7 (36.8%) were male, 12 (63.2%) were female, and ranged in age from 36 to 74 years. All (100%) study subjects had undergone CT evaluation, 3 (16%) had undergone trans-bronchial biopsy, 11 (58%) surgical lung biopsy, and 4 (21%) bronchoscopy prior to referral. Four individuals were classified as 'inflammatory' HP and 15 were classified as having 'fibrotic' HP. Time between onset of symptoms to HP diagnosis ranged from less than 1 year (10.5% of individuals) to greater than 3 years (Table 1.) with the largest percentage of individuals (47.4%) falling into the 1–3 year category.

Up to 54 (range 6–54, median = 15) 'bulk' samples were collected per patient from homes and/or workplace(s) (Table 3), with up to 54 discrete isolates (antigens) cultured for each 'bulk' sample. Seven of the 19 patients (36.8%) had a positive response to one or more sample(s). All were from their home or other location of 'significant activity' but none were from the workplace. Six of these seven individuals tested positive for 1 to 9 different microorganisms isolated from their environmental samples on immunoassay (Fig. 1); no reactive microorganisms were isolated from the positive environmental sample for the seventh patient. A total of 16 discrete antigens were identified as yielding a positive reaction with patient sera (Table 2).

Table 1
Characteristics of study population.

Gender:		(%Total, n = 19)
Male	7	36.8%
Female	12	63.2%
Age (range)	36–74	
HP diagnosis		
Definite	12	63.2%
Probable	7	36.8%
Type:		
Inflammatory	4	21.1%
Fibrotic	15	78.9%
Time between onset of symptoms and diagnosis (in years):		
<1	2	10.5%
1–3	9	47.4%
>3	8	42.1%

Table 2
Microorganisms identified as antigens testing positive against patient sera in present study and whether or not a positive antibody response was previously associated with HP.

Microorganism	Prior reports of positive antibody response	Number of patients with positive reactions
Bacteria		
Acinetobacter calcoaceticus	+ [28]	1
Arthrobacter spp.	+ [29]	1
Bacillus spp. (including *B. pumilis, megaterium, safensis, subtilis, and thuringiensis)*	+ [30,31,32]	4
Brevibacterium spp.	–	1
Enterobacter spp.	–	1
Enterobacteriaceae (unidentified)	+ [29]	1
Paenibacillus spp.	–	1
Pseudomonas spp (including *P. fluorescens, putida,* and *rhizosphaerae*	+ [30,33]	2
Rahnella aquitilis	–	1
Staphylococcus spp. (including *S. saprophyticus* and *succinus*)	–	2
Thermoactinomyces vulgaris	+ [34–50]	1
Fungi and Yeasts		
Cladosporium spp.	+ [51–55]	2
Fusarium spp.	+ [56–61]	1
Penicillium spp.	+ [56,61–66]	2
Rhodotorula spp.	+ [35,67,68]	1
Scopulariopsis spp.	+ [69,70]	1

No positive samples were identified in the two individuals for whom workplace exposure was suspected by the referring physician *and* where workplace sampling took place.

Nineteen individuals had serum tested against a 'standard' HP panel utilized by commercial laboratories (Table 3). Twelve (63.2%) had positive test results to one or more antigens (Table 3). Of the 12 that had a positive test on the 'standard' HP panel(s), 7 (58.3%) tested positive to more than 1 antigen (range 1–5 pos. antigens) (Table 3). Among these 12, six had a positive response to antigen isolated from environmental samples (Table 4).

If a subject was positive on a standard HP panel, there was a non-statistically significant increased chance (OR = 6.0, 95% CI (0.54–66.17), p = 0.17) that they were also positive on a home visit sample. In only one instance was there an antigen match between the 'standard' HP panel with the antigen isolated from home/workplace sampling (*Penicillium* spp.). Among the seven individuals with negative results on the commercial panel, one individual had a positive result to an antigen isolated from environmental sampling (Table 4).

There were eight subjects based on the interview and site visit for which it was thought access to antigen (sample collection) was possible at the time blood was collected for antibody testing. Of these eight, seven individuals had a positive antibody response to samples collected from their environment. In the nine individuals that it was unlikely the patient had ongoing exposure to the offending antigen (e.g. job cessation or home remediation prior to visit) and in the two individuals where antigen exposure although intermittently ongoing was not occurring at a location that could be sampled (e.g. tropical rain forest), none had a positive antibody response to samples collected from their home or work environment ($p < 0.001$) (Table 3).

Of the seven control subjects matched with the seven cases who tested positive to antigens collected in their environment, one tested positive on the 'standard' HP panel, and one tested positive to an antigen isolated from samples collected in the home. In both of the two control subjects with a positive result the antigen was different than the positive result in their matched HP case.

Because there was frequently a discrepancy between our immunodiffusion results and the commercial HP panel results, we sought to further characterize the immune response of seven patients to a subset of the antigens commonly identified. Immunoblots were performed with selected patient sera against *Cladosporium, Penicillium,* or *Thermoactinomyces vulgaris* (Fig. 2) cultured from environmental samples. Our results showed a correlation between results of immunodiffusion and immunoblot tests ($p \leq 0.04$), and no correlation between immunodiffusion and immunoblot results with those of the HP panel (Table 5). In three cases, a reaction against an antigen that was not cultured from the patient environment (*Thermoactinomyces*) was detected by immunoblot.

4. Case report

A nonsmoking man in his forties developed respiratory symptoms in his early 30s. Initially, he was diagnosed as having asthma although no pulmonary function testing was performed. He used a bronchodilator intermittently. Fifteen years later he experienced shortness of breath when he was in the Rocky Mountains at which time he had a chest radiograph, which was abnormal and he was diagnosed with high altitude pulmonary edema. His shortness of breath persisted after his return to Michigan. Further testing continued to show an abnormal chest radiograph. Pulmonary function tests and open lung biopsy was performed. The pathology was consistent with HP.

He had a management position, which required frequent travel to printing plants. He noted increased symptoms when he was home. His home had two episodes of water damage.

On spirometry, his FVC was 3.25 L (63% of predicted), his FEV_1 was 2.78 L (72% of predicted). The FEV_1/FVC ratio was 86% (114% of predicted). His residual value was 1.14 L (52% of predicted) and total lung capacity was 3.77 L (51% of predicted). His diffusion capacity was 21.56 ml/min/mmHg (66% of predicted). On chest radiograph he had "hazy increased interstitial ground glass opacities" in both mid-lungs and subtle diffuse ground glass opacities in both lungs on CT.

On a commercial hypersensitivity panel IgG antibodies were not elevated to *Aspergillus fumigatus*, *Micropolypora faeni* or *Thermoactinomyces vulgaris*. Tests for rheumatoid factor, anti-nuclear antibody and anti-scleroderma antibody were negative. Bronchial lavage fluid showed 11% neutrophils, 54% lymphocytes and 35% histocytes.

Review of his job description, time spent in the field, assigned tasks (primarily sales), and chemicals identified by review of material safety datasheets from printing plants he visited did not identify a likely association between an exposure at the workplace and HP. Travel (time spent in a hotel) was noted as a potential source of antigen exposure as the interview between the patient and physician indicated that symptoms and a diagnosis of high altitude pulmonary edema occurred during a ski trip in which the patient was residing in an older "musty smelling" cabin. He mentioned he had not experienced issues with altitude on previous ski vacations, and that indeed symptoms did not improve on return to lower elevation.

Water damage and mold was not apparent during a tour of his home. The home was clean and little dust was present. Water remediation in the basement occurred prior to the home sampling visit. The patient reported that he did not spend much time in the basement, however it was noted that the furnace was located in the basement and therefore return air moving through the area would be captured by the ventilation system and dispersed throughout the home. As a result, and while environmental samples were taken throughout the home, the focus of antigen collection occurred in the basement.

Table 3
Summary of likelihood of diagnosis, exposure suspected by physician, results of standard HP panel, and home/workplace antigen collection outcome in subjects with disease.

ID	Likelihood of diagnosis	Type	Exposure suspected by physician	Std HP panel outcome (#Pos)	# bulk samples	Suspected antigen (home/ Wkplace visit)	Home/Workplace antigen collection (#pos)	Comments
01	Definite	Fibrotic	Metalworking Fluids (work) or Aspergillus (early sputum smear)	3	54	Metalworking Fluids or Mold (Farm)	3 Bulk Samples 7 Antigens (Arthrobacter, Bacillus, Enterobacter, Pseudomonas, Staphylococcus, Penicillium, Scopulariopsis	Pos Antigen ID on Farm 1 common antigen ID between HP Panel and Home/Workplace Collection
04	Probable	Fibrotic	None	3	20	Country Farmhouse (Remodeling/Landscaping)	1 Bulk Sample 1 Antigen (Bacillus)	Positive Antigen Collected from Garden at Secondary Home (Farmhouse)
07	Probable	Fibrotic	None	1	30	Family Farm	5 Bulk Samples 9 Antigens (Bacillus, Pseudomonas, Paenibacillus, Rahnella, Fusarium, Rhodotorula, Cladosporium, Penicillium, unidentified yeast	Currently still exposed (declining PFT)
08	Definite	Fibrotic	Parrot	0	22	Mold in home	1 Bulk Sample 0 Antigens cultured from positive bulk sample	Not able to collect samples from suspected source
09	Probable	Fibrotic	Bird Exposure (Home)	1	30	Mold in Silage (Clean out of Relatives Farm), Bird Exp	5 Bulk Samples 4 Antigens (Bacillus, Brevibacterium, Staphylococcus, Thermoactinomyces)	Several positive samples taken from farm (hayloft, floors), and personal garden. Straw from farm was mulched and used in home garden.
*10	Definite	Fibrotic	None	1	10	Isocyanates, Metalworking Fluids	0 Bulk Samples	Not able to collect samples from suspected source
*11	Definite	Fibrotic	None	0	10	Mold in home	0 Bulk Samples	Not able to collect samples from suspected source
12	Probable	Fibrotic	None	4	18	Mold in home	2 Bulk Samples 1 Antigen (Acinetobacter)	Positive samples taken from bathroom and household vacuum cleaner
*13	Probable	Fibrotic	None	0	13	Mold in Travel Trailer	0 Bulk Samples	Not able to collect samples from suspected source
14	Definite	Fibrotic	None	0	17	Exposure to Chemicals in R&D Lab	0 Bulk Samples	Not able to collect samples from suspected source
15	Probable	Fibrotic	None	1	14	Vacation site (rainforest), frequently visited attraction	0 Bulk Samples	Not able to collect samples from suspected source
*16	Definite	Fibrotic	None	0	15	Workplace exposures (cleaning of homes and businesses)	0 Bulk Samples	Not able to collect samples from suspected source
17	Definite	Fibrotic	Farm	0	12	Garage/Barns (tractor hobbyist)	0 Bulk Samples	Sampling took place in winter months, likely not able to collect sufficient antigen.
*18	Probable	Fibrotic	None	3	23	Basement prior to mold remediation/School building	0 Bulk Samples	Not able to collect samples from suspected source
*03	Probable	Fibrotic	None	3	10	Garden	0 Bulk Samples	Not able to collect samples from suspected source (unable to access garden in winter months)
	Total (Fibrotic)	**15/19 (79%) Fibrotic**		**9/15 (60%) pos HP Panel**			**6/15 (40%) pos Home/ Workplace Sampling**	**9/15 (60%) Unable to collect samples from suspected source.**

(*continued on next page*)

Table 3 (*continued*)

ID	Likelihood of diagnosis	Type	Exposure suspected by physician	Std HP panel outcome (#Pos)	# bulk samples	Suspected antigen (home/ Wkplace visit)	Home/Workplace antigen collection (#pos)	Comments
02	Definite	Inflamm	None	1	6	Mold in Home	1 Bulk Sample 1 Antigen (Cladosporium)	
*05	Definite	Inflamm	None	0	10	Daughter's Home	0 Bulk samples	Not able to collect samples from suspected source
*06	Probable	Inflamm	None	2	11	Metalworking Fluids (Machinist)	0 Bulk samples	Not able to collect samples from suspected source
*19	Probable	Inflamm	Cardboard dust (work)/ Mold on Shed Roof	3	18	Possible cardboard dust (workplace), mold in home	0 Bulk samples	Not able to collect samples from suspected source
	Total (Inflamm)	**4/19 (21%) Inflamm**		**3/4 (75%) pos HP Panel**			**1/4 (25%) pos Home/ Workplace Sampling**	**3/4 (75%) Unable to collect samples from suspected source.**

* *Indicates patient likely not exposed at time of environmental sampling based on follow-up with referring physician.*

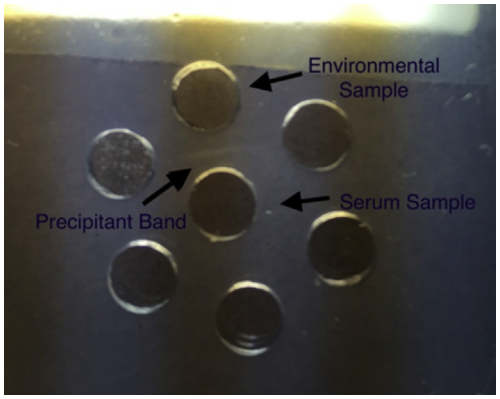

Fig. 1. Precipitant band indicating a positive immune response to a single antigen is shown in conjunction with placement of patient sera and discrete antigen(s) collected from the patient's environment.

Table 4
Subjects with one (or more) positive antigen result from samples collected during Home/Workplace Sampling and/or on the Standard HP Panel.

	Standard HP Panel		
Home/workplace antigen collection	Negative	Positive	Total
Negative (%total, n = 12)	6 (50.0%)	6 (50.0%)	**12 (63.2%)**
Positive (%total, n = 7)	1 (14.3%)	6 (85.7%)	**7 (31.2%)**
Total (%)	**7(36.8%)**	**12 (63.2%)**	**19 (100%)**

Swab samples were collected from doors and ledges capturing settled dusts that potentially contained antigen. Vacuum samples were taken from the areas of the carpet the patient noted were most heavily involved in the previous water damage.

The patient's serum reacted with one of the carpet vacuum samples from the basement. Antigen isolation and a second immunoassay determined that the patient reacted to *Cladosporium* spp. from the carpet sample.

The patient had all carpeting removed from the basement. In addition, he was advised as to common sources *Cladosporium* spp. The referring physician had submitted serum for an HP Avian Panel, to which the patient tested positive to pigeon proteins. While not routinely exposed to pigeons, he was advised to eliminate feather sources from his home (comforters, pillows, etc.).

Post remediation the patient successfully tapered off steroids and pulmonary function returned to normal.

5. Discussion

Identification of the etiologic agent of HP in standard medical care consists of a history taken by the clinician and measurement of

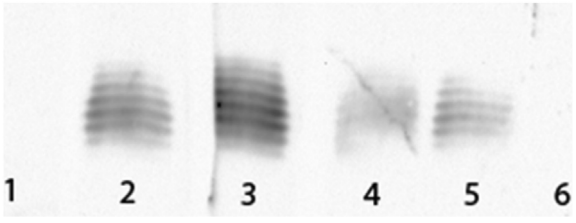

Fig. 2. Immunoblot showing reactivity of patient sera to an antigen preparation from a *Thermoactinomyces vulgaris* isolated from an environmental sample from patient #11 numbers). Patient sera used were: lane 1, #07; lane 2, #09; lane 3, #11; lane 4, #14; lane 5, #18; and lane 6, no primary antibody.

Table 5
Immunoblot, Immunodiffusion, and Standard HP Panel results for antigen (*Cladosporium, Penicillium*, and *Thermoactinomyces vulgaris*) in seven patients. 'N/A' indicates that the antigen was not part of the Standard HP Panel for that patient.

Antigen	ID	Immunoblot	Immunodiffusion	Standard HP Panel
Cladosporium	02	+	+	N/A
	07	+	+	N/A
	11	−	−	N/A
	18	−	−	+
Penicillium	01	+	+	+
	07	+	+	N/A
	18	−	−	+
Thermoactinomyces	09	+	+	−
	18	+	−	+
	11	+	−	−
	14	+	−	−
	07	−	−	−

IgG antibodies to a standard HP panel. This was a 'proof of concept' study to determine whether a site visit and sampling of a patient's home/workplace would be useful in identifying the etiologic agent of a patient's HP so as to better be able to provide advice on antigen avoidance. Seven of 19 (36.8%) subjects had positive IgG results to organisms collected during environmental sampling while 12 of 19 (63.2%) had positive IgG to the standard HP panel. Although there was a non-statistical increase in the likelihood that those individuals who tested positive on the standard HP panel would test positive to samples taken from their environment, 7 of 19 subjects had divergent results (Table 4). Even among the six subjects who reacted to both the standard panel and environmental samples, only one reacted to the same antigen.

Within the limited scope of this pilot study, it is difficult to say which of the results were of primary clinical importance in individuals testing positive to multiple antigens. However, when multiple antigens were identified in environmental samples, they were likely to come from the same or a similar source (i.e. isolated from bulk samples taken from a garden). Given this, we believe that a benefit to our 'patient-centered' environmental assessment was the feedback provided to referring physicians as to possible sources of exposures not identified during routine patient-provider discussions and/or to help interpret the results of the commercially available HP panel. One example includes the seasonal cutting/baling of hay (a 'hobby') in an individual where MWFs in the workplace were the incorrect suspected exposure of interest. The absence of positive results from the patient's environmental samples suggests that the antigen to which the patient tested positive on the standard panel may not currently be present in the patient's home/workplace environment. Conversely, a discrepancy between HP panel results and a positive antibody response to collected samples suggests they may be reacting to other/additional antigens that were not included on the 'standard' panel. Another benefit was the ability to discuss with patients the source(s) of offending antigen. Rather than discussing the need to 'stay away' from a specific antigen (i.e. *Cladosporium* spp.) positive on the 'standard' panel, this type of assessment allows for a practical discussion based on the antigen identified and *specific location* as to where the antigen(s) were found in the subjects' environment. The case described in detail in this paper clearly indicated the usefulness of the personalized sampling in identifying *Cladosporium* in the basement carpet of the patient.

Four of the six individuals in this study for which there was a positive result on the 'standard' HP panel that did not test positive on an environmental sample from their home/workplace were likely not still exposed to the offending antigen at the time sampling took place. The fact that antibodies may be present after exposure has ceased lends an additional layer of complexity. Conversely, one individual serving as a control tested positive to antigen on a standard HP panel with absence of disease. It is generally accepted that a positive result, although suggesting importance in the etiology of the patient's HP, may be a marker of exposure and not be involved in pathophysiology of the disease.

Most commercial HP panels use immunodiffusion techniques, and as such we chose this method for our assays. Discrepancies between our results and those from commercial panels could be due to differences in strains of bacteria and fungi used as antigens; in the patient serum samples used, which were often collected at different times; or the sensitivity of the assays. Therefore, we utilized a more sensitive technique, immunoblot, which allows visualization of multiple reactive antigens from a single bacterial or fungal isolate rather than a single precipitin band. For the fungi *Cladosporium* and *Penicillium*, results with immunoblots match those with immunodiffusion. However, with the bacterium *Thermoactinomyces*, immunoblots detected antibodies in three additional patients for whom *Thermoactinomyces* was not isolated from environmental samples (Table 5). We will continue to assess the utility of immunoblots as a tool to develop more sensitive assays.

Environmental sampling of the patient's environment has its own limitations. The source of the patients for this pilot study were tertiary referral centers, so at the time of recruitment, 90% of the subjects had symptoms for at least a year and 42% for over three years before environmental sampling was initiated (Table 2). This may explain why environmental sampling in the homes and workplaces in 12 of these 19 individuals (63%) did not identify an antigen for which the patient had antibodies. Of those 12 subjects, 9 were deemed (during the interview) 'likely to not still be exposed' to the offending antigen (i.e. one individual with a history of isocyanate exposure who had retired and one the sale of a visibly 'moldy' camper and cessation of routine traveling/camping using said camper). These findings were corroborated by the stability or improvement of their PFT's and HRCT, symptoms, and successful tapering and/or cessation of steroid therapy in those 9 patients (Table 3). This problem in antigen identification emphasizes the importance of early recognition of the etiologic antigen and assessment of the patient's environment in order to provide recommendations for exposure avoidance, thus halting disease progression. For the three individuals with active HP but for which environmental sampling was unsuccessful in isolating an antigen of interest, we hypothesize based on the temporal pattern of their symptoms that there was a seasonal component and that the environmental sampling took place in the winter when exposures would be expected to be highest in spring/summer (2 individuals), or the likely exposure of concern occurred in one individual routinely vacationing in a rainforest (Table 3). Although it would be possible for clinicians to obtain an extensive history, time constraints in clinical practice and the inability to observe the patient's environment, it is more likely that our evaluation - even when we could not collect a sample related to a possible exposure - would at a minimum facilitate collection of this detailed and potentially clinically useful information.

The environmental sampling would need to be streamlined, i.e. fewer samples, and reimbursed by insurance companies to become clinically useful. Insurance companies are paying for home visits for patients with asthma, and if we are able to prove cost-effectiveness through the early identification of antigen related to the subject's disease and provide meaningful exposure avoidance/elimination strategies, there could be substantial cost-savings related to the minimization of need for medication(s), hospitalizations, routine testing (PFT, HRCT), etc., as well as potentially a reduction of time away from work, and improved quality of life. We estimate that it takes five days from the date environmental sampling takes place

to initially determine whether a patient reacts to the sample(s) (bulk) collected, at which time clinical recommendations for exposure avoidance can be initiated and another 10–20 days to identify the discrete antigen(s) within the sample(s) to which the patient reacts.

Another limitation was that if testing was due to a low molecular weight chemical our environmental sampling and antibody testing could have identified such a substance in the bulk environmental samples, but we would have been unable to identify the specific substance in the bulk sample causing the positive response to the bulk sample. That said, this information is still useful as the source (location/characteristics) of the sample can be documented and the composition of the bulk sample investigated in terms of HP (i.e. isocyanate exposure).

Finally even when a positive response was identified to an environmental sample, further evaluation is needed to determine if the antigen is causing the inflammatory response responsible for disease or is a marker of exposure. Current practice is to advise the patient to cease exposure to the antigen and assess clinical progression/remission and radiographic and pulmonary function changes after removal from exposure. To assess additional approaches to determining the clinical significance of a positive immunodiffusion response to an environmental sample we plan to expand this study with a focus on subjects with acute onset of symptoms (within the previous 3 months) who are expected to be 'currently' exposed to the offending antigen as evidenced by continued symptoms and ongoing changes on PFT's and/or HRCT's. For research purposes, or if specific antigen challenge testing for HP ever became part of clinical practice [16], our environmental sampling and immunoassays could be the source for antigen used for this type of testing. In the absence of the availability of specific antigen challenge testing, a possible alternate approach to determine when a positive antibody response is a marker of exposure versus when it is an indication that the antigen is involved in the disease process is to determine if the antigen induces cell-proliferation, clonal expansion of antigen-specific T cells, and cytokine production. Successful identification of the etiologic antigen would likely be a powerful tool for physicians to treat their patients as not only the offending antigen would be identified, but the *source* of antigen (exposure) identified as well – optimizing suggestions for exposure avoidance to patients, potentially affording an opportunity for the patient to stabilize or reverse their disease.

Conflict of interest

The authors declare no conflict of interest.

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References

[1] J.N. Fink, H.G. Ortega, H.Y. Reynolds, Y.F. Cormier, L.L. Fan, T.J. Franks, et al., Needs and opportunities for research in hypersensitivity pneumonitis, Am. J. Resp. Crit. Care Med. 171 (2005) 792–798.
[2] Y. Cormier, Hypersensitivity pneumonitis, Lippincott-Raven, Philadelphia, 1998.
[3] M. Selman, A. Pardo, T.E. King, Hypersensitivity pneumonitis, Am. J. Respir. Crit. Care Med. 186 (4) (2012) 314–324.
[4] Y. Lacasse, M. Selman, U. Costabel, J.-C. Dalphin, M. Ando, F. Morell, et al., Clinical diagnosis of hypersensitivity pneumonitis, Am. J. Respir. Crit. Care Med. 168 (8) (2003) 952–958.
[5] L.C. Mohr, Hypersensitivity pneumonitis, Curr. Opin. Pulm. Med. 10 (2004) 401–411.
[6] T. Ismail, C. McSharry, G. Boyd, Extrinsic allergic alveolitis, Respirology 11 (2006) 262–268.
[7] M. Lopez, J.E. Salvaggio, Epidemiology of hypersensitivity pneumonitis/allergic alveolitis, Monogr. Allergy 21 (1987) 70–86.
[8] M. Ando, K. Hirayama, K. Soda, R. Okubo, S. Araki, T. Sasazuki, HLA-DQw3 in Japanese summer-type hypersensitivity pneumonitis induced by Trichosporon cutaneum, Am. Rev. Respir. Dis. 140 (4) (1989) 948–950.
[9] A. Aquino-Galvez, A. Camarena, M. Montano, A. Juarez, A.C. Zamora, G. Gonzalez-Avila, et al., Transporter associated with antigen processing (TAP) 1 gene polymorphisms in patients with hypersensitivity pneumonitis, Exp. Mol. Pathology 84 (2008) 173–177.
[10] J. Kokkarinen, H. Tukainen, E.O. Terho, Mortality due to farmer's lung in Finland, Chest 106 (2) (1994) 509–512.
[11] M. Solaymani-Dodaran, J. West, C. Smith, R. Hubbard, Extrinsic allergic alveolitis: incidence and mortality in the general population, Q. J. Med. 100 (2007) 233–237.
[12] M.B. Bang, D.N. Weissman, G.A. Pinheiro, V.C. Antao, J.M. Wood, G. Syamlal, Twenty-three years of hypersensitivity pneumonitis mortality surveillance in the United States, Am. J. Ind. Med. 49 (2006) 997–1004.
[13] E.O. Terho, Diagnostic criteria for farmer's lung disease, Am. J. Ind. Med. 10 (3) (1986), 329–29.
[14] H.B. Richerson, I.L. Bernstein, J.N. Fink, G.W. Hunninghake, H.S. Novey, C.E. Reed, et al., Guidelines for the clinical evaluation of hypersensitivity pneumonitis: report of the subcommittee on hypersensitivity pneumonitis, J. Allergy Clin. Immunol. 84 (5, Part 2) (1989) 839–844.
[15] M. Schuyler, Y. Cormier, THe diagnosis of hypersensitivity pneumonitis, Chest J. 111 (3) (1997) 534–536.
[16] X. Muñoz, M. Sánchez-Ortiz, F. Torres, A. Villar, F. Morell, M.-J. Cruz, Diagnostic yield of specific inhalation challenge in hypersensitivity pneumonitis, Eur. Respir. J. 44 (6) (2014) 1658–1665.
[17] J.S. Vourlekis, M.I. Schwarz, R.M. Cherniack, D. Curran-Everett, C.D. Cool, R.M. Tuder, et al., The effect of pulmonary fibrosis on survival in patients with hypersensitivity pneumonitis, Am. J. Med. 116 (10) (2004) 662–668.
[18] G. Raghu, H.R. Collard, J.J. Egan, F.J. Martinez, J. Behr, K.K. Brown, et al., An official ATS/ERS/JRS/ALAT Statement: Idiopathic pulmonary fibrosis: evidence-based guidelines for diagnosis and management, Am. J. Respir. Crit. Care Med. 183 (6) (2011) 788–824.
[19] E.J. Hapke, R.M. Seal, G.O. Thomas, M. Hayes, J.C. Meek, Farmer's lung. A clinical, radiographic, functional, and serological correlation of acute and chronic stages, Thorax 23 (5) (1968) 451–468.
[20] Y. Lacasse, M. Selman, U. Costabel, J-c Dalphin, F. Morell, R. Erkinjuntti-pekkanen, et al., Classification of hypersensitivity pneumonitis, Int. Archives Allergy Immunol. 149 (2) (2009) 161–166.
[21] Y. Ohtani, S. Saiki, M. Kitaichi, Y. Usui, N. Inase, U. Costabel, et al., Chronic bird fancier's lung: histopathological and clinical correlation. An application of the 2002 ATS/ERS consensus classification of the idiopathic interstitial pneumonias, Thorax 60 (8) (2005) 665–671.
[22] J.J. Barboriak, A.J. Sosman, C.D. Reed, Serological studies in pigeon breeders disease, J. Lab. Clin. Med. 65 (4) (1964) 600–604.
[23] J.N. Fink, Hypersensitivity pneumonitis, J. Allergy Clin. Immunol. 74 (1) (1984) 1–9.
[24] B. Lowe, T. Marsh, N. Isaacs-Cosgrove, R. Kirkwood, M. Kiupel, M. Mulks, Microbial communities in the tonsils of healthy pigs, Vet. Microbiol. 147 (3–4) (2011) 346–357.
[25] J. Cole, B. Chai, R. Farris, Q. Wang, S. Kulam, D. McGarrell, et al., The ribosomal database project (RDP-II): sequences and tools for high-throughput rRNA analysis, Database, Nucleic Acids Res. 33 (2005) D294–D296.
[26] J. Cole, Q. Wang, E. Cardenas, J. Fish, B. Chai, R. Farris, et al., The Ribosomal Database Project: improved alignments and new tools for rRNA analysis, Nucleic Acids Res. 37 (2009) D141–D145.
[27] W.T. Cruz, Y.A. Nedialkov, B.J. Thacker, M.H. Mulks, Molecular characterization of a common 48-kilodalton outer membrane protein of Actinobacillus pleuropneumoniae, Infect. Immun. 64 (1) (1996) 83–90.
[28] P. Dawkins, A. Robertson, W. Robertson, V. Moore, J. Reynolds, G. Langman, et al., An outbreak of extrinsic alveolitis at a car engine plant, Occup. Med. 56 (8) (2006) 559–565.
[29] J. Milanowski, J. Dutkiewicz, H. Potoczna, L. Kus, B. Urbanowicz, Allergic alveolitis among agricultural workers in Eastern Poland: a study of twenty cases, Ann. Agric. Environ. Med. 5 (1998) 31–43.
[30] D.I. Bernstein, Z.L. Lummus, G. Santilli, J. Siskosky, I.L. Bernstein, Machine operator's lung: a hypersensitivity pneumonitis disorder associated with exposure to metalworking fluid aerosols, Chest J. 108 (3) (1995) 636–641.
[31] A. Tripathi, L.C. Grammer, Extrinsic allergic alveolitis from a proteolytic enzyme, Ann. Allergy Asthma & Immunol. 86 (4) (2001) 425–427.
[32] C.L. Johnson, I.L. Bernstein, J.S. Gallagher, P.F. Bonventre, S.M. Brooks, Familial hypersensitivity pneumonitis induced by Bacillus subtilis, Am. Rev. Respir. Dis. 122 (2) (1980;August) 339–348.
[33] D. Fishwick, P. Tate, J. Elms, E. Robinson, B. Crook, F. Gallagher, et al., Respiratory symptoms, immunology and organism identification in contaminated

metalworking fluid workers. What you see is not what you get, Occup. Med. 55 (3) (2005) 238–241.
[34] T. Takahashi, M. Munakata, Y. Ohtsuka, A. Satoh-Kamachi, R. Sato, Y. Homma, et al., SErum kl-6 concentrations in dairy farmers, Chest J. 118 (2) (2000) 445–450.
[35] R. Erkinjuntti-Pekkanen, J.I. Kokkarinen, H.O. Tukiainen, M. Reiman, E.O. Terho, IgG antibodies, chronic bronchitis, and pulmonary function values in farmer's lung patients and matched controls, Allergy 54 (11) (1999) 1181–1187.
[36] K. Konishi, S.-I. Murakami, K. Kokubu, H. Itoh, T. Mouri, M. Tamura, Determination by enzyme-linked immunosorbent assay (ELISA) of specific IgG antibody activities for diagnosis of farmer's lung disease, Tohoku J. Exp. Med. 147 (2) (1985) 135–144.
[37] F.L. Babbott Jr., D.W. Gump, D.L. Sylwester, B.V. MacPherson, R.C. Holly, Respiratory symptoms and lung function in a sample of vermont dairymen and industrial workers, Am. J. Public Health 70 (3) (1980) 241–245.
[38] Y. Cormier, J. Belanger, P. Durand, Factors influencing the development of serum precipitins to farmer's lung antigen in Quebec dairy farmers, Thorax 40 (2) (1985) 138–142.
[39] Z.U. Khan, M.D. Richardson, D.W. Warnock, Rapid enzyme-linked immunosorbent assay (ELISA) for the detection of IgG antibodies against micropolyspora faeni and thermoactinomyces vulgaris, Clin. Allergy 14 (3) (1984) 295–305.
[40] E.O. Terho, P. Lindstrom, R. Mantyjarvi, H.O. Tukiainen, O. Wager, Circulating immune complexes and rheumatoid factors in patients with farmer's lung, Allergy 38 (5) (1983) 347–352.
[41] E.O. Terho, K. Husman, M. Kotimaa, T. Sjoblom, Extrinsic allergic alveolitis in a sawmill worker. A case report, Scand. J. Work Environ. Health 6 (2) (1980) 153–157.
[42] L. Romeo, K. Dalle Molle, G. Zanoni, A. Peretti, G. Marangi, L.G. Conrado, et al., Respiratory health effects and immunological response to thermoactinomyces among sugar cane workers in Nicaragua, Int. J. Occup. Environ. Health 15 (3) (2009) 249–254.
[43] M. Gangwar, Z.U. Khan, S.N. Gaur, H.S. Randhawa, Occurrence and significance of precipitating antibodies against thermophilic actinomycetes in the sera of dairy herd workers, Nangali, Delhi, Ant. Van Leeuwenhoek 59 (3) (1991) 167–175.
[44] Y.E. Shen, V.P. Kurup, J.N. Fink, Circulating antibodies against thermophilic actinomycetes in farmers and mushroom workers, J. Hyg. Epidemiol. Microbiol. Immunol. 35 (3) (1991) 309–316.
[45] T. Ojanen, E.O. Terho, H.O. Tukiainen, R. Mantyjarvi, Class-specific antibodies during follow-up of patients with farmer's lung, Eur. Respir. J. 3 (3) (1990) 257–260.
[46] H. Thorshauge, I. Fallesen, P.A. Ostergaard, Farmer's lung in infants and small children, Allergy 44 (2) (1989) 152–155.
[47] R. Mantyjarvi, V.P. Kurup, Dot-immunobinding assay in the detection of IgG antibodies against farmer's lung antigens, Mycopathologia 103 (1) (1988) 49–54.
[48] V.P. Kurup, R. Mantyjarvi, E.O. Terho, T. Ojanen, J.H. Kalbfleisch, Circulating IgG antibodies against fungal and actinomycete antigens in the sera of farmer's lung patients from different countries, Mycopathologia 98 (2) (1987) 91–99.
[49] E.O. Terho, K. Tupi, I. Vohlonen, K. Husman, Serum precipitins against microbes in mouldy hay with respect to the geographical location of the farm and to the work of farmers, Eur. J. Respir. Dis. Suppl. 152 (1987) 128–138.
[50] Y. Homma, T. Terai, M. Matsuzaki, Incidence of serum-precipitating antibodies to farmer's lung antigens in Hokkaido, Respiration 49 (4) (1986) 300–306.
[51] Z. Watanuki, S. Okada, S. Chiba, K. Kamei, Y. Suzuki, N. Yamada, Increased prevalence of high anti-*Cladosporium* antibody titers in interstitial lung diseases, Tohoku J. Exp. Med. 226 (4) (2012) 287–291.
[52] S. Chiba, S. Okada, Y. Suzuki, Z. Watanuki, Y. Mitsuishi, R. Igusa, et al., *Cladosporium* species-related hypersensitivity pneumonitis in household environments, Intern. Med. 48 (5) (2009) 363–367.
[53] N. Akizuki, N. Inase, N. Ishiwata, Y. Jin, K. Atarashi, M. Ichioka, et al., Hypersensitivity pneumonitisamong workers cultivating *tricholoma conglobatum* (Shimeji), Respiration 66 (3) (1999) 273–278.
[54] R.L. Jacobs, R.E. Thorner, J.R. Holcomb, L.A. Schwietz, F.O. Jacobs, Hypersensitivity pneumonitis caused by cladosporium in an enclosed hot-tub area, Ann. Intern. Med. 105 (2) (1986), 204–04.
[55] K. Reijula, S. Sutinen, T. Tuuponen, R. Lahti, P. Karkola, Pulmonary fibrosis, with sarcoid granulomas and angiitis, associated with handling of mouldy lichen, Eur. J. Respir. Dis. 64 (8) (1983) 625–629.
[56] R. Merget, I. Sander, P. Rozynek, M. Raulf-Heimsoth, T. Bruening, Occupational hypersensitivity pneumonitis due to molds in an onion and potato sorter, Am. J. Ind. Med. 51 (2) (2008) 117–119.
[57] S.K. Lee, S.S. Kim, D.H. Nahm, H.S. Park, Y.J. Oh, K.J. Park, et al., Hypersensitivity pneumonitis caused by Fusarium napiforme in a home environment, Allergy 55 (12) (2000) 1190–1193.
[58] J. Bickhardt, P. Kunze, A. Rolle, A. Matthiessen, Histologically proven extrinsic allergic alveolitis with severe obstructive pulmonary emphysema, Eur. J. Med. Res. 5 (3) (2000) 127.
[59] S. Lappalainen, A.-L. Pasanen, M. Reiman, P. Kalliokoski, Serum IgG antibodies against wallemia sebi and fusarium species in Finnish farmers, Ann. Allergy, Asthma & Immunol. 81 (6) (1998) 585–592.
[60] X. Baur, G. Richter, A. Pethran, A.B. Czuppon, M. Schwaiblmair, Increased prevalence of IgG-induced sensitization and hypersensitivity pneumonitis (humidifier lung) in nonsmokers exposed to aerosols of a contaminated air conditioner, Respiration 59 (4) (1992) 211–214.
[61] A. Tiotiu, C. Metz-Favre, G. Reboux, R. Kessler, F. de Blay, Hypersensitivity pneumonitis related to penicillium chrysogenum and mesophilic streptomyces: the usefulness of the medical indoor environment councelor (MIEC), Rev. Pneumol. Clin. 69 (5) (2013) 278–282.
[62] F. Morell, M.J. Cruz, F.P. Gomez, F. Rodreguez-Jerez, A. Xaubet, X. Munoz, Chacinero's lung - hypersensitivity pneumonitis due to dry sausage dust, Scand. J. Work Environ. Health 37 (4) (2011) 349–356.
[63] S. Yoshikawa, K. Tsushima, M. Yasuo, K. Fujimoto, K. Kubo, T. Kumagai, et al., Hypersensitivity pneumonitis caused by Penicillium citrinum, not enoki spores, Am. J. Ind. Med. 50 (12) (2007) 1010–1017.
[64] S. Yoshikawa, K. Tsushima, T. Koizumi, K. Kubo, T. Kumagai, Y. Yamazaki, Hypersensitivity pneumonitis induced by spores of *penicillium citrinum* in a worker cultivating enoki mushroom, Intern. Med. 45 (8) (2006) 537–541.
[65] J.C. Winck, L. Delgado, R. Murta, M. Lopez, J.A. Marques, Antigen characterization of major cork moulds in suberosis (cork worker's pneumonitis) by immunoblotting, Allergy 59 (7) (2004) 739–745.
[66] K. Nakagawa-Yoshida, M. Ando, R.I. Etches, J.A. Dosman, Fatal cases of farmer's lung in a canadian family: probable new antigens, penicillium brevicompactum and p olivicolor, Chest J. 111 (1) (1997) 245–248.
[67] P.A. Greenberger, Mold-induced hypersensitivity pneumonitis, Allergy Asthma Proc. 25 (4) (2004) 219–223.
[68] J.A. Alvarez-Fernandez, S. Quirce, J.L. Calleja, M. Cuevas, E. Losada, Hypersensitivity pneumonitis due to an ultrasonic humidifier, Allergy 53 (2) (1998) 210–212.
[69] N. Akizuki, N. Inase, N. Ishiwata, Y. Jin, K. Atarashi, M. Ichioka, et al., Hypersensitivity pneumonitis among workers cultivating Tricholoma conglobatum (shimeji), Respiration 66 (3) (1999) 273–278.
[70] H.G. Grieble, J.W. Rippon, N. Maliwan, V. Daun, Scopulariopsosis and hypersensitivity pneumonitis in an addict, Ann. Intern. Med. 83 (3) (1975) 326–329.