

Respiratory and immunological reactions among Shiitake (*Lentinus edodes*) mushroom workers

J. SASTRE, M. D. IBANEZ, M. LOPEZ and S. B. LEHRER*

Section of Allergy and Clinical Immunology, Department of Medicine, Tulane University Medical School, New Orleans, Louisiana, U.S.A.

Summary

Four workers, the total work force employed at a Shiitake farm, developed cough and sputum production following a variable period of exposure to Shiitake mushrooms. All four had abnormal diffusing capacity and three had abnormal spirometry values. Chest roentgenograms demonstrated an interstitial pattern in one worker. Pulmonary function tests performed before and during several days of work demonstrated a significant decrease ($>20\%$) in forced vital capacity (FVC) and/or maximal mid-expiratory flow (MMEF) in three workers. Although specific antibodies to an extract of Shiitake spores were detected in sera from three workers none were IgE. High levels of Shiitake spores were detected in growing rooms ($>10^6/\text{m}^3$) as well as other locations at the farm. Shiitake spore airborne antigen, detected by an immunochemical assay, was present in dust collected with a volumetric sampler from different locations at the farm. Antigenic determinants of Shiitake spore antigens, in common with antigens from other cultivated mushrooms (*Agaricus* and *Pleurotus*) were demonstrated by ELISA inhibition assay. This study demonstrates that workers exposed to high levels of Shiitake spores develop symptoms and laboratory findings suggestive of hypersensitivity pneumonitis (HP). Strict environmental control and the wearing of a face mask is probably needed to reduce the high risk of sensitization and possible development of immunological lung disease. Shiitake spores must be considered as an aetiological agent of mushroom workers' lung.

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Introduction

The mushroom industry is expanding in many countries of the world. At the present time, *Agaricus bisporus* (Lange) Imbach (= *A. brunescens* Peck) is the only mushroom produced in the U.S.A. in significant quantities. However, there is increasing interest in diversification of the industry with the introduction of new mushrooms [1]. Production of *Lentinus edodes* (Berk) Sing. commonly called Shiitake or Japanese mushroom, has increased in the U.S.A. due to its excellent culinary properties. This gilled mushroom has generally been grown outdoors in logs inoculated with mycelium or indoors under more controlled conditions in sawdust, wood chips or other plant debris in the absence of compost. Recently, indoor cultivation, which has the advantage of producing Shiitake throughout the year, has

been used to increase yields greatly through the manipulation of substrate and environment.

It has been reported that inhalation of basidiospores may induce immunological lung diseases, such as allergic rhinitis, asthma and hypersensitivity pneumonitis (HP) [2–6]. When cultivating mushrooms such as *Pleurotus*, *Agaricus* or Shiitake, spores are released into the atmosphere and inhaled by workers. In particular, *Pleurotus* and Shiitake release tremendous numbers of spores into the atmosphere, because they have no veil; thus spores are released during cap growth prior to attaining a marketable size. Hypersensitivity pneumonitis due to spores of cultivated mushrooms, such as *Pleurotus* 'florida' or *Agaricus bisporus* has been described [6,9] and reported to cause mushroom workers' lung [9].

In Japan and China, where Shiitake is extensively cultivated both outdoors and indoors, up to 9% of the exposed workers developed asthmatic symptoms [8]. In some cases skin tests and inhalation challenge tests have

* To whom correspondence should be addressed.

been positive [7,8]. In this report we describe respiratory and immunological findings in workers employed at a farm where Shiitake mushroom are cultivated indoors, under controlled conditions.

Materials and methods

Spore extract preparation. Spore collection and extract preparation have been described previously [2]. Briefly, caps from *Agaricus bisporus*, *Pleurotus ostreatus* (Jacq.: Fr) Kumm (Oyster mushroom) and *Lentinus edodes*, harvested from different mushroom farms, were placed gills down on, and covered with, Whatman no. 1 filter paper for 24–48 hr. The resulting spore prints were scraped from the paper. Purity of each spore sample as determined by light microscopy was at least 98%.

Prior to extraction spores were defatted with ethyl ether (100 ml per g spores). One gram of defatted spores was homogenized in a Braun homogenizer in 20 ml 0.125 M ammonium bicarbonate buffer, pH 8. The homogenate was centrifuged (48 000 g), the supernatant lyophilized and stored at room temperature under desiccation. Protein determination for each antigen extract was performed according to the method of Lowry *et al.* [10].

Environmental study. The atmosphere was sampled with a Rotorod sampler (Ted Brown Ass., Los Angeles, U.S.A.). This device was placed at a height of 180 cm in different locations of the farm (growing rooms, hallway and packing area) on different days and for different periods of time. For identification, spores were stained with lactophenol cotton blue, and rods of the sampler were examined by light microscopy. The number of spores per m³ was calculated as recommended by the manufacturer with a correction of efficiency for particles of less than 10 µm.

For determination of Shiitake airborne antigen, the volumetric air sampler, Air Sentinel [11] was used. The fibre glass filter (GMW 810, General Metal Works, Redwood City, U.S.A.) retains particles of 0.3 µm or larger. Sampling was performed in the same areas and for the same time periods as with the Rotorod sampler. The air flow was 260 m³/24 hr. Following sampling, air filters from Air Sentinel were cut and extracted for 24 hr with 15 ml of 0.125 M NH₄HCO₃ at 4°C under constant shaking. The extract was centrifuged (10 000 g) and the supernatant was frozen at -20°C. These extracts were assayed for antigen content at several dilutions (1/1 to 1/10 000) in an ELISA inhibition, the sera pool used was from three workers with antibodies to Shiitake spores as determined by ELISA and precipitation.

Mushroom processing. The mushrooms were cultivated in artificial logs made of compressed wood chips. Logs were

inoculated with mycelium and stored on shelves in closed rooms with humidity and temperature artificially controlled. After harvesting, mushrooms were selected and packeted in an adjacent room. An organo-phosphate insecticide (vapone) was automatically sprayed into the growing rooms which were kept closed for at least 18 hr.

Study Population. The farm had been in operation for 8 months with a total workforce of five. At the time of study four workers were employed at the farm. Two months prior to the study one worker left his job with symptoms of cough and sputum production as reported by a local physician. All workers were involved in harvesting and packing of mushrooms. All gave informed consent prior to participation in the study.

Workers 1 and 2 were employed for 8 months. During the first 3 months, they worked without a face mask. Worker 3 was employed for 6 months and worked without a mask for only 1 month. For the next 3 months, all three workers wore a 8710 Dust and Mist respirator (3M, St Paul, U.S.A.) during the harvesting process. Finally, for the last 2 months workers 1, 2 and 3 wore a fitted face mask with two cartridges, each containing a filter cover and a Type F filter for dust and mist (MSA, Pittsburg, U.S.A.). Worker 4, employed for a total of 4 months, wore the first mask for 2 months and second mask for the subsequent 2 months.

Double diffusion (DD). The DD test for precipitating antibodies was performed according to the method of Ouchterlony [12]. Fifty microlitres of antigen and/or serum were added to opposite wells of 1% agarose plates and incubated for 72 hr at room temperature. Gels were monitored at 24, 48 and 72 hr. Gels were washed with 5% sodium citrate for 72 hr, washed with phosphate-buffered saline (PBS), dried and stained with Coomassie brilliant blue. The following antigens: *Micropolyspora faeni*, *Thermoactinomyces vulgaris*, *T. candidus*, *T. viridans*, *Criptomastix corticale*, *Aspergillus fumigatus*, *A. niger*, *A. flavus* (Greer Lab., Lenoir, U.S.A.) were tested at 20 mg/ml; *L. edodes*, *P. ostreatus* and *A. bisporus* (prepared in our laboratory) were tested at 10 mg/ml.

Enzyme linked immunosorbent assay. IgG antibodies were detected by the microplate method of Voller *et al.* [13] and the double-antibody assay of Sepulveda *et al.* [14] with some modifications. Wells of the microtiter plates (Immulon 1, Dynatech Labs, Chantilly, U.S.A.) were coated with antigen, 100 µl/well at 0.1 mg/ml in carbonate buffer (pH 9.6) overnight at 4°C as determined previously by checkerboard titration using known negative and positive sera. Plates were then incubated with a solution of 1% bovine serum albumin (BSA) in PBS for 1 hr and washed

three times with 0.05% Tween 20 in PBS. Sera from the subjects (100 μ l), at 1:250 in 1% BSA-PBS, was added in duplicate to each well, and incubated for 60 min at room temperature. After washing three times with PBS-Tween buffer, 100 μ l antihuman IgG (0.1 mg/ml) labelled with peroxidase (Kirkegaard & Perry Labs., Gaithersburg, U.S.A.) was added and incubated for 1 hr. Plates were washed as previously before adding the substrate hydrogen peroxide and 100 μ l 2,2'-azino-di-3-ethyl-benzthiazoline sulphonic acid (ABTS). The reaction was stopped by the addition of 50 μ l of 3 N NaOH. Absorbance at 690 nm was measured on a BIO-TEK model EL 307 spectrophotometer (Vermont, U.S.A.). The spectrophotometer was adjusted to zero for each plate with the absorbance produced by using PBS instead of serum.

The dose-response curve of a positive serum demonstrated maximal binding of antibodies between 1/1 and 1/500 dilutions and reached the value of the negative control at 1/5000 serum dilution.

Twenty-five sera of normal unexposed laboratory workers of the Tulane University Hospital had an optical density (OD) of 216 ± 60 (mean $\pm$ s.d.). None of these sera contained precipitating antibodies to Shiitake spore antigens. Serum IgG, IgA and IgM concentrations were within normal values. An optical density of an individual sera exceeding the mean + 2 s.d. of that for normal individuals was regarded as positive.

ELISA inhibition. A sera pool was prepared from equal volumes of three individual sera, selected on the basis of elevated ELISA values and positive DD. Fifty microlitres of pooled serum and 50 μ l increasing concentrations (0.001, 0.01, 0.1, 1 and 10 mg/ml) of inhibiting antigen, peanut extract (negative control) or PBS were simultaneously added to duplicate wells coated with *L. edodes* antigen. For evaluation of results, the concentration of extract inhibiting ELISA by 50% (IC-50) was determined. Regression lines and equations were calculated and compared [15]. Antigenic potency of the extract of Air Sentinel filters was calculated in terms of ELISA inhibition units (EIU) where: $\text{EIU/ml} = 1/\text{IC-50}$.

Radioallergosorbent test (RAST). Shiitake spore extract at 25 mg/ml or human serum albumin (HSA; Sigma, St Louis, U.S.A.) at 10 mg/ml were coupled to cyanogen bromide-activated paper discs as described by Ceska *et al.* [16]. The RAST was performed as described previously [17]. The percentage of total [^{125} I]anti-IgE added that bound to spore-coated discs was calculated by dividing spore c.p.m. by total c.p.m. added, $\times 100$. The positive control, serum from a patient allergic to other basidiospores as demonstrated by positive RAST, skin test and bronchial challenge, bound 10% of the added label and yielded at RAST ratio of 8.4.

Total IgE. Serum IgE levels were measured by Kallestad Quanti Clone kit using ^{125} I-labelled anti-IgE (Kallestad, Austin, U.S.A.).

Skin-prick test. Prick tests were performed with 16 common aero-allergens (Hollister-Stier, Spokane, U.S.A.), *L. edodes*, *P. ostreatus* and *A. bisporus* (10 mg/ml in 50% glycerol-PBS). Tests were read at 15 min, 6 and 24 hr.

Pulmonary function testing. Spirometric measurements were carried out at work using a Jones Datamite III spirometer (Oak Brook, U.S.A.). Each subject performed three technically satisfactory forced expiratory manoeuvres, and the best values were used for analyses. Normal predicted values were those of Ruanton *et al.* [18]. Diffusing capacity was measured by the single breath method and expressed as a percentage of predicted values and 95% confidence limits. Normal values were those of Crapo *et al.* [19].

Spirometric follow-up in the work place. Spirometry was obtained prior to, and following, several days away from work. During the first day back at work, peak flow was recorded hourly with a portable peak flow meter during the shift and following work until bed time. Subsequent days pre, mid- and post-shift spirometry was determined.

Results

Description of cases. The clinical and laboratory data of workers are summarized in Table 1 and their pulmonary function tests and radiographical findings in Table 2. Only worker 2 had a relevant occupational history prior to employment at the farm. This individual worked for 27 years in the steel industry and 5 years ago had a documented 25% loss of forced vital capacity (FVC). All workers noticed that respiratory symptoms tended to improve following 1 or 2 days away from work but did not completely resolve. Although workers wore face masks during harvesting operations, their symptoms persisted at the time of this study. None had a family (as determined by questionnaire) or personal (as determined by absence of elevated serum IgE levels and skin-prick reactivity to common aero-allergens) history of allergy or atopy (Table 1).

Natural challenge in the work place. After several days away, workers returned to work without wearing a mask for the first day. There was no significant difference in pre-/post-shift spirometric values, except in worker 3, who had a decrease of 19% in the peak flow at 6 hr, which persisted for 24 hr. Workers continued for several days working in the area wearing a face mask during

Table 1. Summary of clinical and laboratory findings in Shiitake farm workers

Worker	Symptoms	Blood/sputum eosinophilia	Skin test	Total IgE	RAST	ELISA*	DD results
No. 1, male (64 yr)	Cough, sputum, s.o.b	No	+ <i>D. farinae</i>	12 IU/ml	—	1025	Shiitake
No. 2, male (50 yr)	Cough, sputum, s.o.b.	No	All (—)	7 IU/ml	—	820	Shiitake
No. 3, female (31 yr)	Cough, sputum, wheezing, s.o.b.	No	All (—)	9 IU/ml	—	810	Shiitake
No. 4, male (17 yr)	Cough, sputum	No	+ <i>D. farinae</i>	7 IU/ml	—	327	All (—)

* Mean OD of 25 normal individuals was 216 ± 60 .

—, Negative.

s.o.b. Shortness of breath.

Table 2. Pulmonary function tests and radiological data of workers

	Workers			
	1	2	3	4
FVC (l)	3.19 (65)	2.26 (52)	3.69 (91)	5.66 (101)
FEV ₁ (l)	2.43 (72)	1.71 (54)	3.06 (96)	4.12 (79)
FEV ₁ /FVC	76%	77%	82%	72%
MMEF (l/sec)	2.02 (66)	1.41 (41)	3.15 (88)	3.24 (65)
PEFR (l/sec)	5.18 (56)	5.38 (62)	5.82 (87)	7.45 (75)
After inhaling bronchodilator	NV*	NV	FEV ₁ + 14%	NV
DLCO _{Hgb}	21.48 (62)†	11.90 (38)†	26.16 (80)†	30.59 (66)†
KCO _{Hgb}	3.37 (72)†	2.68 (52)†	4.19 (66)†	4.11 (67)†
Smoker	No‡	No‡	Yes	Yes
Chest X-ray	Minimal increase in interstitial markings	Diffuse interstitial disease in both lungs	Minimal increase in interstitial markers	Normal

* No variation (<10%).

† Value under 95% confidence limits of normal values.

‡ Never smokers.

Values in parentheses are percentages of predicted values.

DLCO_{Hgb}, diffusing capacity, haemoglobin corrected.

KCO, DLCO/aleolar volume, haemoglobin corrected.

Table 3. Work place challenge: percentage difference in spirometric values on the first and last day of work

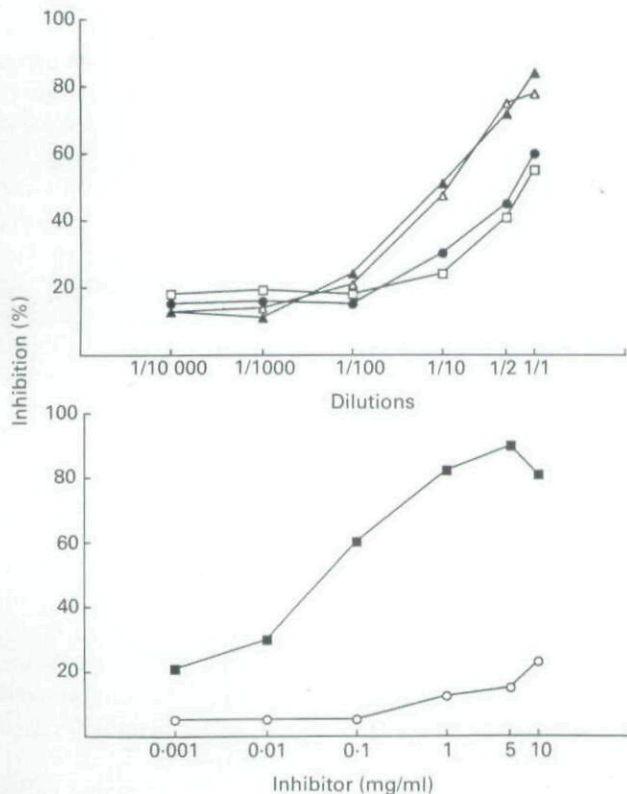
Worker	FVC	FEV ₁	FEV ₁ /FVC	MMEF	PEFR	Number of days
1	-26	-28	-2	-37	-16	5
2	-8	-3	4	5	-5	5
3	-11	-15	-3	-27	13	3
4	-4	-10	-4	-22	-27	3

harvesting operations, the difference between the best spirometric values the first and last day at work are summarized in Table 3.

Environmental study. The concentration of Shiitake spores in the growing room was greater than $10^6/\text{m}^3$. The exact number could not be determined due to the saturation of spores on the rods of the Rotorod sampler. As shown in Table 4 the concentration of Shiitake spores in the packing area was $7500/\text{m}^3$ and in the hallway of the growing rooms was $1500/\text{m}^3$. Spores other than Shiitake

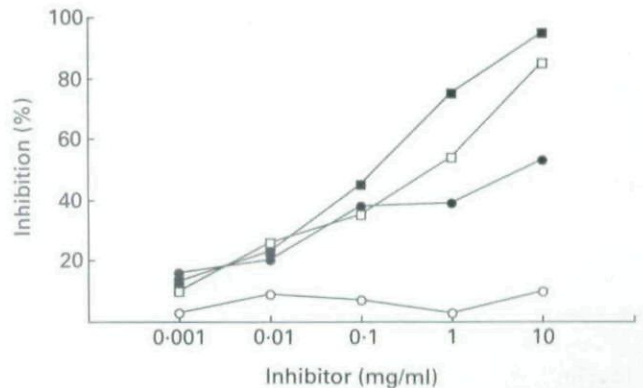
Table 4. Concentration of Shiitake spores at different farm locations

Growing room	$> 10^6/\text{m}^3$
Packing room	$7500/\text{m}^3$
Hallway	$1500/\text{m}^3$

**Fig. 1.** ELISA inhibition curve for *L. edodes* using filter extracts of the volumetric sampler (a) from (□) the hallway, (●) the packing area, (Δ) the growing room (9 hr), and (▲) the growing room (24 hr). (b) For (■) *L. edodes* and (○) peanut extract.

on the Rotorod sampler were few, corresponding to less than $25/\text{m}^3$. The mean size and shape of *L. edodes* spores was $6.8 \mu\text{m} \times 3.4 \mu\text{m}$. Shiitake spores are therefore clearly respirable.

Protein content of extracts from Air Sentinel filters following 24-hr exposure was $120 \mu\text{g}/\text{ml}$ in growing rooms, $38 \mu\text{g}/\text{ml}$ in the packing area and $12.5 \mu\text{g}/\text{ml}$ in the hallway. The 24-hr Air Sentinel filter extract from the growing rooms produced a IC-50 of Shiitake ELISA at a dilution of 1/10, in the packing area at 1/1.7 and in the hallway at 1/1.3 (Fig. 1). When expressed as EIU, 24-hr samples from the growing rooms contained 10 EIU ($260/\text{m}^3$), 9-hr samples also contained 10 EIU ($97.5/\text{m}^3$), and 5-hr samples contained 1.72 EIU ($54/\text{m}^3$). Apparently the 24-hr samples are under-estimating Shiitake antigen

**Fig. 2.** Inhibition of *L. edodes* ELISA with basidiospore, *L. edodes* (■), *A. bisporus* (□), *P. ostreatus* (●), and peanut extract (○).

levels due to a saturation effect. Eluate from the packing area 24-hr sample contained 1.72 EIU and from the hallway 1.3 EIU. A Shiitake spore extract at 0.05 mg/ml produced 25 EIU. These findings support the results of Shiitake spore levels determined by the Rotorod sampler (Table 3).

Crossreactivity of spore antigens. Protein concentrations of the spore extracts tested were $18 \mu\text{g}/\text{ml}$ for *L. edodes*; $13 \mu\text{g}/\text{ml}$ for *P. ostreatus* and $8 \mu\text{g}/\text{ml}$ for *A. bisporus*. Both *Agaricus* and *Pleurotus* spore extracts exhibited significant inhibitory activity of the Shiitake ELISA (Fig. 2). *Agaricus* gave IC-50 at a concentration of 0.6 mg/ml and a maximal inhibition of 77%. *Pleurotus* gave IC-50 at 1.6 mg/ml and a maximal inhibition of 52%. Analysis of linear regression lines of Shiitake and *Agaricus* ELISA inhibition demonstrated statistically equivalent slopes and the same intercepts. Similar results were obtained by comparing regression lines and intercepts of Shiitake with *Pleurotus*. These findings suggest the presence of common antigenic determinants in these mushrooms. Peanut extract, a negative control, did not demonstrate any inhibitory activity.

Discussion

Workers in the mushroom growing industry are frequently cited as being at risk of developing immunologically mediated lung disease [5–9]. Of the studies reported in the literature, most have been concerned with workers who grow *Agaricus* mushrooms. It has been reported that the majority of these workers are sensitized to actinomycetes and spores of other fungi growing in the compost rather than those from *Agaricus* mushrooms. This is probably due to the fact that these mushrooms are harvested prior to the veil being broken, thus minimizing spore release. More recently, other mushrooms have been

introduced to the U.S.A. and are becoming quite popular. In particular, *Pleurotus* and the Shiitake mushrooms are being grown more frequently for a developing market. These mushrooms have no veil and thus spores are released during cap growth prior to caps attaining marketable size. Shiitake mushrooms in particular release tremendous numbers of spores into the atmosphere.

Our study of workers at the Shiitake farm demonstrated that all workers develop cough and sputum production after a variable exposure to Shiitake mushrooms. A fifth worker, who left his job 2 months prior to the study for unrelated reasons, also developed similar respiratory symptoms. Thus, the incidence of respiratory symptoms at the farm is extremely high.

In three out of four workers studied, a significant specific immunological response to Shiitake spores was present. The intensity of the immunological response in each worker seems to be related to the duration of exposure to Shiitake spores. An IgE-mediated response to the spores was not demonstrated in our study in contrast to that reported by other authors [8,9]. Perhaps the absence of an IgE response to the spores is due to the absence of an atopic predisposition as demonstrated by the lack of atopic antecedents and a normal value of total IgE in these workers.

Only worker 2 clearly had an abnormal chest X-ray, although we did not have the benefit of a radiography prior to work at the farm. As this subject had worked in the steel industry for many years it was difficult to determine if the radiographical alterations were due to his contact with mushrooms or his prior employment. The diffusing capacity was abnormal in all workers. In addition, two workers showed a decrease of FVC and MMEF (1 and 2). In another worker (3) there was a response to an inhaled bronchodilator. Finally, worker 4 showed evidence of an abnormal MMEF.

Spirometric studies during several days at work demonstrated changes in lung function in three of the workers. In worker 2 we could not demonstrate any significant variation in lung function after 5 days of work. These changes consisted of a restrictive impairment and a small airways obstruction associated with exposure to the environment at work. However, the physiopathology of the workers' respiratory symptoms is not clear at present. An IgE-mediated mechanism is unlikely. Subjects had normal IgE levels and negative RAST to Shiitake allergens. The clinical presentation, immunological changes and decrease in diffusing capacity are suggestive of hypersensitivity pneumonitis (HP). The presence of a normal chest X-ray does not rule out the diagnosis of HP since in the non-acute phases of hypersensitivity pneumonitis, chest X-rays can be normal [20,21] or show minimal changes along with varying values of diffusing capacity

[22]. This may be the case of workers 1, 3 and 4 with minimal or no changes in chest X-ray and abnormal diffusing capacity. In three workers some airway obstruction was observed following several days of work. This is not unusual in HP since histological evidence of involvement of small bronchioles with features of bronchiolitis has been reported by several authors [23-25]. The results of pulmonary function and X-ray studies in subject 2 are more consistent with HP. However, this subject had a history of previous exposure to industrial materials capable of inducing pulmonary fibrosis. A more definitive diagnosis of HP would require lung biopsy, broncho-alveolar lavage and controlled provocation challenge.

High concentrations of Shiitake spores and Shiitake spore antigens were detected not only in the growing rooms but also in other areas of the building such as the packing room and even in the hallway leading to the growing rooms. As workers only wore masks during harvesting in the growing rooms, the lack of clinical efficacy of masks in these workers may be due to exposure to significant amounts of spores in other working areas where masks were not worn.

A mycotoxicosis could explain the respiratory symptoms observed in these workers due to massive inhalation of spores. However, in mycotoxicosis the immunological response is generally absent and a permanent reduction in the functional parameters of the lungs does not occur [26]. Also, these mushroom are eaten without any reaction, suggesting the absence of harmful toxins. Furthermore, none of the workers present the characteristic symptoms of intoxication by the organo-phosphate insecticide used in the farm.

Cross-antigenic reactivity was demonstrated by ELISA inhibition with other cultivated mushrooms, *A. bisporus* and *P. ostreatus*, suggesting that these workers should not be transferred to other mushroom farms. This recommendation is tempered in the case of *Agaricus* by the fact that the number of spores released in the atmosphere may be significantly less than in the case of Shiitake, as they are usually harvested prior to maximal spore liberation.

A strict environmental control of Shiitake spores is reasonable in this industry to reduce the high risk of sensitization and possible development of immunological lung diseases. Wearing appropriate face masks in all work places where significant levels of spores are detected and monitoring environmental spore levels may be useful in the prevention of the disease as has already been demonstrated in other cases of HP [27]. A periodic monitoring of workers by pulmonary function tests, especially the diffusing capacity along with the detection of specific antibodies will also help to assess reactivity and thus prevent the disease in such workers by removal from the work place.

In conclusion, our results suggest that these workers may have developed a hypersensitivity pneumonitis due to spores of *Lentinus edodes* and Shiitake spores should therefore be considered as an aetiological agent of the mushroom workers' lung.

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