



Respiratory syndrome very similar to extrinsic allergic alveolitis due to *Penicillium verrucosum* in workers in a cheese factory

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Abstract

A respiratory syndrome very similar to extrinsic allergic alveolitis due to *Penicillium verrucosum* was recognized in 4 workers employed in a Gorgonzola cheese factory. A mycogen allergy to *P. verrucosum*, used as starter in the production, was demonstrated by positive sputum culture and detection of specific antibodies in the blood. Intense and prolonged exposure to inhalation of fungal spores could have lead to the development of this allergic response. The fact that 2 of the subjects are siblings seems to indicate host susceptibility or immunological constitution in the pathogenesis of the respiratory allergy.

Key words: extrinsic allergic alveolitis, *P. verrucosum*, cheese-factory

Introduction

Moulds of the genus *Penicillium* have rarely been found as etiological agents in mycoses in humans. The species implicated have been considered opportunistic pathogens [1, 2], with the infections being produced in immunologically compromised hosts. The only exception is *P. marneffei*, a pathogenic species first isolated in bamboo rats from Vietnam by Segretain [3]. This fungus is the only known thermally dimorphic *Penicillium* species. Naturally acquired human infections, all originating from Southeast Asia and Indonesia, have been reported since 1973. A larger number of cases in recent years have been reported in association with HIV infection [4, 5]. Certain species of the genus *Penicillium* are associated with the spoilage of food and some have been found to be potential mycotoxin producers and implicated in causing serious health problems in man and other animals [6, 7].

But some species of *Penicillium* are involved in the pathogenesis of some respiratory diseases, such as bronchitis, bronchial asthma and extrinsic allergic alveolitis (EAA) also known as hypersensitivity pneumonitis (HP), a lung disease occurring in a variety of occupational environments.

In current food microbiology *Penicillium* species such *P. roqueforti* Thom, *P. camemberti* Thom are used as fungal starters for the production of different types of cheeses; they are also important in the maturation of some cheeses and sausages and may grow abundantly on their surfaces. When these are cleaned prior to sale, the spores are easily dispersed and they may be implicated in occupational asthma or extrinsic allergic alveolitis in exposed workers [8–11].

This paper reports respiratory symptoms in 4 cheese workers employed in a Gorgonzola cheese making factory in which *Penicillium weidemannii* var. *fuscum* is used in the processing of this blue cheese.

The aim of the study was to establish whether there was a relationship between respiratory symptoms and indoor fungal spores of this mould within the factory. The subjects of the study work in a seasoning chamber at a low temperature and with conditioned air. The surface of the cheeses they process are covered with an abundant growth of *Penicillium*, which we identified as *P. verrucosum*.

Materials and methods

This study was performed in a Gorgonzola factory in Pavia Province in Lombardia. Italian Gorgonzola is similar to the type of blue Roquefort cheese made in southern France; however, the various types of blue cheese have different methods of production. In Lombardia, pasturized whole cow's milk is used. Strains of bacteria (*Streptococcus thermophilus*, *Lactobacillus bulgaricus*), lyophilized fungi (*Geotrichum candidum*, *Saccharomyces cerevisiae* and *Penicillium weidemanni* var. *fuscum*) and rennet are added to the milk. In this factory *P. weidemanni* var. *fuscum* are added to the milk before curdling. The setting temperature is 32 °C. When the curd reaches the proper firmness (70 minutes) it is cut, separated from the whey and put into metal hoops. The hoops are placed on a draining rack without pressure at 22 °C for 3 days and turned to facilitate drainage, then stored at 4 °C. After 15 days the cheese form is punctured with about 240 long needles to admit the air needed for a good growth of the mould and to permit carbon dioxide to escape. The cheese is stored at a temperature of 6 °C and a humidity of 95–98% for 60 days. The cheeses are then removed, cleaned and scraped.

In the cold room the ventilation is regulated by an air conditioning system with partial recycle of indoor air. The air circulation allow the fungal spores to become airborne.

The cheese factory employs six hundred workers; a group of 43 people (26 males and 17 females) work in the seasoning chamber in which the ripening cheese is stored.

Of these, 8 suffer from rhinitis, cough, malaise and 4 of whom have been hospitalized in recent years. A survey of the fungal airspores in the work environment was performed to determine whether their presence might be related to a respiratory syndrome in these workers.

Mycological investigations

Isolation and identification of the fungi from sputum of the workers

During the three aeromycological environmental investigations, sputum cultures from all 43 workers were made in duplicate in Sabouraud agar and Czapek agar (SAB 1 + CZ 1). The mycological identification of fungal taxa was conducted by standard methods.

The yeasts were identified with the ID 32 C bio-Mérieux system and classified according to Kurtzman [21] taxonomy. Analysis of total proteins and of immunoglobulin IgE, IgG, IgA, IgM and an eosinophilic leucocytes count were conducted for all 43 workers as well as a Roentgenological examination of the chest and lung function evaluation.

Antigen preparation

Ouchterlony's immunodiffusion technique in agar [12] was carried out on samples of serum to detect precipitating antibodies. Somatic antigens of *Penicillium verrucosum*, *Geotrichum candidum* and *Saccharomyces cerevisiae* strains, isolated from workers' sputum cultures, were prepared according to the methods described in "Serology of fungal infection and farmer's lung disease" edited by Evans [13], at present considered to be the most satisfactory. A spore suspension made from a pure culture of *P. verrucosum* grown on Czapek agar (Difco) slants was transferred to a 2 litre Erlenmeyer flask containing 1 litre of liquid Czapek medium. The flask in static culture was incubated at 27 °C for 3 weeks. The fungus was killed by addition of phenol to a final concentration of 0.5% (w/v) and allowed to remain for 48 hours at 4 °C. After determining that no viable organism remained, the mycelium was harvested after filtration. An equal volume of phosphate buffered saline (P.B.S.) containing 1.0 mg/ml sodium azide was added to the mycelium. This was disrupted in a Sorvall omnimixer homogenizer. The insoluble debris were removed by centrifugation at 3000 × g for 10 minutes at 4 °C and the supernatant filtered through a 0.45 µm millipore filter. The filtrate was dialysed in dialysis tubing for 24 h and finally lyophilised. The lyophilised antigen was reconstituted to 10 mg/ml in sterile P.B.S. and used in agar gel diffusion studies.

Antigens were prepared from *S. cerevisiae* and *G. candidum* as follows. A 72 hour culture on 250 ml of Wickerham medium broth (malt extract, yeast extract, glucose, peptone: MYGP) was all used to inoculate 1 litre of Wickerham broth medium. Individual yeast strains were grown at 28 °C for 3 days. The cells were harvested by centrifuging the broth culture at 3000 × g for 10 minutes at 4 °C, and washed twice with saline. Ten g of yeast paste were resuspended in 10 ml of 0.154 M NaCl; yeast cells was disrupted by sonication with 20 g of glass beads (0.1–0.11 mm) at 100 W for 5 minutes at 4 °C. After disruption, the glass beads and cell debris were removed by two centrifu-

gations at 12,000 rpm for 10 minutes at 4 °C and at 90,000 × g for 90 minutes (Spinco ultracentrifuge, rotor 30). The fluid supernatant was collected and finally freeze-dried.

Precipitating antibodies were detected by Ouchterlony's immunodiffusion technique in agar gel using McIlvaine's citrate buffer on 10 × 10 cm glass filter slides with a gel thickness of 1 mm. The diameter of the serum well was 12.5 mm and that of the peripheral antigen wells 4 mm. The antigen and patient serum were then allowed to react for 72 h, and the slides washed and stained with Coomassie blue. The areas that formed were rated strong (+++) or weak (+--) for precipitin arcs and negative where no precipitin arcs were present.

Aeromycological investigation of the work environment

Fungal analysis of the indoor air of the seasoning chamber was conducted by the sedimentation method: exposure to the air of sterile media in petri dishes and identification of colonies that developed after incubation at room temperature (23–25 °C). Petri dishes (16 cm diameter) containing Sabouraud agar and Czapek agar (SAB 2 + CZ 1) supplemented with antibiotics were exposed in triplicate for 10 minutes in 10 sites of the seasoning chamber. A few days after incubation the fungi appearing on each plate were counted and subsequently identified. Fungal genus and fungal species were identified from microscopic appearance in accordance with the taxonomic keys for *Penicillia* of Pitt [14], Samson et al. [15–17] and Stolk et al. [18]. For *Geotrichum*, the yeast and fungi imperfecti, monographs by De Hoog et al. [19], Domsch et al. [20] and Kurtzman and Fell [21] were consulted. Mucorales were identified following Zycha and Siepmann [22].

Fungal analysis of the air of same seasoning chamber was performed in October and December 1996 and February 1997. The plates were exposed during work hours in the same sites of the seasoning chamber, in which thousands of Gorgonzola cheese are stored. The drawbacks of the plate exposure method have been reviewed by Gregory [23], but the method does provide information on the relative abundance and fluctuation of numerous genera, particularly unspecialised saprophytes. Strains of *Penicillia* in *P. verrucosum* Dierckx were identified in accordance with the description of morphological characters of the conidiogenous structures proposed by Samson et al. [15], but also in

Table 1. Fungi isolated from sputum of 43 workers

Workers	Female (17)	Male (26)	Total (43)
Fungal species			
<i>P. verrucosum</i>	5	4	9
<i>P. roqueforti</i>	–	4	4
<i>P. verrucosum</i> +	3	5	8
<i>G. candidum</i>			
<i>P. verrucosum</i> +	3	–	3
<i>C. albicans</i>			
<i>P. verrucosum</i> +	–	11	11
<i>S. cerevisiae</i>			
Case negative	6	2	8

accordance with the morphological and physiological criteria used by Pitt [14].

Results

Aeromycological data

Total number of fungal colonies did not differ significantly either between the three sampling times (October, December, February), or between the sites where plates were exposed. 1050 were recorded in October, 1134 in December and 1098 in February. *Penicillium*, *Saccharomyces*, *Geotrichum*, *Cladosporium* and *Mucor* were the most common genera.

Species composition did not differ substantially between the three sampling months either. *Penicillium verrucosum* was always isolated in significantly higher proportions: 72% in October, 74% in December and 72% in February. *Penicillium roqueforti* was only occasionally isolated in February. *Saccharomyces cerevisiae* and *Geotrichum candidum* were also frequently isolated, but in lower percentages, at 16–15–17% and 3–4–4.5% respectively. *Mucor racemosus* and *Cladosporium cladosporioides* were present less than 2–2.5% respectively.

Fungi isolated from sputum of the 43 workers

The fungi data for the sputum secretion of the 43 workers, collected once at each sampling time (October, December, and February) are summarized in Table 1.

Penicillium verrucosum was isolated from the sputum of 31 workers (72.1%), prevalently in association with the arthrosporeous species *G. candidum* and

the yeasts *S. cerevisiae* and *C. albicans*. *Penicillium roqueforti* was isolated only from the sputum of four male workers.

Most of the strains of *Penicillium* isolated from the air and from the sputum of the workers exhibited growth rates and morphology typical of *P. verrucosum*. Characters were: slower growth rate than other species in all media, especially on MEA, conidia yellow-green, exudate clear to pale yellow, reverse on CYA yellow brown; stipes robust, rough walled, penicilli usually terverticillate; conidia usually spherical, smooth walled, 2.5–2.8 μm diam., in a few cases ellipsoidal 3.0–3.5 $\times$ 2.5–2.8 μm .

Serological test for detection of antibodies

Double gel immunodiffusion detected serum precipitating antibodies in the serum of four of the 43 workers: 1 female and 3 male workers exposed to environmental antigens had developed precipitating antibodies against *P. verrucosum* and in one case also against *G. candidum*.

The histories of these 4 workers were very similar.

Case histories

1. Worker D.V.G. is a non smoking female born 1952 and has worked in this factory since 1967. She had had a dry cough and desultory dyspnoea since 1996. Her symptoms had disappeared on hospitalization in January 1997 and at testing, her lung function, estimated by forced expiratory capacity was found normal. Hematochemical data, specific IgG and IgE antibodies were also found to be normal; precipitating antibodies to standard Lofarma antigens (Milan, Italy) *Alternaria tenuis*, *Aspergillus fumigatus*, *Cephalosporium acremonium*, *Cryptostroma corticale*, *Mucor mucedo*, *Penicillium notatum*, *Trichoderma viride*, *Micropolyspora faeni*, *Termoactinomyces vulgaris*, *Sitophilus granarius*, pigeon droppings and serum were negative.

Penicillium verrucosum and *G. candidum* were isolated from cultured sputum of D.V.G., and serum screened by Ouchterlony double gel diffusion technique showed precipitating antibodies to *P. verrucosum* and *G. candidum* (Figure 1).

2. Worker D.V.A., D.V.G.'s brother, is a smoker and was born 1949. He had been working in this factory since 1968 and was hospitalized in January 1997 for asthma attacks and rhinitis. At the time, a chest radiograph, lung function tests, bronchial reactivity with methacholine and β_2 -agonista (salbutamol) were normal. Prick tests for common allergens were

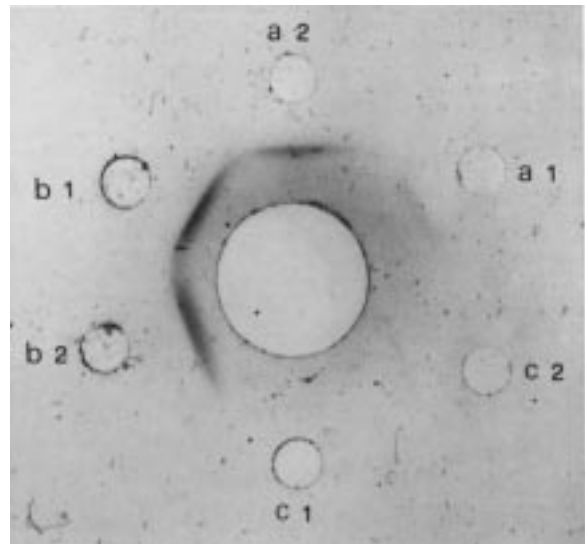


Figure 1. Double diffusion (Ouchterlony) in agar showing patient serum (S 60 μl) reacting with 10 μl of *P. verrucosum* antigen (b₂ 2% and b₁ 1%), *G. candidum* antigen (a₂ 2% and a₁ 1%) and *S. cerevisiae* antigen (c₂ 2% and c₁ 1%).

negative. Precipitating antibody tests to *A. fumigatus*, *A. nidulans*, *M. faeni*, *P. notatum*, *P. roqueforti*, and *T. vulgaris* were negative. IgE total, and IgE specific (CAP system) were negative.

Penicillium verrucosum and *G. candidum* were isolated from cultured sputum and Ouchterlony double gel diffusion screening of serum showed precipitating antibodies to *P. verrucosum*.

3. Worker F.G. is a male, a smoker, was born in 1940, and started working in this factory in 1972. He suffers from rhinitis and sinusitis and was hospitalized in 1996 for recurrent dyspnoea, cough, fever and rhinitis. Hematochemical and clinical examinations were negative. *Penicillium verrucosum* was isolated from cultured sputum in our research and Ouchterlony double gel diffusion screening of serum showed precipitating antibodies to *P. verrucosum*.

4. Worker G.P. is a male, a smoker, was born 1967 and started working in this factory in 1994. He suffers from rhinitis and sinusitis and was hospitalized in 1996 because of an acute attack of pulmonary disorder and bronchitis. Lung function tests, and prick test to common allergens were negative: *P. verrucosum* was isolated from sputum and the serum tested by Ouchterlony double gel diffusion technique showed precipitating antibodies to *P. verrucosum*.

Discussion

A respiratory syndrome thought to be EAA was diagnosed in 4 subjects who had been working in a cheese-making factory for long periods. Mycogenic allergy to *P. verrucosum* was demonstrated by positive sputum culture and by specific antibody formation in the blood of the subjects. Intense and prolonged exposure to fungal spore inhalation probably lead to the development of this allergic response in these workers. *Penicillium* sp. are ubiquitous in nature and their etiological significance when isolated from humans is normally subject to doubt. In our circumstances, the mould that caused respiratory syndrome has been recognised as *P. verrucosum*. This species prevailed in the air, as well as in the sputum cultures of many workers.

However, in this cheese factory the fungal starter, used in the Gorgonzola production, was *P. weidmanni* var. *fuscum*, a species placed in synonymy with *P. roqueforti* Thom by Samson et al. [15] and Stolk et al. [18]. *Penicillium verrucosum* is somewhat similar to *P. roqueforti*; both species are characterized by roughened, two-stage branched conidiophores. Ignored by Raper and Thom [24], who considered Dierckx's species as a synonym of *P. viridicatum* Westling, *P. verrucosum* was revived by Samson et al. [15], who separated the *P. verrucosum* complex into 6 varieties on the basis of colony colour differences. In a clear classification of *Penicillium* subgenus *Penicillium*, Pitt and Cruickshank [25] provide a definitive listing of the currently known and accepted species, included *P. roqueforti* and *P. verrucosum* as distinct species. In a reinvestigation of a species of the terverticillate *Penicillia* delimited on the basis of morphological criteria and mycotoxin profiles, Stolk et al. [18] also consider *P. verrucosum* and *P. roqueforti* to be distinct species in series *Viridicata*.

That a high number of spores of *P. verrucosum* in this cheese plant was responsible for the development of a syndrome resembling EAA in all these workers was not unequivocally shown, but it nevertheless seems plausible that in addition to this extrinsic factor, intrinsic factors in some workers might facilitate the settling of moulds in the tissues and consequent pathogenic activity [26]. Sputum cultures were positive for *P. verrucosum* in 72.1% of the 43 subjects studied, but only 8 suffered from bronchitis and rhinitis. Of these, specific antibodies against *P. verrucosum* were found in the blood of 4 workers, 2 of whom, a male and female, were siblings. This seems to implicate

host susceptibility or immunological constitution of the subject in the pathogenesis of the respiratory allergy. The role of airborne spores in causing bronchitis is uncertain, but their incidence among atopic subjects suggests an allergic mechanism.

Evaluations of the hazard of respiratory symptoms believed to be associated with occupational exposure to airborne *Penicillium* species used in cheese factory have been reported. Two cases of recurrent bronchitis are described by De Weck et al. [8] in cheese workers who presented clinical symptoms similar to those of farmer's lung. The fungus incriminated was *P. casei* developing on the surface of stored cheese. *Penicillium casei* related respiratory syndromes in cheese workers have also been recorded by Minning and De Weck [27]. Asthma resulting from professional exposure to *P. candidum* in a patient employed in a cheese dairy was reported by Gari et al. [10]. In a clinical epidemiological study of allergic manifestations in cheesemongers, Molina et al. [9] demonstrated that a small mite living on the surface of the cheese was the allergen truly responsible for the disorders.

Hypersensitivity pneumonitis induced by *P. roqueforti* in cheese manufacturing workers has been reported by Campbell et al. [11] and by Galland et al. [28]. *Penicillium verrucosum* has not been reported in the literature as a species used in cheese production. It is common on cereals, on processed meats and fish in temperate regions [29]. The species is characterised by production of ochratoxin A and verrucolone and the chemotype II by citrinin production [6]. Nevertheless, it may be identified as the most important allergenic fungus in this cheese factory in which the surface of cheeses is covered with an abundant fungal growth of *P. verrucosum*. It is not surprising that *Geotrichum* and *Saccharomyces* occur in this environment in addition to *P. verrucosum*: all derive from the same indoor source and are all implicated in respiratory allergy. The term "allergy" is interpreted here as a hypersensitivity to the fungus developed by certain individuals who show associated clinical symptoms. Very little is known about the numbers of spores required to produce allergic disease. Estimation of the indoor concentrations of the spores of individual species of fungi presents difficulties and little information is available for those fungi which are allergenic. It has been estimated that average concentrations of 10^6 spores/m⁻³ air may sensitize atopic individuals, who will react immediately to the allergens to which they are exposed [30]. Non-atopic subjects require more intensive exposure (10^6 – 10^9 spores/m⁻³) for sensitization, and develop-

ment of extrinsic allergic alveolitis (hypersensitivity pneumonitis) is delayed.

In a list of fungi grouped according to spore size and the type of disease they may cause, Lacey et al. [31] include 8 species of the genus *Penicillium*, among them *P. casei*, and *P. roqueforti*, held to be responsible for rhinitis, asthma and alveolitis. All spores smaller than 5 μm , whether viable or not, may be regarded as potential allergens. Fungi whose spores are frequently inhaled and which may be found in sputum or bronchial secretion, e.g. *Penicillium*, although rarely causing tissue lung's damage, can provoke an allergic response.

Very few studies have been undertaken on xerophilic species of the genus *Penicillium*. Although *Penicillium* are frequently found in the atmosphere and have been shown to contain allergens [32], no studies have examined the characteristics of the mycelial or conidial allergens of this genus. Airborne fungi have been postulated as the cause of symptoms associated with non industrial indoor environments [33]. In one study [34], the office environment was found to be an indoor fungal reservoir, particularly of airborne *Penicillium* species. The relationship between symptoms and IgG and IgE antibodies in the office workers was evaluated and 51% of the participants found to have high *Penicillium* IgG levels. *Penicillium* being the predominant organism found in the study area. None of the participants had a positive IgE antibody test to *Penicillium* or other fungi such as *Candida* and *Aspergillus*. *Penicillium* sp. are never endogenous but in cases of extrinsic allergy alveolitis is usually diagnosed from the sputum. We concluded that host factors contribute to disease pathogenesis, since of all the antigen-exposed individuals only a relatively small number (8 workers) developed respiratory symptoms similar to those of hypersensitivity pneumonitis: only four workers exposed to *P. verrucosum* of whom two were siblings, also developed precipitating antibodies. This suggests that as yet undefined genetic factors may play a role in the development of hypersensitivity pneumonitis. Genetic mechanisms were also suggested to influence the occurrence of pigeon breeder's disease in patients having hypersensitivity pneumonitis due to exposure to avian antigens [35, 36]. Knowledge of the indoor environment and immunological investigation of patients affected by a suspected occupational disease are fundamental to linking the disease with the environment.

As with other allergic conditions, once the etiological agent is identified, appropriate ventilation

procedures or modifications of the work process may be carried to prevent further exposure and enhance recovery as well as to prevent further illness.

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