

The role of *Penicillium frequentans* in suberosis (Respiratory disease in workers in the cork industry)

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Summary

The widespread occurrence of spores of *Penicillium frequentans* in the air of a Portuguese cork factory was associated with the presence of precipitins against it in 38% of exposed workers and a high incidence of radiological and functional changes in their lungs.

Patients with suberosis gave positive reactions in skin tests with extracts of the fungus, and their inhalation as aerosols provoked characteristic symptoms.

Cork particles were less numerous in the air and larger than *P. frequentans* spores, but numbers were correlated with the incidence of precipitins in workers and it is suggested that they may have a role in sensitization and the pathology of the disease. However, *P. frequentans* seems to be the source of antigens in suberosis and accounts for the antigenicity of mouldy cork.

Introduction

Workers in factories where cork bark is processed may become affected by an often incapacitating disease of the respiratory tract known as suberosis, a form of extrinsic allergic alveolitis caused by inhaling dust from mouldy cork (Ávila & Villar, 1968; Cancelli, 1955, 1963). Recently, Pimentel & Ávila (1973) reported precipitins to *P. frequentans* Westling in sera from suberosis patients.

P. frequentans was first isolated from cork that had been incubated in humid conditions in England. However, it has now been shown to be common in the air of cork factories (Lacey, 1973).

In this paper we compare the air spora in different parts of a cork factory at Seixal, near Lisbon, with the incidence of precipitins against *P. frequentans* in 648 workers and the radiological and functional changes in 630 and 568 workers respectively. Statistical correlations were usually poor and reasons for this are discussed while results of a detailed epidemiological study will be described by the Department of Chest Diseases, Lisbon University Faculty of Medicine (Ávila *et al.*, 1973). We also present the results

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of skin and inhalation tests with *P. frequentans* antigens in twenty-six patients with suberosis.

At the factory where these observations were made the freshly stripped cork bark was usually stacked outside for about a year. It was then cut to size, baled, boiled in water and re-stacked outdoors until required. Some cork was reboiled and stacked while still hot in a poorly ventilated, damp warehouse (fundão 1) for several days before use. This cork was mostly used to make discs, but sometimes also corks for wall coverings and stoppers. Other cork was wetted with cold water and stacked in a larger, drier warehouse (fundão 2). This was steamed before it was used to make stoppers. Tiles were made from unprocessed cork, while balls, fishing rod handles and other items were made from resin-bonded granulated cork, shaped by sanding machines.

Methods

The general spora in different parts of the factory was assessed using a cascade impactor (May, 1945) and a modified Andersen sampler (May, 1964). Exposure of individual workers was determined with a small suction trap, the 'Casella' personal sampler, worn on a harness, with the sampling orifice on the shoulder so that the air sampled represented that inhaled. Slides from the cascade impactor and membrane filters from the personal sampler were examined microscopically. Spores were classified as described by Lacey (1971). Some slides were stained with cochineal red (Pimentel, 1971), to identify cork particles which were also counted.

Isolations were made on 2% malt extract agar and half-strength 'Oxoid' nutrient agar using an Andersen sampler (Gregory & Lacey, 1963). Malt plates were incubated at 25 and 37°C, nutrient plates at 25, 37 and 55°C, and counted respectively after 7, 6 or 3 days incubation.

In preparing the antigens from *P. frequentans* the technique of consecutive freezing and thawing of the plates was used, followed by extraction with Coca's fluid, filtration, dialysis and freeze-drying. The freeze-dried extract was then dissolved to give a concentration of 40 mg/ml in normal saline solution for double diffusion tests with the worker's sera, as described by Pimentel & Ávila (1973).

Skin tests were done by injecting 0.2 ml of a solution containing 5 mg of the freeze-dried *P. frequentans* extract/1 ml Coca's fluid into the forearm. Tests were assessed at 20 min and 3, 4, 5, 6 and 7 hr after the injection.

In inhalation tests, patients were submitted to an aerosol of 1 ml of a solution of 5 mg/ml of freeze-dried *P. frequentans* extract in Coca's fluid. The patients were examined, the axillary temperature taken and ventilatory parameters (VC, FEV₁ and %VC/FEV₁) measured before the test and 20 min, 3, 4, 5, 6 and 7 hr afterwards. Whenever possible CO transfer factors were determined before and 7 hr after provocation.

High quality 100 mm photo-fluorograms of the chest were made of 630 of the cork workers. The ventilatory function was studied with a Vitalograph in 568 workers. Vital capacity (VC), FEV₁ and VC/FEV₁ × 100 were determined and corrected to BPTS.

Different work-stations during the processing of cork were classified as follows: *open air*, workers sorting and grading, work in the open air and those boiling cork; *cork storage*, those working in the warehouses (fundões); *cutting and production*, disc-, stopper-, paper- and tile-making sections; *finishings*, ball makers, sanders, etc.;

grading and packing, grading of finished products, washing, sterilization and packing; others, posts where cork is not directly handled, e.g. machinists, electricians, smiths, etc.

Results

Incidence of airborne Penicillium frequentans spores and cork particles in the cork factory

Air within the factory buildings contained 35×10^3 to 54.2×10^6 spores/m³, when there were $36-76 \times 10^3$ spores/m³ outdoors. Numbers of spores varied according to the mouldiness of the cork and the handling it received (Table 1). Fewest occurred in the boiling department, where ventilation was good and the cork clean. Spore numbers differed little from outdoors, contrasting with fundão 1 where the cork was stacked while still hot, after boiling. This fundão was small, dark and humid, with poor ventilation. Mould was visible on the cork 4 days after stacking and extensive after 7 days, when the cork was usually removed. Many spores became airborne when the cork was moved, producing more than 50×10^6 spores/m³ air. Most of this cork was used to make discs and many spores were again released as the cork was cut into strips and

Table 1. Spore concentrations in different sections of the cork factory

	10 ⁶ spores/m ³	Proportion of <i>Penicillium</i> spores (%)
Open air	<0.13	72
Cork storage	23.8	80
Cutting and production	6.2	82
Finishings	0.8	89
Grading and packing	1.2	97

Table 2. Incidence of airborne *Penicillium* spores, cork particles and precipitins against *P. frequentans* (592 smokers and non-smokers) in different parts of the cork factory

Sections of the factory	Airborne concentrations		No. of workers examined	Proportion with precipitins against <i>P. frequentans</i> (%)
	<i>Penicillium</i> spores (10 ⁶ /m ³ air)	Cork particles (10 ⁶ /m ³ air)		
Open air	<0.1	<0.1	66	18
Cork storage	17.6	<0.1	—	—
Cutting and production	5.1	0.6	118	36
Finishings	0.7	1.3	106	40
Grading and packing	1.3	1.5	255	46
Others	—	—	47	26

Significant relationship between cork particles and precipitins ($P < 0.05$).

sliced thinly. By contrast, cork in fundão 2 remained free from mould and there were many fewer airborne spores. In most parts of the factory more than 80% of the spores were classified as *Penicillium* type (Table 2).

The predominant organisms isolated from the air were fungi. Most common, occurring in more than 75% of air samples, were *P. frequentans*, *P. granulatum* Bain., *Aphanocladium album* (Preuss) W. Gams, *Monilia sitophila* (Mont.) Sacc. and *Mucor plumbeus* Bon. Other species occurred infrequently and have been listed by Lacey (1973). *P. frequentans*, *A. album*, *M. sitophila* and *M. plumbeus* were all abundant in fundão 1 and the disc department, but of these only *P. frequentans* was numerous in other departments. *P. granulatum* was most numerous in fundão 2 and the ball department, but never so abundant as *P. frequentans*. More than 10^5 colonies/m³ grew on Andersen sampler plates from air sampled at most sites, with more than 10^6 colonies/m³ air in fundão 1 and the disc department, and a maximum of 5.6×10^6 colonies/m³ air. By contrast, the maximum number of *P. granulatum* isolated was 6.5×10^5 colonies/m³ air, and usually there were fewer than 5×10^3 colonies/m³ air. Actinomycetes and bacteria were never abundant.

Cork particles were often present (Table 2), but except near sanding machines, there were seldom more than 2×10^6 particles/m³ air. Cork particles were fewer but larger than *Penicillium* spores, only about 22% penetrating the second stage of the cascade impactor. The median cut-off diameter for this stage at the sampling rate used is about 4.5 µm, so that this figure indicates the proportion of cork particles small enough to reach the alveoli. By contrast, 68% of *Penicillium* spores penetrated this stage, while 64% of colonies of *P. frequentans* occurred on stages 3–6 of the Andersen sampler, which Andersen (1958) considered would trap particles that could penetrate to the alveoli. Although *P. frequentans* spores are 3.0–3.5 µm diameter, and individually might all be expected to penetrate to the alveoli, they often remain aggregated.

In most departments the numbers of airborne fungus spores and cork particles were similar on different sampling occasions. Greatest differences occurred in fundão 1 where there were only 2×10^6 spores/m³ when cork was not being handled, but over 20 times more spores when mouldy cork was being moved.

Exposure of workers to fungus spores

Workers were often exposed to more spores than indicated by general air samples. In fundão 1, for example, the personal sampler trapped 128×10^6 spores/m³, when carried by a worker moving mouldy cork. Even this is probably an underestimate of maximum exposure, since part of sampling period was spent taking the cork on

Table 3. Exposure of workers to fungus spores

Section	Mean spore concentration (10^6 spores/m ³)
Open air	—
Cork storage	72.4
Cutting and production	13.9
Finishings	0.9
Grading and packing	1.2

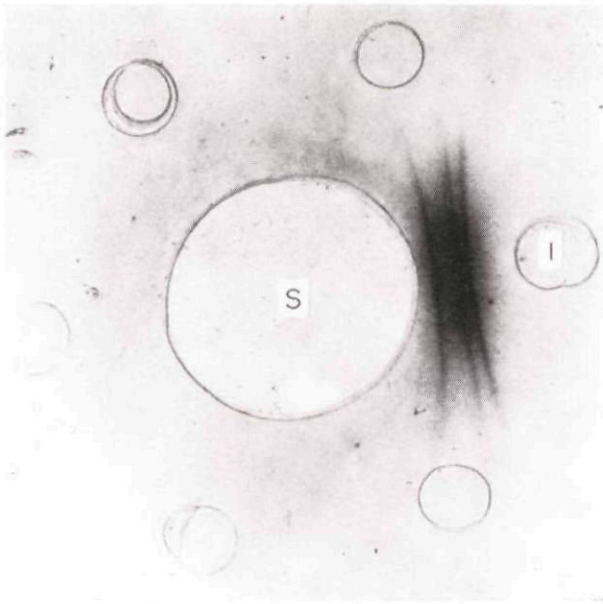


Fig. 1. Double-diffusion slide. S, Patient's serum; I, *P. frequentans* antigen at 40 mg/ml in saline solution.

trolleys to the disc department. The average results obtained in each section are shown in Table 3.

Immunological studies

Extracts of the fungi isolated from air most frequently were tested against sera of workers exposed to cork dust. Ávila & Villar (1968) had shown that extracts of mouldy cork yielded multiple precipitin arcs with sera from suberotic cork workers. Extracts of *P. frequentans* alone reacted similarly to mouldy cork (Fig. 1).

Precipitins to *P. frequentans* were found in the sera of 38% of exposed cork workers but only once in control studies of 157 normal human sera. Seven per cent of unaffected

Table 4. Incidence of precipitins against *P. frequentans* and restrictive ventilatory changes after different periods of exposure to the factory environment (568 smokers and non-smokers)

Years of exposure	With precipitins against <i>P. frequentans</i> (%)	With restrictive syndrome (%)
0-5	38	8
6-10	45	5
11-30	42	17
>30	37	29

No significant relationship between precipitins and years of exposure ($P>0.05$). Significant relationship between restrictive syndrome and years of exposure ($P<0.05$).

cork workers also had precipitins to *P. frequentans*. The incidence of precipitins was greatest at work stations where most *Penicillium*-type spores and cork particles were airborne (Table 2) but only the number of airborne cork particles showed a significant correlation ($P < 0.05$) with the incidence of precipitins.

The period workers had been exposed to the factory environment seemed to influence the incidence of precipitins very little. Less than 5 years' exposure had sensitized 38% of workers, compared with 45% in those exposed for 6–10 years (Table 4).

Skin tests

Dual skin test reactions (type 1, immediate wealing reaction followed by type 3 late reaction) to extracts of *P. frequentans* were obtained in twenty-five of the twenty-six patients with suberosis tested. In the other subject, there was a late positive reaction but no immediate urticarial reaction.

Inhalation tests

Inhalation tests with aerosols containing extracts of *P. frequentans* caused febrile reactions and malaise together with cough, dyspnoea, the appearance of basal crepitations and a decrease in diffusion capacity that developed after 6–7 hr in eighteen of the nineteen suberotic patients studied. All subjects showed a late bronchial reaction while seventeen also showed an immediate bronchial reaction.

Radiological aspects

The results of X-ray examinations of 630 cork factory workers are shown in Table 5. The incidence of the different types of X-ray changes was significantly different in smokers and non-smokers ($\chi^2 = 6.62$; $P = \pm 0.01$). Therefore, only the non-smokers were considered further.

In non-smokers, the incidence of reticulo-nodular changes correlated significantly with the concentration of fungal spores in the atmosphere of the different sections of the factory ($P < 0.01$). There was, however, no significant relation between the radiologic appearance and the presence of precipitins against *P. frequentans* in the workers' sera ($P > 0.05$). No correlation was found between the years of exposure to the factory environment and the X-ray changes ($P > 0.05$).

Ventilatory studies

There were ventilatory changes in 42% of the smokers and in 32% of the non-smokers, but the difference between the two groups was not significant ($\chi^2 = 1.47$; $P > 0.05$).

Table 5. Radiological appearances found in cork factory workers

	Smokers (157) (%)	Non-smokers (473) (%)
Normal X-ray	19.4	34
Increased lung markings	13	7
Miliary shadows	6	2.5
Reticulo-nodular shadows	61	55
Infiltrates	0.6	1.5

Table 6. Incidence of ventilatory changes in cork factory workers

	Smokers (159) (%)	Non-smokers (409) (%)
Normal function	57	69
Restrictive defect	24	23
Obstructive defect	8	5
Mixed defect	11	4

No relationship was found between the incidence of ventilatory disturbances and the concentrations of fungal spores or cork particles in different sections of the factory.

There was also no significant relationship between the occurrence of ventilatory defects and the incidence of precipitins against *P. frequentans* ($P > 0.05$), but the ventilatory changes increased significantly with period of exposure to the factory environment ($P < 0.05$), especially in relation to the restrictive syndrome (Table 4).

Discussion

Pimentel & Ávila (1973) first suggested that *P. frequentans* might be important in the etiology of suberosis in Portuguese cork factories. Our work strongly suggests that it causes the disease. It fulfils many of the criteria of organisms causing forms of extrinsic allergic alveolitis. It is widespread in the environment, usually in numbers greater than the normal outdoor concentrations of all fungus spores and often many times greater than these; its spores are small enough to penetrate to the alveolar region of the lungs; precipitins against *P. frequentans* are present in the sera of exposed workers; reticulo-nodular radiological changes are significantly correlated with numbers of *P. frequentans* spores; patients reacted positively in skin tests with extracts of the fungus, while inhalation of these provoked symptoms typical of suberosis. It can be argued that precipitins only indicate exposure to the fungus; however, they were found in 98% of affected workers but in only 7% of unaffected. The latter might indicate subclinical disease that could perhaps worsen with continued exposure to the dust (Pimentel & Ávila, 1973). A similar situation is found in farmer's lung (Pepys & Jenkins, 1965).

Airborne cork particles seem less likely to cause the disease because they are fewer and most are larger than *P. frequentans* spores. The precipitin reactions with extracts of mouldy cork can be accounted for by the presence of the fungus. However, two factors suggest that cork dust may play a part. First, the incidence of precipitins correlated significantly with numbers of airborne cork particles and second, cork particles are characteristic in granulomatous lung lesions (Pimentel, 1971; Pimentel & Ávila, 1973). It is difficult to place much emphasis on significant correlations in the factory, as workers had often been employed in different parts of the factory previously, and the degree of significance was never great. However, cork particles may possibly persist longer in the lungs than *Penicillium* spores, and they may carry metabolites and hyphae of the fungus (although fungal elements have not been identified in them) that could lead to more efficient sensitization and the granulomatous reaction.

The lack of correlation between the incidence of suberosis and numbers of fungus

spores and cork particles at different work-stations could result from their abundance throughout the factory and the frequency of movement of workers from one section to another. The incidence of precipitins varied little with the period of exposure suggesting that most workers who were going to become sensitized, did so within the first 5 years. There were too few workers to show how precipitins developed during the first 5 years exposure.

As suberosis may present under various clinico-pathologic forms, from subclinical through asthma-like disease, allergic alveolitis, chronic bronchitis with bronchiectasis to progressive pulmonary fibrosis (Pimentel & Ávila, 1973), it is not surprising that there is not a clear correlation between the ventilatory and X-ray changes and the presence of precipitins.

The lack of correlation between radiological changes and length of exposure to the factory environment suggests that the changes may appear early in disease development. On the other hand, the ventilatory changes increased with prolonged exposure showing that functional involvement of the respiratory system is the last to become apparent as might be expected.

The incidence of suberosis in the factory could probably be decreased by increased hygiene, better ventilation and the prevention of moulding. The contrast between the two fundões shows that improvement is possible, providing that steaming is an acceptable procedure for disc manufacture. Alternatively a fungicide added to the boiling water (Sardica, 1971) or to a post-boiling dip could be used, providing these do not interfere with subsequent use of the cork.

Suberosis is the third form of allergic alveolitis in which *Penicillium* species have been implicated. Workers handling surface cultures of *Aspergillus niger* van Tieghem used to produce citric acid became sensitized to contaminating *Penicillium* spp. (Hořejší *et al.*, 1960), while cheese washers in Switzerland were affected by *P. casei* Staub (Weck, Gutersohn & Bütikofer, 1969). It emphasizes the need to suspect any prevalent airborne spores of fungi or actinomycetes, when allergic alveolitis occurs.

Acknowledgments

We gratefully acknowledge assistance from a large team of physicians, nurses, technicians and medical students from the Department of Chest Diseases, Lisbon Faculty of Medicine, carrying out the associated epidemiological survey.

We thank Professor Thome Villar, Director of the Department of Chest Diseases, for his great assistance and encouragement, the Department of Dermatology, Lisbon Faculty of Medicine (Director, Professor Juvenal Esteves) for facilities in the Mycology Laboratory, and Dr Telles de Araújo, Dr Julia Cabrita and Mrs Maureen E. Lacey for technical assistance.

Part of this work was supported by the Instituto de Alta Cultura, Portugal (Research Project LMC 5).

References

- ANDERSEN, A.A. (1958) New sampler for the collection, sizing and enumeration of viable airborne particles. *Journal of Bacteriology*, **76**, 471.
- ÁVILA, R., ARAÚJO, A.T., ALMEIDA, A.B. & VILLAR, T.G. (1974) Respiratory disease in a cork factory. *British Journal of Industrial Medicine*, **31** (in press).
- ÁVILA, R. & VILLAR, T.G. (1968) Suberosis, respiratory disease in cork workers. *Lancet*, **i**, 620.

- CANCELLA, L.C. (1955) On a special kind of pneumoconiosis: the suberosis. *Medicina Contemporânea*, **73**, 325.
- CANCELLA, L.C. (1963) Suberosis: a pneumoconiosis due to cork dust. *Industrial Medicine and Surgery*, **32**, 435.
- GREGORY, P.H. & LACEY, M.E. (1963) Mycological examination of dust from mouldy hay associated with farmer's lung disease. *Journal of General Microbiology*, **30**, 75.
- HOŘEJŠÍ, M., ŠACH, J., TOMŠÍKOVÁ, A. & MECL, A. (1960) A syndrome resembling farmer's lung in workers inhaling spores of aspergillus and penicillia moulds. *Thorax*, **15**, 212.
- LACEY, J. (1971) The microbiology of moist barley storage in unsealed silos. *Annals of Applied Biology*, **69**, 187.
- LACEY, J. (1973) The air spora of a Portuguese cork factory. *Annals of Occupational Hygiene*, **16**, 223.
- MAY, K.R. (1945) The cascade impactor: an instrument for sampling coarse aerosols. *Journal of Scientific Instruments*, **22**, 187.
- MAY, K.R. (1964) Calibration of a modified Andersen sampler. *Applied Microbiology*, **12**, 37.
- PEPYS, J. & JENKINS, P.A. (1965) Precipitin (FLH) test in farmer's lung. *Thorax*, **20**, 21.
- PIMENTEL, J.C. (1971) Fibrose pulmonar difusa idiopática (síndrome de Hamman & Rich). Revisão de dois casos em que foi possível identificar o provável agente etiológico. *Journal da Sociedade das Ciências Médicas de Lisboa*, **135**, 445.
- PIMENTEL, J.C. & ÁVILA, R. (1973) Respiratory disease in workers in the cork industry (suberosis): new trends and diagnostic possibilities. *Thorax*, **28**, 409.
- SARDICA, L. (1971) Estudo de eficácia do ácido salicílico como agente inibidor de fungos. *Boletim Junta Nacional da Cortiça*, **33**, 217.
- WECK, A.L. DE, GUTERSOHN, J. & BÜTIKOFER, E. (1969) La maladie des laveurs de fromage. *Schweizerische Medizinische Wochenschrift*, **99**, 872.

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