

From Kommunehospitalet, Medical Department III, and  
Rigshospitalet, Laboratory for Clinical Allergy, Medical Department TA,  
Copenhagen, Denmark.

## PRECIPITATING ANTIBODIES AGAINST *MICROPOLYSPORA PHAENI* IN SERA FROM MUSHROOM WORKERS

By

BIRGER BROCH MØLLER, POUL HALBERG, SUSANNE GRAVESEN  
& BENT WEEKE

Since Brighurst (2) in 1959 described a respiratory disease in mushroom workers, it has been known that the cultivation of mushrooms carries a risk of a pulmonary disease with the same pathogenetic mechanism as farmer's lung.

After exposure to compost from mushroom cultures a free interval of 4-8 hours will pass, after which the patient may experience general malaise, fever, dyspnoea and sometimes chills. Leukocytosis and diffuse pulmonary infiltrates may be found. The disease is limited, but following repeated exposures it may progress to a chronic respiratory disease undistinguishable from chronic obstructive lung disease (6). *Micropolyspora phaeni* (*M. phaeni*) has been incriminated by several authors (5, 6, 7) as the cause of this condition. The symptoms are those compatible with an allergic reaction of type III, i.e. alveolitis due to the formation of complexes consisting of inhaled microorganisms and precipitating antibodies from the circulation.

The object of the present study is to investigate the occurrence of antibodies against *M. phaeni* in Copenhagen mushroom workers. The antibodies were demonstrated by means of counterelectrophoresis (CE) and crossed immunoelectro-

phoresis (CRI) with intermediate gel in order to compare the two techniques.

*Mushroom cultivation:* *M. phaeni* belongs to the mycelial enbacteria. It is a gram-positive and thermophilic organism. Compost of plant material that undergoes spontaneous heating contains thermophilic actinomycetes as a characteristic component of the microflora (3).

Mushrooms—*Agaricus hortensis*—are grown on natural compost consisting of a mixture of wheat straw and fresh horse manure, which is allowed to decompose. This takes place in two phases. In phase 1 the compost is exposed to the atmosphere for 3 weeks. In phase 2 the compost is subjected to a temperature of 55–60° C and a humidity of 100 per cent for several days. Thermophilic organisms find this environment excellent for their growth. The spawning process is carried out by hand. The spawn is then mixed mechanically with the compost and after approximately 6 weeks the mushrooms can be picked. After picking, the sheds are heated to 60° C and the trays are emptied of compost. Large quantities of dust containing *M. phaeni* are liberated during the spawning process and particularly when the sheds are emptied.

## MATERIAL AND METHODS

The material consists of 22 mushroom workers from the vicinity of Copenhagen. We have tried to contact all available mushroom workers in this area. The controls are 15 persons who have had no contact with mushroom cultivation and have no known pulmonary disease. Age and sex for the two groups are shown in Table 1. None of the mushroom workers investigated had experienced the symptoms compatible with mushroom worker's disease.

*Test extract and preparation.* The bacteria (a subculture from Baarn) were incubated on V-8 agar at pH 7.2 in Petri dishes and the surface carefully harvested after 5 days of incubation in 45° C with sterile, distilled water, and immediately frozen. They were then thawed and resolved in a buffer consisting of 0.1 M NaCl and 15 M NaN<sub>3</sub> with pH 8.2. In a glass-grinder, extracts were produced by four consecutive shakings for 1 min at 0° C. The extracts were centrifuged, decanted and placed at -20° C. The supernatant was used as reference antigen *M. phaeni* 1973 (*M. ph.* 73) for the immunoelectrophoretic techniques and for the immunization of the rabbits.

Antibodies were produced in rabbits in the following way: three rabbits were

immunised with 100  $\mu$ l *M. ph. 73*, mixed with 100  $\mu$ l incomplete Freund adjuvant. After four immunizations at 2-week intervals, blood was drawn from an ear-vein and the serum isolated. Monthly samples were taken preceded by a booster dose 8 days prior to tapping. In this way a pooled rabbit antiserum against *M. ph. 73* (Ra. *M. ph. 73*) was obtained. Finally, the gammaglobulins were isolated from the serum (4).

#### *Sera from Mushroom Workers*

All sera were tested in the following ways:

*Counterelectrophoresis* was done in a mixture of equal amounts of agarose 1 per cent (Litex Denmark) and agar 1 per cent (Bacto-Difco) in a barbital buffer pH 8.6 and ionic strength 0.02. Fifty  $\mu$ l of serum was placed at the anodic punch, and by electroendosmosis, the gammaglobulins moved towards the cathodic punch where 50  $\mu$ l *M. ph. 73* was placed in 6 mm punches in the gel. *M. ph. 73* moved towards the anode where it met the gammaglobulins and gave rise to a precipitation line in the positive sera. Electrophoresis was performed at 2 V  $\text{cm}^{-1}$  overnight. The gel was pressed, and non-bound proteins were removed from the gel by repeated washings in saline. After this, the gel was dried and the proteins coloured with Coomassie brilliant blue.

*Crossed immunoelectrophoresis with intermediate gel* was performed according to Svendsen & Axelsen (1) on 5  $\times$  5 cm glass plates as modified by Weeke (9). First dimension electrophoresis was done with 50  $\mu$ l *M. ph. 73* in a 1 per cent (w/v) agarose gel in barbital buffer pH 8.6 and ionic strength 0.02 at 15° C for 30 min with 10 V  $\text{cm}^{-1}$ . The second dimension electrophoresis was done with 2 V  $\text{cm}^{-1}$  at 15° C overnight carried out in a 1 cm intermediate gel containing patient serum (100  $\mu$ l per 5  $\text{cm}^2$  gel, i.e. 20  $\mu$ l serum  $\text{cm}^{-2}$  gel) and finally in a gel with 200  $\mu$ l Ra. *M. ph. 73* per 15  $\text{cm}^2$ , i.e. 13  $\mu$ l  $\text{cm}^{-2}$  gel. After this, the gel was pressed, and non-bound proteins were washed away by saline—three times—the whole procedure lasting 24 hours. After a new pressure the gel was dried in warm air and the proteins coloured with Coomassie brilliant blue. A pooled serum, stored at -20° C from fresh blood donors was used as a normal reference serum.

By this method, not only the precipitation is shown, but also the protein(s) in the *M. ph. 73* extract responsible for the precipitation.

## RESULTS

Fig. 1 shows the reference picture from CRI of *M. ph. 73* against Ra. *M. ph. 73*. The precipitation curves are numerated from anode to cathode as shown in the drawing. We have found 35 precipitation curves.

Twelve of the 22 sera from mushroom workers contained

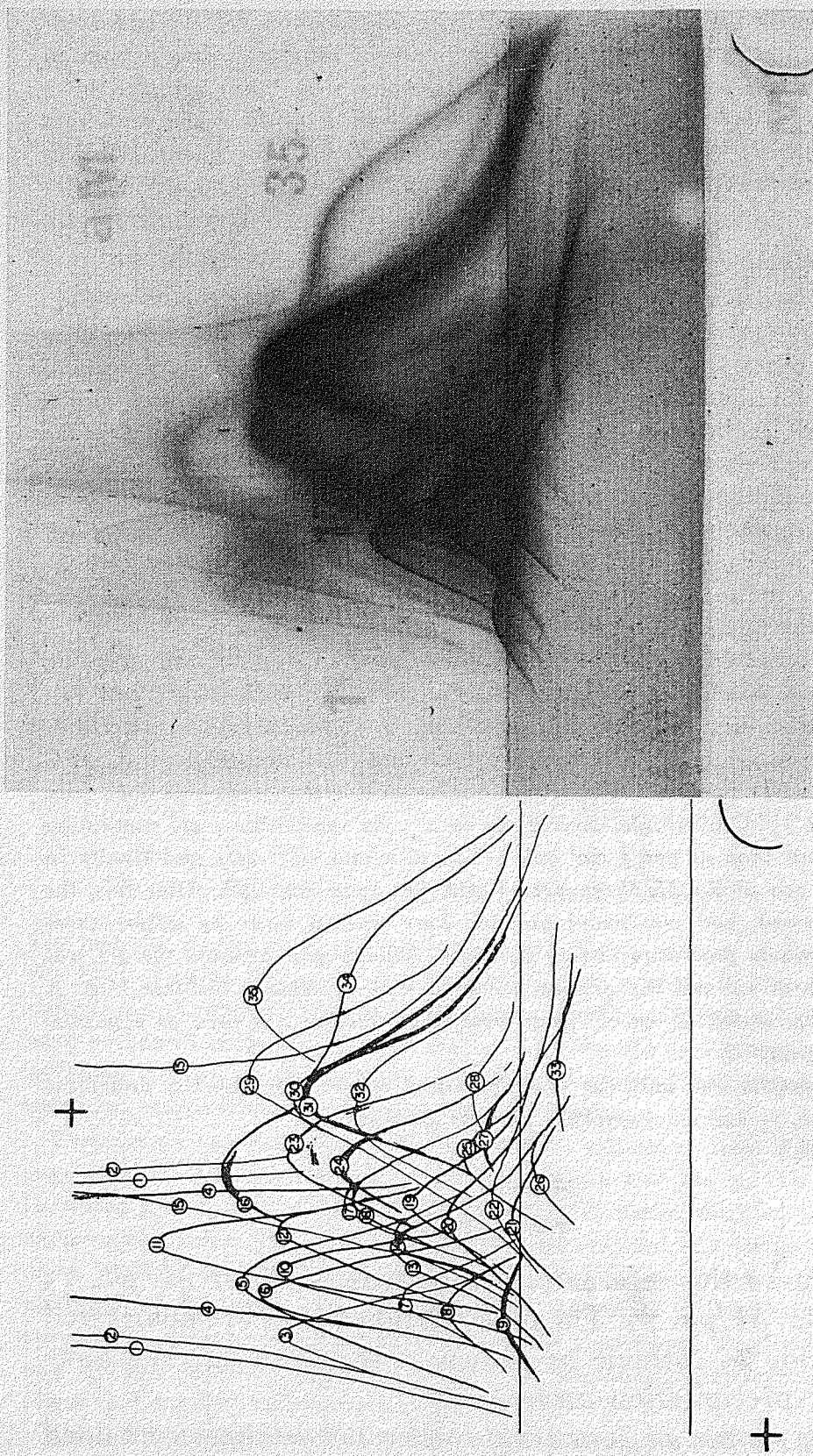


Fig. 1.

The appearance of the reference picture of crossed immunoelectrophoresis with *M.ph.73* and *Ra.M.ph.73* is shown in the drawing (left) and the plate (right). The precipitation curves are numbered from 1-35.

TABLE 1

*Age and Sex of 22 Mushroom Workers and 15 Control Persons Investigated for Precipitating Antibodies Against Micropolyspora phaeni.*

|                         | Sex |   | Age in years<br>(Mean and range) |
|-------------------------|-----|---|----------------------------------|
|                         | m   | f |                                  |
| Mushroom workers (n=22) | 16  | 6 | 42 (27-64)                       |
| Control group (n=15)    | 9   | 6 | 36 (25-59)                       |

TABLE 2

*Number of Sera with Precipitating Antibodies Against M. phaeni in Mushroom Workers and Control Subjects Demonstrated with Counter-electrophoresis (CE) and Crossed Immunoelectrophoresis (CRI).*

|                         | Total number<br>of sera with<br>precipitin(s) | Number of sera with<br>precipitin(s) in |     |
|-------------------------|-----------------------------------------------|-----------------------------------------|-----|
|                         |                                               | CE                                      | CRI |
| Mushroom workers (n=22) | 12                                            | 9                                       | 12  |
| Controls (n=15)         | 1                                             | 1                                       | 0   |

precipitating antibodies against *M. ph. 73*. Table 2 shows the results obtained with both CE and CRI. Nine mushroom workers had precipitating antibodies (41 per cent of the material) demonstrated with CE, whilst all the twelve mushroom workers (55 per cent of the material) with precipitating antibodies against *M. ph. 73* were found with CRI. The control group exhibited no precipitating antibodies against *M. ph. 73* except in one instance.

Fig. 2 demonstrates the counter-electrophoretic pattern from sera from patients 2, 4, 8 and 14. In patients 2 and 8 the precipitant lines are marked, while patients 4 and 14 demonstrate only slightly visible precipitant lines. In Fig. 3 the crossed immunoelectrophoretic pattern for *M. ph. 73* against Ra. *M. ph. 73* with patient serums 2 and 8, respectively, in the intermediate gel. In both instances, precipitate no. 17 decreased compared with the reference pattern (see Fig. 1). Patient

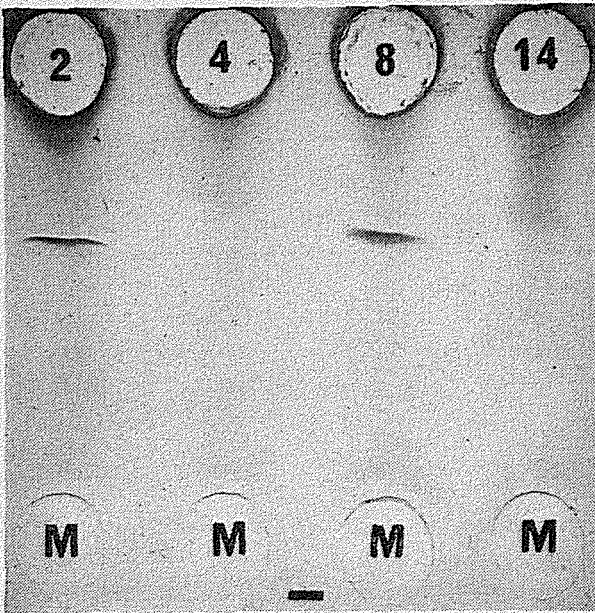


Fig. 2.

The pattern of counterelectrophoresis with *M. ph. 73* (M) and positive sera from patients 2, 4, 8 and 14. Marked precipitation lines are seen from patients 2 and 8.

no. 2 showed a marked precipitate, while patient no. 8 only demonstrated a faint precipitate.

In the nine patients with antibodies demonstrable with CE it was possible to identify the specific precipitation curve at CRI. In every instance, precipitate no. 17 was found (see Fig. 3). In four of the patients more than one precipitate was found at CRI, but it was impossible to identify the specific

TABLE 3

*The Relationship Between the Existence of Precipitating Antibodies Against M. phaeni and Employment in the Mushroom Trade for more than 5 Years.*

| Mushroom workers             | Working less<br>than 5 years | Working more<br>than 5 years |
|------------------------------|------------------------------|------------------------------|
| Precipitin(s) present (n=12) | 1<br>(8%)                    | 11<br>(92%)                  |
| Precipitin(s) absent (n=10)  | 7<br>(70%)                   | 3<br>(30%)                   |

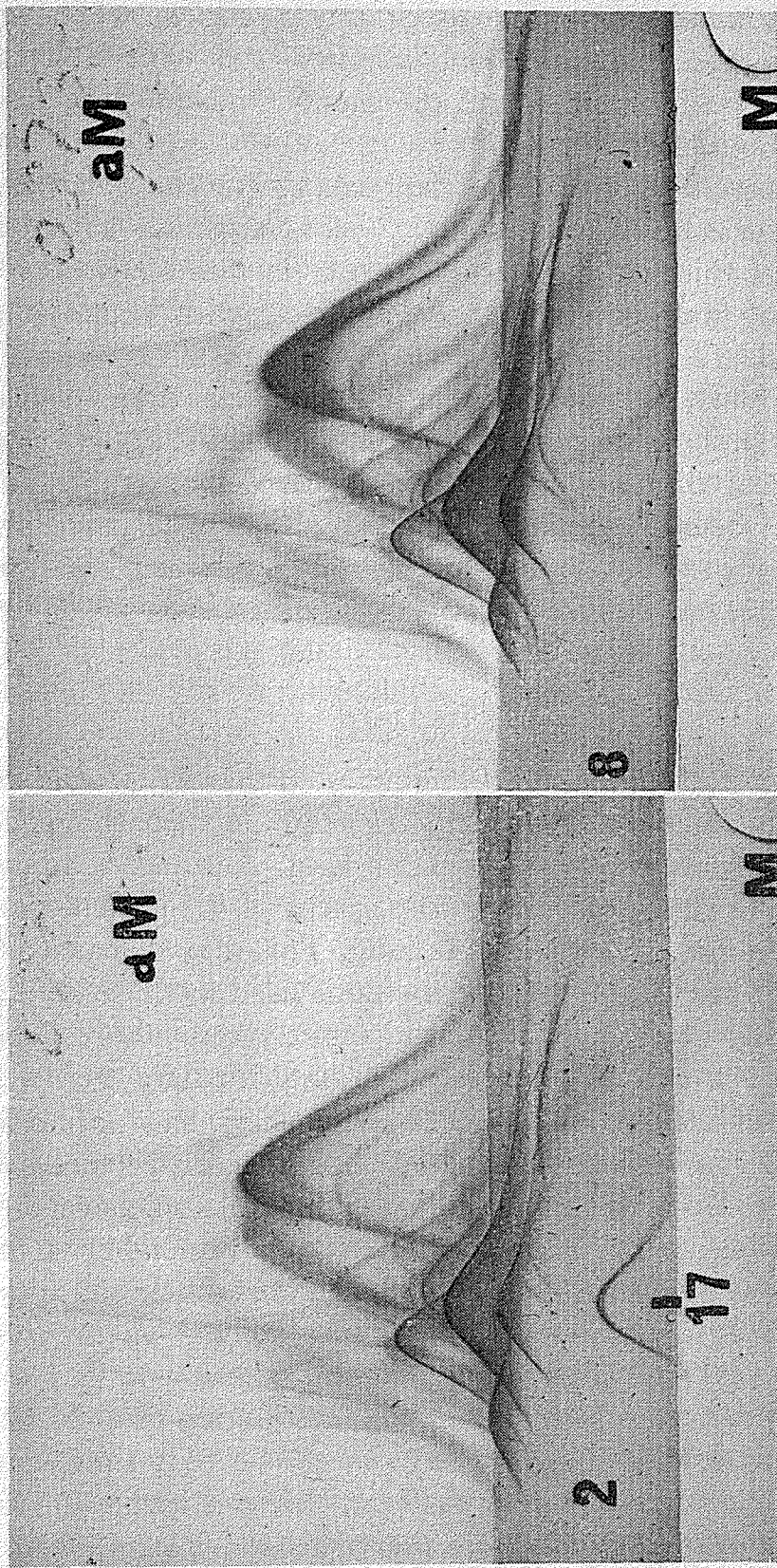


Fig. 3.

The crossed immunoelectrophoretic pattern of *M. ph. 73* (M) and Ra. *M. ph. 73* (aM) with patient sera containing precipitins against *M. ph. 73*. In both instances, precipitate no. 17 decreased.

precipitate number, as the precipitation curves were very faint. The three patients found positive only with CRI had weak precipitation curves and the number of the precipitate could not be identified.

As discussed later, a significant change in the cultivation procedure was introduced some 5 years ago and as a consequence of this, we have tried to correlate the existence of precipitating antibodies against *M. ph. 73* with employment in the trade for more than 5 years. The result is shown in Table 3. Only one person working for less than 5 years exhibited antibodies against *M. ph. 73*, whilst 11 persons (91 per cent of the positive sera) with demonstrable antibodies had been in the trade for more than 5 years. Three persons, 21 per cent of the total number working for more than 5 years, had no precipitating antibodies.

#### DISCUSSION

Both Pepys (5) and Sakula (7) suggested that *M. phaeni* and occasionally *Micropolyspora vulgaris* might be of pathogenetic importance for mushroom worker's lung. Antibodies against *M. phaeni* were found only in 50 per cent of the patients with documented mushroom worker's lung. This may be caused by the relative insensitivity of the methods used in these studies. In the present study, in which both counterelectrophoresis and crossed immunoelectrophoresis with intermediate gel were used, 55 per cent of the mushroom workers had antibodies against *M. phaeni*, although no-one had ever experienced the symptoms compatible with the disease in question. Several of those working in the trade for many years remembered that the symptoms used to be quite common amongst their colleagues some years ago. Only one worker, who started in the trade less than 5 years ago was sensitized, while those who had been in the trade for more than 5 years had antibodies in their sera. The explanation of these findings might be that the second phase in the compost process has been changed within the last 5-10 years in this country. The heating is now augmented to

80° C at which temperature the microorganisms are killed. At the same time mechanization has been promoted with a consequent decrease in the antigenic load. Only one of the controls had precipitating antibodies against *M. phaeni*, even though this is an ubiquitous microorganism. This could suggest that sensitization takes place only if the antigenic load is heavy and the organisms actively growing.

In the present study crossed immunoelectrophoresis with intermediate gel proved to be a little more sensitive than counterelectrophoresis. On the other hand, there was some doubt about the validity of the results obtained with crossed immunoelectrophoresis, when the counterelectrophoresis was negative for precipitating antibodies against *M. ph.* 73. On the basis of the present study, it is our opinion that counterelectrophoresis is, in any case, an excellent screening procedure for precipitating antibodies against *M. phaeni*. The two methods presented might be found useful in further work on the subject of type III reactions in the lungs.

#### SUMMARY

A new method for determining precipitating antibodies in allergic alveolitis is presented. The principle used is immunoelectrophoresis, either as a counterelectrophoresis or as a crossed immunoelectrophoresis with intermediate gel. Twenty-two mushroom workers without anamnestic signs of allergic alveolitis and 15 control persons were investigated. Twelve mushroom workers (55 per cent) had precipitating antibodies as determined by crossed immunoelectrophoresis, and nine of these persons had antibodies demonstrated with counterelectrophoresis. Thus, the sensitivity seems greater in the crossed immunoelectrophoresis, but we find that the simpler counterelectrophoresis is an excellent screening procedure in patients suspected of allergic alveolitis. In the controls only one person had precipitating antibodies, though the antigen tested for *Micropolyspora phaeni* exists ubiquitously. It seems probable from this study that the antigenic load is important, although

it is remarkable that no mushroom worker with precipitating antibodies against *M. phaeni* had ever experienced symptoms compatible with allergic alveolitis.

#### ACKNOWLEDGEMENTS

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*Author's address:*

*Birger Broch Møller*  
 Aprilvej 109  
 DK-2730 Herlev  
 Denmark

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