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by Heikkila P, Salmi T, Kotimaa M

Affiliation: Uusimaa Regional Institute of Occupational Health, Helsinki, Finland.

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Identification and counting of fungal spores by scanning electron microscopy

by Pirjo Heikkilä, MSc,[1] Tuula Salmi,[2] Marjut Kotimaa, LSc[3]

In the analysis of bioaerosols two different types of approaches can be employed. Either viable spores only can be used, or both viable and nonviable spores can be counted.

The most commonly used methods of the first category are based on impaction of the bioaerosols on surfaces containing a culture medium. The exposed plates are then incubated to develop colonies, after which the number of colony-forming units is determined. The widely used Andersen impactor is only suitable for stationary sampling however; at high spore concentrations its sampling time is on the order of minutes or even shorter.

Various types of filters have also been used to collect airborne microorganisms. The polycarbonate filters have proved useful for viable count estimation. The filters may be rinsed after sampling and the rinsing fluid spread on culture media, whereafter the plates are incubated and the number of colony-forming units counted (2). The total number of microorganisms (viable and nonviable) collected on the filter can be estimated with optical microscopy, with epifluorescence microscopy after fluorochrome staining (6), or with scanning electron microscopy (SEM).

Spore diameter is typically 0.7—9 μm; thus identification based on typical surface ornamentation is not easy with the light microscope because of the low magnification. With the fluorescence microscope nonspecific staining of organic material makes it difficult to distinguish microorganisms from other organic particles in the sample.

SEM has great focal depth and good resolution, and it thus allows a detailed examination of the shape and surface structure of spores. In this study we have compared sampling with Nuclepore filters combined with counting by SEM with sampling with an Andersen impactor combined with the counting of colony-forming units.

Materials and methods

Fungal spores were collected by the two methods in parallel in eight cow barns during foddering of the cattle.

Polycarbonate filters (37 mm, 0.2 μm; Nuclepore Corp, USA) in open-face plastic filter cassettes were used. Air volumes of 100—500 l were sucked through the filters at rates of 2—10 $l \cdot min^{-1}$. A 90° sector of each filter was gold-coated (JEOL JFC 1100) for the analysis. The microscopic specimen was taken from the middle of the sector and examined with an electron microscope (JEOL 100 CX ASID4) in SEM mode (40 kV). The number of spores was counted from at least 100 fields of view at a magnification of 3 000—5 000 × depending on the spore density on the filters.

Air samples for determining the concentration of viable microbes were taken with a six-stage fractionating impactor (model 10—800, Andersen Inc, Georgia, USA). The method included the determination of five mold groups (xerophilic fungi, mesophilic bacteria and fungi, thermotolerant fungi and thermophilic actinomycetes) from successive air samples. The volume of each sample was 15 l. The culture media and incubation temperatures were selected so as to favor the preparation of known or possible causative agents of allergic alveolitis. Pure cultures were made from five main species and groups. The positive hole correction method was used for the colony counts in the calculation of the concentrations (1).

Spores are released into the air both as single spores and as aggregates of two or more spores. An aggregate may form only one colony during cultivation. For a comparison of the results of the two methods both the number of aggregates and the number of total spores were counted. Asbestos counting criteria were used for the counting (3).

For the identification of different spores, reference samples of the five dominant groups grown in pure culture were obtained after a Nuclepore filter was pressed gently onto the culture, and the filter was then prepared for SEM analysis as usual. Spores collected in the cowbarns were identified by the SEM method in comparisons of their size, shape, and surface structure with those of the reference species. When required, we viewed the spores at a higher magnification than that applied for the spore counting.

Results

The number of colony-forming units obtained by the cultivation method was only 1—17 (mean 6) % of the

[1] Uusimaa Regional Institute of Occupational Health, Helsinki, Finland.
[2] Institute of Occupational Health, Helsinki, Finland.
[3] Kuopio Regional Institute of Occupational Health, Kuopio, Finland.

number of aggregates and 0.7—10 (mean 4) % of the total number of spores counted by SEM (table 1).

The median values of the intermethod concentration ratios were 1—11 % for the five dominant groups and the pooled other/unidentified species (table 2). The range of these ratios was very wide between the various barns. The large ranges may indicate different viability of the main species at different locations.

Discussion

A 10- to 100-fold difference existed between the results of the two methods regarding the total number of spores. The correlation of the logarithms of the total spore concentration given by these two methods was high. The correlation coefficient for the total spore concentration was 0.88, and the equation of the regression line was $y = 0.94\ x + 1.5$, where y is the logarithm of the concentration of aggregates measured with SEM and x is the logarithm of the concentration measured by the cultivation method.

The difference between the methods may be due either to differences in sampling efficiency or to the counting procedure itself. The sampling efficiency of a 37-mm open-face filter cassette is 90—100 % (4), and that of an Andersen impactor 100 % for particles with an aerodynamic diameter of less than 10 μm (1). Thus the difference would appear to result from a different method of counting, a finding indicating that only a small proportion of spores in the air is viable when collected with an Andersen impactor. Similar results have been obtained in a museum where the concentration of spores was 100 times higher when measured with epifluorescence microscopy than when measured with the cultivation method (5).

Spore identification by SEM is possible only with the use of pure cultures of the dominant species and through comparisons of their morphology with that of spores collected in a work environment. Species such as *Alternaria species, Absidia species, Aspergillus niger, Botrytis cinerea, Cladosporium,* and *Mucor* were also identified in the air of the cow barns by the cultivation method. The concentrations of each of these species were below 10 000 colony-forming units per cubic meter of air. Pure cultures of these species were not made, and thus they were not identified by the SEM.

For any morphological characteristic to have taxonomic value, its interspecific variation should exceed the intraspecific variation. All five of the main species groups, *Aspergillus sp, Aspergillus umbrosus, Aspergillus fumigatus, Penicillium sp* and *Actinomycetes,* showed characteristic surface ornamentation, and it was possible to distinguish them on the filters.

Table 1. Total concentrations of fungal and actinomycetous spores in eight cow barns ($10^3/m^3$). (cfu = colony-forming units, SEM = scanning electron microscopy)

Cow barn	I Cultivation (cfu)	SEM II Aggregation	SEM III Single	Ratio I/II (%)	Ratio I/III (%)
1	4 600	57 000	85 700	8	5
2	3 700	22 400	36 300	17	10
3	1 200	83 000	183 000	1	0.7
4	130	4 300	12 600	3	1
5	120	800	1 300	14	9
6	50	1 200	2 000	4	2
7	20	2 800	3 200	0.7	0.6
8	20	500	600	4	3
Mean	1 200	21 000	40 000	7	4

Table 2. Mean concentrations of the main groups of species in eight cow barns ($10^3/m^3$). (cfu = colony-forming units, SEM = scanning electron microscopy)

Species	I Cultivation method (cfu)	SEM II Aggregation	SEM III Single	Median ratio I/II (%)	Median ratio I/III (%)
Aspergillus sp	350	9 400	22 300	10	2
Asp umbrosus	310	3 000	3 200	6	6
Asp fumigatus	110	55	55	1	1
Actinomycetes	110	870	4 400	7	2
Penicillium	30	720	920	11	8
Others	61	7 400	9 200	4	1
Total	1 300	21 000	40 000		

This study indicates the potential usefulness of SEM in the identification of fungal spores. As both viable and nonviable spores have been implicated as causative agents of allergy, the SEM method would be better than the cultivation method for estimating total spore concentrations.

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