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Indirect Immunodetection of Fungal Fragments by Field Emission Scanning Electron Microscopy

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Submicronic fungal fragments have been observed in *in vitro* aerosolization experiments. The occurrence of these particles has therefore been suggested to contribute to respiratory health problems observed in mold-contaminated indoor environments. However, the role of submicronic fragments in exacerbating adverse health effects has remained unclear due to limitations associated with detection methods. In the present study, we report the development of an indirect immunodetection assay that utilizes chicken polyclonal antibodies developed against spores from *Aspergillus versicolor* and high-resolution field emission scanning electron microscopy (FESEM). Immunolabeling was performed with *A. versicolor* fragments immobilized and fixed onto poly-L-lysine-coated polycarbonate filters. Ninety percent of submicronic fragments and 1- to 2-μm fragments, compared to 100% of >2-μm fragments generated from pure freeze-dried mycelial fragments of *A. versicolor*, were positively labeled. In proof-of-concept experiments, air samples collected from moldy indoor environments were evaluated using the immunolabeling technique. Our results indicated that 13% of the total collected particles were derived from fungi. This fraction comprises 79% of the fragments that were detected by immunolabeling and 21% of the spore particles that were morphologically identified. The methods reported in this study enable the enumeration of fungal particles, including submicronic fragments, in a complex heterogeneous environmental sample.

Personal exposure to fungal aerosols in damp buildings has been associated with respiratory morbidity (1, 2). Fungal spore exposure levels in residential indoor environments seem too low to explain such an association (3). Submicronic fungal fragments observed *in vitro* during aerosolization experiments with fungal cultures are believed to contribute to the respiratory health problems observed in moldy indoor environments (4, 5). However, this role of submicronic fragments has remained unclear due to limitations associated with their quantification.

Airborne fungal particles have been shown to include spores in addition to larger and smaller (submicronic) fragments of spores and hyphae. These fragments may constitute a significant reservoir for antigens, allergens, and toxins in addition to spores. To date, the quantification of submicronic fungal fragments has remained technically challenging in environmental samples due to the lack of adequate detection and enumeration methods (6, 7). In this regard, the evaluation of the exposure burden of fungal submicronic fragments in fungally contaminated environments has been underestimated. *In vitro* studies that have evaluated the release of submicronic fragments have provided insight into the aerodynamic characteristics as well as the abiotic factors that influence the release of these particles. These laboratory studies of common indoor fungal isolates have shown the need to include the enumeration of submicronic fragments in addition to spores and larger fragments during exposure assessment of mold-contaminated environments (5, 8, 9).

Methodological advances have been made in a number of studies by using fungal membrane constituents such as ergosterol, phospholipid fatty acids, and (1→3)-β-D-glucans to demonstrate the presence of fungal biomass in size-fractionated fungal aerosols (9–16). Further, sugar alcohols (arabitol and mannitol) (17), enzymes (N-acetyl hexosaminidase and N-acetyl-D-glucosaminidase) (18–22), antigens, allergens (23–26), and DNA (27–29) have been used as proxies for total fungal exposure or occurrence of airborne fungal particles. However, none of these detection approaches enabled the detection or enumeration of fungal particles in the submicrometer size range. Quantifying particles in this size range will provide a more accurate assessment of fungal exposure due to the toxicological properties of very fine particles (<2.5 μm). In this regard, the toxicological properties of such particles has been shown to be more strongly correlated to their number and overall surface area than to their mass (30).

The immunostaining of allergens and surface antigens for microscopic visualization has enabled detection and quantification of large fungal particles (>1 μm), including spores and fragments (23, 31–33). However, the detection and morphological characterization of submicronic fragments by this technique have not been possible due to the methodological limitations associated with microscopic resolution (34). The adaptation of this technique for field emission scanning electron microscopy (FESEM) has contributed to an improved microscopic resolution (35) and

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has enabled the detection of immunolabeled particles in the submicrometer size.

In the present study, we describe a novel indirect immunostaining technique that utilizes FESEM to resolve and identify fungal fragments in the submicrometer size range. This method was further tested in proof-of-principle experiments with indoor air samples from a mold-contaminated school building.

MATERIALS AND METHODS

Preparation of fungal material for immunization. An isolate of *Aspergillus versicolor* (VI03554) was provided by the Section of Mycology, Norwegian Veterinary Institute. *A. versicolor* was selected because this species is a common contaminant of water-infiltrated building materials in indoor environments (36). The frozen isolate stock was revitalized on 2% malt extract agar (MEA) and allowed to grow for 14 days at 25°C. Conidia were collected by submerging the cultures in phosphate-buffered saline (pH 7.4; Sigma-Aldrich GmbH, Schnelldorf, Germany) containing 0.05% (vol/vol) Tween 20 (PBST) for 5 min. Spores were then gently scraped into the buffer solution. To dissociate aggregates, the conidial suspension was vortexed for 30 s, followed by sonication (Sonorex RK 510H; Bandalin Electric, Berlin, Germany) at 35 kHz for 5 min. After filtration through a 10-μm mesh using a Steriflip (Millipore), the filtrate was washed three times in PBS by centrifugation at 4,100 × *g*. The washing step was introduced to remove soluble antigens on the conidial surface.

Production of pIgY. A suspension of *A. versicolor* containing approximately 10^7 ml^{-1} conidia was sent to Norwegian Antibodies (Kroer, Aas, Norway) for the production of custom polyclonal chicken IgY (pIgY). Briefly, Lemon race hens (Oraug Killing Oppdrett, Askim, Norway) were immunized five times with *A. versicolor* conidia between the ages of 11 and 30 weeks. The eggs were collected, and pIgY antibodies were extracted from egg yolks and purified using chemical precipitation and centrifugation according to previously described manufacturer methods (Norwegian Antibodies). The final pIgY concentration was 12 mg ml^{-1} in PBS (pH 7.4) containing 0.02% NaN_3 to restrict bacterial or fungal contaminants. The pIgY was then used in subsequent development of the immunolabeling assay. The pIgY was not affinity purified.

Poly-L-lysine coating of polycarbonate filters. Each 37-mm-diameter and 0.4-μm-pore-size polycarbonate filter (Millipore, Tullagreen, Cork, Ireland) was placed in a 90-mm-diameter petri dish and covered with 1 ml poly-L-lysine solution (0.01%; molecular weight, 70,000 to 150,000; Sigma-Aldrich) for 15 min in a laminar flow hood at room temperature (RT). The remaining poly-L-lysine solution was removed and the filter air dried (37). The poly-L-lysine-coated filters were then mounted in standard aerosol cassettes (SKC Inc., Eighty Four, PA) prior to sampling. For sampling, particles were vacuumed onto the coated face of the filter.

Preparation of a positive-control aerosol sample for immunostaining. *A. versicolor* mycelial fragments (AVM) were prepared and used as a positive control for pIgY immunodetection experiments. Briefly, 4- to 5-day-old cultures were grown on a cellophane membrane covering MEA (38). The samples were harvested by scraping the mycelial mass into a 15-ml polypropylene centrifuge tube. The biomass was freeze-dried using a Drywinner 1.0 to 6.0 (Heto, Denmark) connected to a RZ2 vacuum pump (Vacuubrand Gmbh, Wertheim, Germany) for 22 h. The dried biomass was then ground in a Retch MM 301 mixer mill (Retsch Gmbh, Haan, Germany), and the resulting mycelial powder was vacuumed through a high-flow respirable-thoracic cyclone (GK2.69 Cyclone; BGI, Waltham, MA, USA) at 4.2 liters min^{-1} onto the poly-L-lysine-coated polycarbonate filters.

Preparation of negative-control aerosol samples for immunostaining. Wood dust (pine dust [PD]) was prepared from an artificially dried, planed, and heat-treated *Pinus sylvestris* wood sample without visible staining (Bergene Holm AS, Kirkenær, Norway). The wood dust was generated by sanding the wood piece with sandpaper (Mirox P120; Mirka, Finland) over a petri dish under sterile conditions. The dust was then vacuumed onto poly-L-lysine-coated filters as described above.

Fungal particle immunolabeling procedure. A 1/8 sector (ca. 1.2 cm^2) of the filter with vacuumed samples was cut and mounted onto a 25-mm double-sided carbon tab (Agar Scientific Ltd., Stansted, Essex, United Kingdom). Thereafter, the carbon tab was glued onto a 25-mm metal grid (NIOH, Norway), which served as a support. The samples were vapor fixed with glutaraldehyde overnight at RT in a fume hood to prevent the loss of soluble antigens (39). A sterile 9-mm-diameter petri dish chamber containing a cellulose pad soaked with 1 ml 25% glutaraldehyde (Sigma) was used during this procedure.

Following the fixation, samples were placed in a sterile 6-well plate (VWR, Norway), and free aldehyde sites were quenched for 2 times 5 min with 1 ml of 0.02 M glycine (Sigma). The samples were then blocked for 1 h at RT in 2 ml Tris-buffered saline (pH 8; Sigma) containing 0.05% Tween 20 and 5% skimmed milk (TBSTSM). Thereafter, the samples were incubated in 1 ml of anti-*A. versicolor* pIgY diluted in TBSTSM (1:100 equivalents to 120 μg ml^{-1}). After incubation for 1 h, samples were washed in 2 ml TBSTSM for 3 times 5 min. Following this washing step, samples were incubated, as above, in 1 ml of secondary antibody of goat anti-chicken H&L conjugated with 25-nm gold particles (Abcam, Cambridge, United Kingdom) diluted in TBSTSM (1:10). Reagent controls incubated with TBSTSM instead of primary antibodies were included in each experiment. In addition, negative controls using chicken IgY from unimmunized chicken (Gallus Immunotech Inc., Canada) at the same concentration were tested in order to evaluate the efficiency of the fungal immunization. After washing in TBSTSM followed by rinsing in 2 ml of BPC grade water (Sigma) for 3 times 5 min at RT, samples were then silver enhanced for 30 min at RT using 1 ml of Aurion kit (Aurion R-gent SE-LM; EMS, Hatfield, PA, USA). The enhancement was stopped by washing the samples in 2 ml water (biotechnology performance certified [BPC] grade; Sigma) for 3 times 3 min. Samples were then dried under sterile conditions for approximately 1 h at RT before being mounted on specimen mounting stubs (25 mm) that were sputter coated with 5- to 6-nm platinum in a Cressington 208HR sputter coater (Cressington Scientific Instruments Ltd., Watford, United Kingdom). During all incubation and washing steps, samples were subjected to gentle agitation.

Efficiency of fragment immobilization and immunolabeling. In order to check for the possible loss of sample particles during the immune labeling procedure, different sizes of *A. versicolor* mycelial fragments (positive controls) enumerated on three unlabeled filters were compared to fragments on three labeled filters. In both cases, the mycelial fragments were vacuumed onto poly-L-lysine-coated filters and vapor fixed as described above.

Fifty particles of each types were randomly selected from the positive controls (3 experiments) and were used to evaluate the efficiency of the immunolabeling procedure based on the number of gold particles per fragment type (40). The labeling efficiency was defined as the percentage of particles, in each size category, labeled in relation to the total particles present on the filter or per volume of air sampled. Fragments in the negative-control samples were similarly assessed in order to determine the maximal level of nonspecific labeling.

Crude protein extraction for cross-reactivity test. The anti-*A. versicolor* pIgY was tested for cross-reactivity toward 24 fungal species, 4 bacterial species, and 2 plant species (see Table 3). The tests were performed on water extracts to assess water-soluble antigens, whereas the insoluble fraction was tested after lysis using yeast protein extraction reagent (YPER; Thermo Fisher Scientific, Norway).

All fungal and 3 bacterial isolates (*Pseudomonas lurida*, *Bacillus subtilis*, and *Streptomyces coelicolor*) were grown in 80-mm petri dishes containing yeast extract sucrose (YES) medium (38) covered with sterile 90-mm polycarbonate membrane (0.22-μm pores; Millipore), as this technique eases the biomass collection. The petri dishes were inoculated with 200 μl of inoculum containing approximately 10^7 spores or bacteria. The dishes were sealed and incubated for 6 days at 25°C in humidified chambers (zip-lock bag and sterile-water-saturated paper). The filter membranes covered with fungal or bacterial biomass were then carefully

removed from the medium and transferred into 50-ml centrifuge tubes. The water-soluble proteins were extracted following the method described by Bridge (41). In short, fungal or bacterial biomass was separated from the filter by vortexing the filter membrane with 5 ml water (BPC grade water; Sigma) containing 1% (vol/vol) protease inhibitor (Thermo Fisher Scientific) and 10 Retsch metal beads (5 mm) for 10 min at room temperature. Aliquots (1.5 ml) of the biomass suspension were prepared and kept at −20°C until extraction of crude proteins. For water-soluble protein extraction, 1 ml of the aliquot was then transferred into an Eppendorf tube with screw cap and centrifuged at 13,000 × *g* at 4°C for 10 min. The supernatant was transferred into 1.8-ml cryotube vials (Nunc, Roskilde, Denmark) and kept at −20°C until crude proteins were quantified.

For the cyanobacterium *Arthrospira platensis*, 200 mg of the organism (obtained as dry powder) was transferred to an Eppendorf tube containing 300 mg borosilicate glass beads (Sigma) and 500 μl water (BPC grade water; Sigma). Samples were then agitated in a Mini Beadbeater (Biospec Products Inc., Bartlesville, OK, USA) for 5 times 1 min. The obtained suspension was then centrifuged as described above, and the supernatant was collected and stored at −20°C until crude protein quantification.

For extracts derived from plants, water-soluble crude proteins were extracted by grinding 600 mg (dry weight) whole-grain wheat flour from *Triticum aestivum* (sammalt mel; Norges Møller, Norway) or fresh needles from pine tree (*Pinus sylvestris*) in Retsch jars containing 5 ml water (BPC grade water; Sigma) with 10 metal beads for 3 times 5 min, with a 1-min break between the 5-min cycles. The obtained suspension was transferred to an Eppendorf tube and centrifuged as described above. The supernatant was kept at −20°C for protein quantification.

The lysates with YPER were prepared according to the manufacturer's instructions (Thermo Fisher Scientific). Briefly, ca. 200 ml of borosilicate solid-glass beads of 2 mm (Sigma) and 500 μl of YPER containing 1% protease inhibitor (vol/vol) were added to the remaining wet pellet (100 to 200 mg) after the water-extracted fraction from fungal and bacterial materials or ca. 500 to 600 mg from plant materials. The tubes were treated by bead beating for 5 times 1 min in a Mini Beadbeater followed by a 20-min treatment on a Vibramax 110 (Heidolph Instruments, Schwabach, Germany). Samples were thereafter centrifuged at 13,000 × *g* and 4°C for 10 min, and the supernatant was collected into cryotube vials. The procedure was repeated once, and the collected supernatants were combined and kept at −20°C until crude protein analysis.

The crude protein concentrations in both water extracts and lysates were determined using the bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific). Serial diluted bovine serum albumin (BSA) was used as a standard following the procedure from the manufacturer. The results of the BCA assay were used to dilute all extracts to a final crude protein concentration of 1 mg ml^{-1} prior to the cross-reactivity test of the *A. versicolor* pIgY.

Sandwich ELISA for cross-reactivity determination. For sandwich enzyme-linked immunosorbent assay (ELISA), 96-well Nunc Maxisorp microplates (Sigma-Aldrich) were coated overnight with 100 μl pIgY (12 μg ml^{-1}) in 0.1 M sodium carbonate buffer, pH 9.6 (Sigma), at 4°C. The plates were washed 3 times with 200 μl PBS containing 0.05% (vol/vol) Tween 20 (PBST with pH 7.4), and the remaining free binding sites were blocked with PBST containing 5% skimmed milk (SM; Sigma) for 1 h at RT. Water extracts or lysates diluted in PBST–5% SM (final protein content, 10 μg in 100 μl) were added to each well, and the mixtures were incubated at 37°C for 2 h. Water (BPC grade; Sigma) and YPER (100 μl) used for extract and lysate preparation were included as negative controls. After plate washing as described above, 100 μl of alkaline phosphatase conjugated to pIgY (stock, 1 mg ml^{-1}; Abcam, Cambridge, United Kingdom) and diluted (1:100) in PBST with 5% SM was added, and plates were further incubated for 2 h at RT. After washing, as above, the wells were incubated with 100 μl p-nitrophenyl phosphate disodium (Sigma-Aldrich) in 0.05 M Na_2CO_3 for 30 min, and the absorbance was measured as optical density at 405 nm (OD_{405}) using a Spectromax i3 instrument equipped with SoftMax Pro 6 software (Molecular Devices, LLC, Sunnyvale, CA, USA). All cross-reactivity experiments were run in triplicate and repeated three times.

Environmental sample analysis. Environmental samples were collected from a school with water infiltration and visible mold growth. Samples were collected during renovation work. Airborne particles were collected onto poly-L-lysine-coated polycarbonate filters in open 37-mm-diameter standard SKC cassettes made of conductive polypropylene. During sampling, the cassettes were mounted 1.5 m above ground level and were connected to a vacuum pump, Gast Model D0A-P109-FD (Gast Manufacturing Inc., Benton Harbor, MI, USA). A mass flow meter (822 Top-Track; Sierra Instruments, Monterey, CA) was placed between the cassette and the pump to determine the flow measurement. The mean flow rates varied between 18.7 liters min^{-1} at the start to 17.2 liters min^{-1} at the end of sampling. Three samples were collected during 1-h intervals. Collected particles were stored at RT until immunostaining.

FESEM analysis. Samples were visualized using an SU 6600 FESEM (Hitachi, Ibaraki-Ken, Japan). The FESEM was operated in low vacuum (25 Pa), and imaging was performed in the back scatter mode. Acceleration voltage of 15 kV, extraction voltage of 2.10 kV, and working distances of 6 to 7 mm were used. Size-stratified counts of fragments were performed for the evaluation of immobilization efficiency using the poly-L-lysine coating followed by glutaraldehyde fixation. In short, fragments defined as submicronic fragments (0.2- to 0.5-μm fragments and 0.5- to 1-μm fragments) were counted at a magnification of ×3,000 in 200 fields of a defined inner counting field (37.833 μm by 28.13 μm) of 1,064 μm^2 each. Larger fragments (1- to 2-μm fragments, 2- to 3.5-μm fragments, and >3.5-μm fragments) were also counted at the same magnification, but only in 100 fields. The lowest detectable number of particles on the filter was 2×10^3 to 4×10^3.

The number of gold particles per fragment was assessed by counting gold particles attached to 50 randomly selected fragment types in the positive and negative controls. The enumeration was performed in back scattered electron (BSE) imaging mode at a magnification of ×15,000 to ×25,000 depending on the fragment type. Different numbers of gold particles counted on fragments in positive and negative controls were used to establish the thresholds to correctly classify fragments as containing fungal antigens in field samples. In this regard, 0.20- to 1-μm, 1- to 2-μm, 2- to 3.5-μm, and >3.5-μm fragments labeled with ≥1, ≥2, ≥2, and ≥4 gold particles, respectively, were considered correctly labeled fragments in the environmental samples. Total numbers of the four types of fragments (submicronic fragments, 1- to 2-μm fragments, 2- to 3.5-μm fragments, and >3.5-μm fragments) as well as the gold-stained fungal fragments were enumerated in each environmental sample. Spore particles (single spores and aggregates of 2, 3, 4, and ≥5 spores) recognized by their morphology were also counted. Enumeration in the environmental samples was performed on 100 fields (inner field of 18.915 μm by 14.065 μm, i.e., 266 μm^2 each) at a magnification of ×6,000. Particle distribution on the filter was homogeneous as confirmed by the Poisson distribution test for each type of particles ($SD^2/AM \approx 1$, where SD is the standard deviation and AM is the arithmetic mean number of counts per field of all counted fields) (42). The lowest number of detectable particles in field samples was 1.5×10^4 m^{-3}.

Data analysis. The Student *t* test was used for arithmetic mean (AM) absorbance comparison in the cross-reactivity experiments and for the AM particle number comparison in the fragment immobilization experiments. STATA SE 13.1 (Statacorp LP, College Station, TX) was used for statistical tests and Microsoft Office Excel 2007 for graphs.

RESULTS

Immunolabeling efficiency and fragments immobilization on filter. The binding of pIgY to *A. versicolor* fragments was demonstrated by the immunogold labeling of fungal fragments in positive-control samples. Overall, fungal fragments with homogenously distributed gold particles on their surface were observed

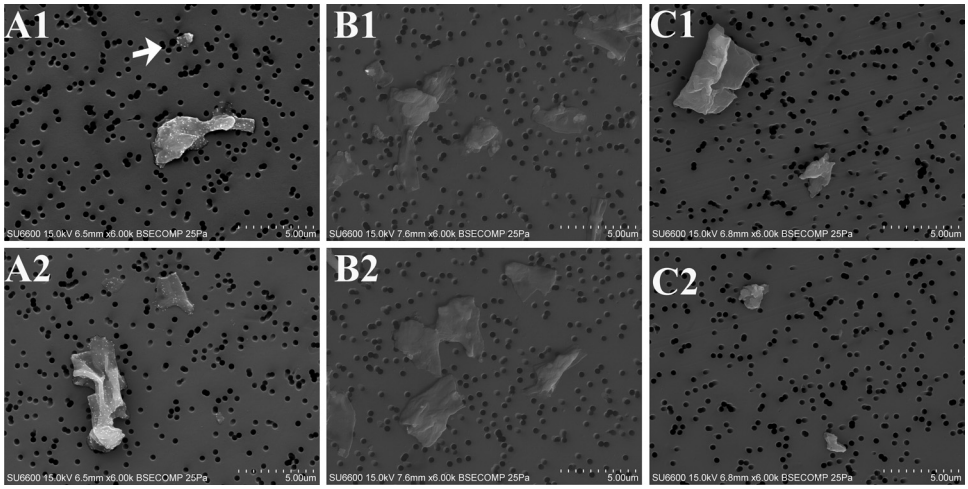

FIG 1 Micrographs of controls: mycelial fragments from *Aspergillus versicolor* as positive controls labeled with IgY anti-*A. versicolor* (A1 and A2) and with IgY isotope (B1 and B2) and pine dust (C1 and C2) as negative controls. Arrows showed immunolabeled submicronic fragments in the positive control. White spots represent silver-enhanced gold particles. Scale, 5 μm.

(Fig. 1A1 and A2). These data indicated the presence of antigens that bind to pIgY.

The average numbers of gold particles on each fragment type in the positive-control samples were significantly higher than those of the negative controls and the background (Table 1). Positive controls stained by chicken IgY isotope showed labeling levels similar to those of the negative controls (Fig. 1B1 and B2). Particles smaller than 1 μm from the negative-control samples (Fig. 1C1 and C2) showed no gold particles binding to the surface. Nonspecific staining was observed for 0%, 10%, 10%, and 14% of enumerated 0.2- to 1-μm, 1- to 2-μm, 2- to 3.5-μm, and >3.5-μm fragments, respectively (Fig. 2B).

The efficiency of immunolabeling of the positive controls is shown in Fig. 2A. We found that 90% of submicronic fragments (0.2 to 1 μm) were stained while 90, 100, and 100% of 1- to 2-μm fragments, 2- to 3.5-μm fragments, and ≥3.5-μm fragments, respectively, were labeled. Minor nonspecific background labeling was additionally observed (Table 1).

TABLE 1 Number of gold particles per fragment type in the positive and negative controls

Sample type, fragment size range, control type	No. of gold particles				No. of sample particles correctly classified
	AM[a]	SD	Min	Max	
Background (n = 100)	0.4	0.64	0	2	
Controls[b]					
0.2–1 μm					
Negative	0	0	0	0	50
Positive	10.8	8.5	0	30	45
1–2 μm					
Negative	0.1	0.3	0	1	45
Positive	21.4	14.4	0	47	45
2–3.5 μm					
Negative	0.1	0.3	0	1	45
Positive	66.02	24.2	19	124	50
>3.5 μm					
Negative	0.24	0.69	0	3	43
Positive	151.9	73.3	50	405	50

[a] AM, arithmetic mean.
[b] n = 50 for each set of negative or positive controls.

The immunogold labeling procedure did not influence the number of immobilized fragments (<3.5 μm) on the filter. In this regard, the comparison of the mean number of fragment particles in the five size ranges on unlabeled filter to the mean number on labeled filters revealed no significant difference for different types of fragments with length of <3.5 μm. However, the number of ≥3.5-μm fragments was significantly higher on labeled filters than on unlabeled filters (2.00×10^6 versus 1.78×10^6, $P = 0.03$) (Table 2). The total number of all fragments on the labeled filters was similar to and not statistically different from the number on the unlabeled filter (2.73×10^6 versus 2.49×10^6; $P = 0.1$).

pIgY cross-reactivity. The results from the cross-reactivity experiments are summarized in Table 3. *A. versicolor* and 19 of the other 23 fungal species reacted with the pIgY raised against *A. versicolor*. The absorbance of the lysates from *Fomitopsis pinicola*, *Mucor mucedo*, *Saccharomyces cerevisiae*, and *Phoma herbarum* did not vary from the control, while the water extracts showed significant differences. Moreover, the lysates from *Eurotium amstelodami* and *Stachybotrys chartarum* showed significantly higher absorbance than the control.

Both water extracts and lysates from *Verticillium lecanii* (Hypocreales), *Geotrichum candidum* (Saccharomycetales), *Botrytis cinerea* (Helotiales), and *Acremonium strictum* (Hypocreales) showed no significant difference compared to the control samples. The absorbance of the water extracts and the lysates derived from the plant materials (wheat flour and pine needles) was similar to that of the controls and showed the absence of antigens that react with the pIgY. Similarly, the pIgY showed no reactivity to the tested bacteria except for the Actinomycetes species *Streptomyces coelicolor*.

Environmental sample analysis. Based on the maximal number of gold particles on the background in the positive- and negative-control samples and on different fragments in the negative controls, a threshold of 2 and 3 gold particles was used to enumerate <3.5-μm fragments and ≥3.5-μm fragments, respectively, as gold labeled. Immunogold-labeled fragments in environmental

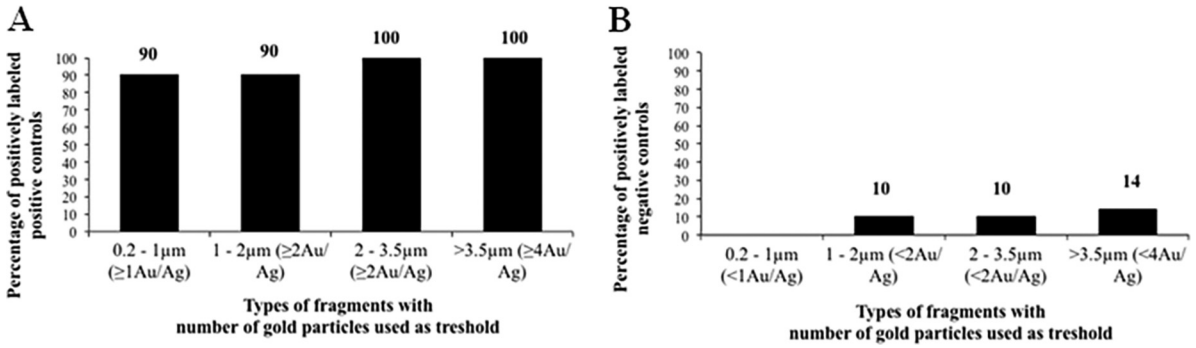

FIG 2 Unspecific labeling with pine dust as a negative control (A) and labeling efficiency with fungal fragments from *A. versicolor* as a positive control (B). Bars represent means from three repetitions. Error bars represent standard deviations.

samples are shown in Fig. 3. The most abundant particle types in the environmental samples were fragments of different sizes and shapes with an arithmetic mean concentration of 10.9×10^6 m^{-3}. These particles represented on average 93% of all particles, while the spore particles constituted only 7% (arithmetic mean, 1.1×10^6 m^{-3}) (Table 4).

In total, 1.2×10^6 m^{-3} fragments, of 12×10^6 m^{-3} total particles, were labeled with gold and represented about 10% of the total number of particles present on the filter. Regarding the larger fragments, 44, 73, and 82% of 1- to 2-μm fragments, 2- to 3.5-μm fragments, and ≥3.5-μm fragments were immunolabeled, respectively (Table 5). Only 6% of all submicronic fragments collected were labeled with gold. Overall, the immunogold labeling showed that 10% the total fragments was of fungal origin. The average number of spore particles identified by their morphology (not labeled by the antibody) was 1.1×10^6 m^{-3}, representing 21% of all estimated fungal particles. The fungal fraction of the aerosols is composed of 39%, 40%, 19%, and 2% of submicronic fragments (0.2 to 1 μm), large fragments (>1 μm), single spores, and spore aggregates, respectively (Table 4).

DISCUSSION

The results show that the novel immunogold labeling method described here is valuable for detection and enumeration of fungal fragments in FESEM. The development of this method comprises four major steps: (i) the production of the egg yolk polyclonal antibodies IgY against *A. versicolor*, (ii) the immobilization of sampled particles onto polycarbonate filters coated with poly-L-lysine, (iii) the immunolabeling of fungal derived particles with the pIgY, and (iv) the detection and enumeration of immunolabeled particles using FESEM. The labeling ability of the pIgY antibodies was confirmed by the immunogold labeling of positive controls (mycelial fragments from *A. versicolor*). The specificity of the antibody was tested in cross-reactivity experiments with materials of various origins, including plant, fungi, and bacteria using a developed sandwich enzyme-linked immunosorbent assay. As proof of principle, the new method was applied to environment samples derived from a built environment with characterized water infiltration and fungal contamination. The results demonstrated that the immunolabeling approach enabled the identification and enumeration of fungal particles other than spores in complex environmental samples through the immunolabeling of exposed surface antigens. Submicronic fungal particles were successfully labeled with the anti-*A. versicolor* pIgY and detected by FESEM. To our knowledge, the immunodetection and enumeration of submicronic fungal fragments have not been possible using preexisting methods of assessing fungal exposure.

TABLE 2 Comparison of numbers of particles immobilized on poly-L-lysine-coated filters without and with immunolabeling procedure[a]

Particle size range (μm) and type	AM (SD) of no. of particles ($\times 10^4$)/filter		*t* test *P* value
	Unstained filters	Stained filters	
0.2–0.5, submicronic fragments	7.0 (0.8)	9.9 (2.8)	0.2
0.5–0.1, submicronic fragments	9.9 (2.8)	8.3 (2.0)	0.5
1–2, fragments	14.4 (1.3)	14.8 (0.3)	0.6
2–3.5, fragments	40 (8.3)	39.4 (6.2)	0.9
>3.5 fragments	178 (8.2)	200 (9.4)	**0.03**
Total	249 (19)	272.7 (12)	0.1

[a] $n = 3$. AM, arithmetic mean.

The results derived from positive-control experiments confirmed that artificially generated fungal fragments, including submicronic fragments, contain surface antigens that could be labeled and detected by FESEM. Our finding is in agreement with Górny et al., who used an ELISA to demonstrate that *in vitro*-aerosolized fungal fragments from *A. versicolor* and *Penicillium melinii* contained antigens on fragments in the submicrometer size (4).

Visually, the gold-labeled antigens were homogeneously distributed on the surface of various types of fragments. The sensitivity of this immunostaining approach using positive controls was 90% for 0.2- to 1-μm and 1- to 2-μm fragments, and 100% for 2- to 3.5-μm and >3.5-μm fragments. The reason why 10% of the submicronic fragments and those of 0.2 to 1 μm were not labeled is unclear. We speculate that this could be due to the density of the accessible antigenic sites, so that the likelihood of particles without binding sites on their surface increases with decreasing particle size. A further explanation could be that glutaraldehyde vapor fixation may denature epitopes and decrease antibody binding events (43).

Negative-control experiments showed that 0%, 10%, 10%, and 14% of 0.2- to 1-μm, 1- to 2-μm, 2- to 3.5-μm, and >3.5-μm fragments, respectively, in the pine dust samples were incorrectly labeled as positive fragments. It is likely that this was due to nonspecific binding. However, the labeling intensity on the negative

TABLE 3 List of organisms and materials tested for cross-reactivity and mean absorbance compared to controls[a]

Kingdom, phylum, order	Species and strain	Water extract		YPER lysate	
		AM (SD)	*P* value	AM (SD)	*P* value
	Controls	0.185 (0.011)		0.186 (0.082)	
Fungi					
Ascomycota					
Eurotiales	*Aspergillus versicolor* VI03554	0.725 (0.234)	**0.02**	0.469 (0.125)	**0.03**
	Aspergillus niger VI06015	1.096 (0.451)	**0.03**	0.668 (0.051)	**0.001**
	Eurotium amstelodami VI05331	0.648 (0.279)	0.05	0.533 (0.056)	**0.004**
	Paecilomyces variotii UMB_AT01	0.779 (0.223)	**0.01**	0.711 (0.190)	**0.01**
	Penicillium chrysogenum VI04528	1.100 (0.421)	**0.02**	0.843 (0.043)	**0.0003**
Dothideales	*Aerobasidium pullulans* VI05029	0.669 (0.205)	**0.02**	0.631 (0.099)	**0.004**
Sordariales	*Chaetomium globoseum* VI05046	0.825 (0.296)	**0.02**	0.432 (0.038)	**0.009**
Capnodiales	*Cladosporium cladosporioides* VI04541	0.518 (0.155)	**0.02**	0.490 (0.143)	**0.03**
Incerta sedis	*Epicoccum nigrum* VI05728	0.433 (0.144)	**0.04**	0.404 (0.106)	0.05
Saccharomycetales	*Candida albicans* VI05945	0.496 (0.127)	**0.01**	0.296 (0.089)	0.2
	Saccharomyces cerevisiae VI05951	0.635 (0.190)	**0.01**	0.309 (0.102)	0.2
	Geotrichum candidum VI03865	0.370 (0.124)	0.06	0.251 (0.066)	0.3
Pleosporales	*Phoma herbarum* VI05764	0.433 (0.140)	**0.04**	0.525 (0.294)	0.1
	Ulocladium chartarum VI04844	0.746 (0.234)	**0.01**	0.662 (0.018)	**0.001**
	Alternaria alternata VI06044	0.673 (0.264)	**0.03**	0.608 (0.108)	**0.006**
Hypocreales	*Stachybotrys chartarum* VI03618	0.754 (0.342)	0.05	0.511 (0.113)	**0.02**
	Trichoderma harzianum VI04074	0.422 (0.097)	**0.01**	0.457 (0.128)	**0.04**
	Verticillium lecanii VI03418	0.299 (0.101)	0.1	0.225 (0.025)	0.5
	Acremonium strictum VI05921	0.314 (0.095)	0.08	0.266 (0,018)	0.2
Helotiales	*Botrytis cinerea* UMB101BC	0.281 (0.085)	0.1	0.218 (0.084)	0.7
Zygomycota					
Mucorales	*Mucor mucedo* UMBM808	0.333 (0.086)	**0.04**	0.254 (0.075)	0.3
	Rhizopus microsporus UMB_AT02	1.214 (0.376)	**0.009**	1.700 (0.275)	**0.001**
Basidiomycota					
Polyporales	*Fomitopsis pinicola* VI06099	0.439 (0.108)	**0.02**	0.274 (0.104)	0.3
Wallemiales	*Wallemia sebi* UMB_AT03	1.088 (0.304)	**0.007**	2.417 (0.348)	**0.0004**
Bacteria					
Proteobacteria					
Pseudomonadales	*Pseudomonas lurida* UMBPSLH406	0.265 (0.061)	0.09	0.230 (0.076)	0.5
Firmicutes					
Bacillales	*Bacillus subtilis* UMB15022013	0.317 (0.102)	0.09	0.325 (0.100)	0.1
Cyanobacteria					
Oscillatoriales	*Arthrospira platensis* (*Spirula nutrex*)	0.180 (0.040)	0.9	0.234 (0.099)	0.6
Actinobacteria					
Actinomycetales	*Streptomyces coelicolor* UMB/ATCCBAA471	0.565 (0.182)	**0.02**	0.498 (0.136)	**0.03**
Plants					
Pinophyta					
Pinales	*Pinus sylvestris* (Pine needles)	0.261 (0.083)	0.2	0.224 (0.087)	0.6
Angiosperms					
Poales	Wheat flour (*Triticum aestivum*)	0.248 (0.045)	0.08	0.253 (0.075)	0.4

[a] Three experiments were conducted in triplicate for water extracts and YPER lysates; *P* values (test versus control) were obtained by a *t* test of the AM of extracts or lysates to respective controls; values in boldface indicate significant differences ($P < 0.05$). Abbreviations: AM, arithmetic mean; UMB, Norwegian University of Life Science, Institute of Chemistry, Biotechnology and food science, Section of Environmental microbiology; VI, Norwegian Veterinary Institute, Section of Mycology.

controls was significantly lower than on the positive controls. There were no gold particles on most of the fragments derived from negative-control samples. Similarly, there was limited gold particle binding on the background. In contrast, positive-control particles showed abundant gold particles binding on the surface of the fragments.

The immobilization of fragments on a membrane has not, to our knowledge, been previously reported. This methodological approach enabled the assessment of a broader range of particle types to be immunolabeled without loss of the particles on the sampling filter. The labeling without extraction of particles from the filter is believed to contribute to a better characterization of the bioaerosol composition, since disturbance of particles after collection is limited. Still, the significant difference between labeled filters and unlabeled filters for larger particles (≥3.5-μm fragments) indicates that aggregates of larger fragments may dissociate into smaller units during the aqueous staining process. It is possible that larger fragments are more unstable than smaller fragments on the filters, and this is likely to cause mechanical stress that can break the fragments into smaller pieces. Further investigation is

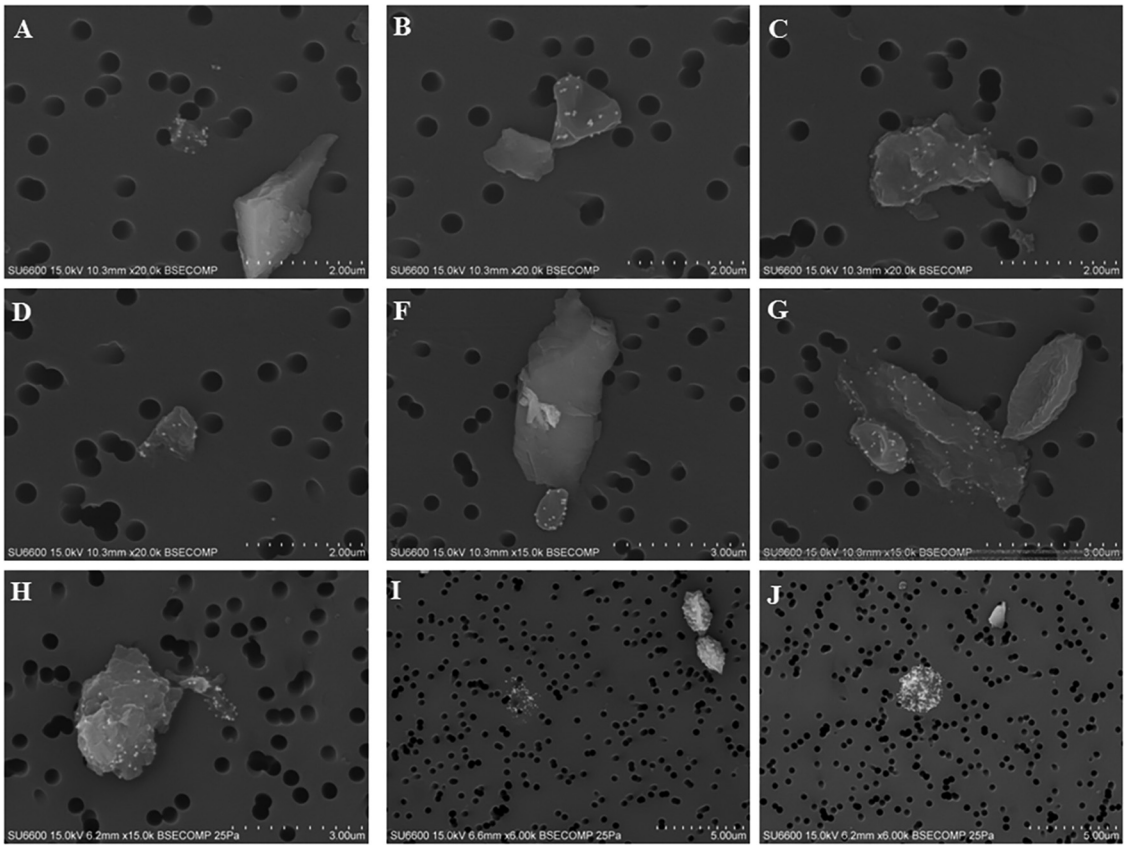

FIG 3 Immunogold labeling of fragments from environmental airborne samples. Micrographs show labeled submicronic fragments (A, B, D, and F), unlabeled submicronic fragment (B), labeled large fragments (C, G, H, I, and J), unlabeled larger fragments (A and F), and unlabeled spores (G and I). White spots represent silver-enhanced gold particles. Scale, 2 μm (A to H) and 5 μm (I and J).

therefore required to better understand the phenomenon behind this significant increase in the number of larger fragments during the immunolabeling procedure.

The determination of the presence of airborne fungal fragments has been based on visualization of hypha-like fragments or fragments with melanization or septation using light or scanning electron microscopy (44–46). The sizes of observed fragments ranged between 2.5 and 100 μm, and concentrations varied between 3 and 3×10^6 m^{-3} in outdoor settings. In indoor environments, proportions between 4 and 33% of the total particle count have been published (6). The application of the halogen immunostaining assay (HIA) has permitted the enumeration of allergen-containing spores and fragments larger than 2.5 μm (33). To date, there is no fungal exposure assessment methodology technique that has enabled the enumeration of fungal particles smaller than 2.5 μm, including submicronic fragments, to evaluate the fungal aerosols within fungal indoor environments.

The application of this novel immunodetection assay to environmental samples has revealed the presence of immunolabeled fungal fragments of various sizes and morphologies. Fungal spores were also present but were identified by their morphology since they were not labeled by the chicken antibodies. In the proof-of-concept experiments, our results showed that the fungal aerosols comprise higher numbers of antigen-containing fragments (79%) than spore particles (21%). The overall quantified fungal aerosols reveal that 39%, 40%, 19%, and 2% were submicronic fragments, large fragments (>1 μm), single spores, and spore aggregates, respectively. In contrast to earlier studies that utilized the HIA, 25% of larger fragments (>2.5 μm) contained fungal allergen (33). In the present study, the immunogold assay labeled 73 to 82% of larger fragments (>2 μm). This difference may be associated to the assay efficiency or the size range that was wider in the present study. Other studies that focused on the occurrence of submicronic fragments in mold-contaminated indoor or outdoor air used methods to detect β-glucan (14, 16, 18, 47) and NAHA (21) as biomarkers of fungal fragments in size-fractionated samples. However, these studies were limited by the imprecise fractionation of particles due to confounding variables such as spore bounce (18, 47, 48). Further, these studies could not predict the number of submicronic fragments present in the air. The results from our proof-of-principle experiments are the first, to our knowledge, that report the number of submicronic fragments in mold-contaminated air samples.

The pIgY raised against *A. versicolor* reacts with water extracts and lysates from 20 of 24 tested fungal isolates. Similar results have been reported with polyclonal antibodies (pAbs) raised against spores from *A. versicolor* (49–51) or other fungal species (52) where high levels of cross-reactivity were observed. Even monoclonal antibodies developed after immunization of animals with spores/conidia were found not to be species specific (49, 53). Common and evolutionarily conserved fungal proteins have been suggested to explain this high level of cross-reactivity between

TABLE 4 Concentration of total particles, labeled fungal fragments, and spore particles in environmental samples[a]

Particle types	Total particles		Labeled fragments and spore particles	
	AM[b] of concn (min–max)	Mean % of total collected particles	AM of concn (min–max)	Mean % of immunolabeled fragments and spores (morphologically identified)
Nonfungal fragment particles				
Submicronic fragments	9.8 (7.4–11.3)	81	0.5 (0.4–0.7)	39
1–2-μm fragments	0.6 (0.4–0.9)	5.0	0.2 (0.2–0.3)	17
3–3.5-μm fragments	0.2 (0.1–0.3)	2.0	0.2 (0.05–0.3)	12
≥3.5-μm fragments	0.3 (0.03–0.7)	2.5	0.2 (0.030–0.5)	11
All fragment particles	10.9 (7.9–12.5)	91	12 (0.9–1.4)	79
Spores				
Single spores	0.9 (0.090–2.1)	7	0.9 (0.090–2.1)	19
Aggregates of 2 spores	0.1 (0–0.4)	1	0.1 (0–0.4)	1
Aggregates of 3 spores	0.03 (0–0.07)	0.2	0.03 (0–0.07)	0
Aggregates of 4 spores	0.01 (0–0.04)	0.1	0.01 (0–0.04)	0.6
Aggregates of ≥5 spores	0.01 (0–0.04)	0.1	0.01 (0–0.04)	0.6
All spore particles	1.1 (0.09–2.6)	9	1.1 (0.09–2.6)	21
All particles	12 (8–15.2)		1.6 (0.9–2)	

[a] Values for concentrations are $\times 10^6$ m^{-3}; the experiments were repeated three times.
[b] AM, arithmetic mean.

fungi. These proteins have been identified as (i) glyceraldehyde-3-phosphate dehydrogenase, (ii) a putative sorbitol or xylose reductase, (iii) catalase A, (iv) enolase, and (v) malate dehydrogenase. These products have been localized both on the outer membrane and in the cytoplasm of many fungi (51, 53). Given the solubility of many fungal antigens in water as shown by the high reactivity of the water extracts, vapor fixation with glutaraldehyde seemed efficient in the preservation of antigens for immunodetection (39). This approach also efficiently prevented nonspecific staining of the background, which was a major problem with aqueous fixation (results not shown).

Our primary goal in this study was to discriminate fungal fragments, including submicronic fragments from plant and bacterial sources, which was achieved except for the actinomycete *S. coelicolor*. Despite the observed cross-reactivity toward most tested fungal materials and the tested actinomycete, the chicken polyclonal antibodies still have potential for achieving this goal. However, the cross-reactivity of the pIgY antibodies prevents the identification of fungal fragments to the species level.

TABLE 5 Proportion of immunolabeled fragments per fragment type in environmental samples[a]

Particle type	AM[b] of proportion of labeled particles (min–max) (%)
Submicronic fragments	5.6 (3.5–7.3)
1–2-μm fragments	44 (31–57)
3–3.5-μm fragments	73 (43–90)
≥3.5-μm fragments	82 (69–100)
All fungal fragments	10.9 (10.7–11.1)

[a] The experiment was repeated three times.
[b] AM, arithmetic mean.

Conclusions. The immunolabeling method and the enumeration of a broad range of airborne fungal particles have the potential to selectively quantify fungal fragments, including submicronic fragments in complex heterogeneous environmental samples. Although identification to the species level is not possible, the present study demonstrated that fragments with exogenous fungal antigens could be labeled and enumerated in environmental samples using the novel immunogold technique and FESEM. Spores were not labeled but could be recognized by morphology. As a result, this methodological approach has the potential to further enable a detailed characterization of the fungal aerosol profile, which has not been possible to date.

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REFERENCES

1. **Institute of Medicine.** 2004. Human health effects, p 189–243. *In* Damp indoor spaces and health. National Academies Press, Washington, DC.
2. **World Health Organization.** 2009. Guidelines for indoor air quality: dampness and mould. WHO Regional Office for Europe, Copenhagen, Denmark.
3. **Eduard W.** 2009. Fungal spores: a critical review of the toxicological and epidemiological evidence as a basis for occupational exposure limit setting. Crit Rev Toxicol **39:**799–864. http://dx.doi.org/10.3109/10408440903307333.
4. **Górny RL, Reponen T, Willeke K, Robine E, Boissier M, Sergey A, Schmechel D, Grinshpun SA.** 2002. Fungal fragments as indoor air biocontaminants. Appl Environ Microbiol **68:**3522–3532. http://dx.doi.org/10.1128/AEM.68.7.3522-3531.2002.
5. **Górny RL.** 2004. Filamentous microorganisms and their fragments in indoor air—a review. Ann Agric Environ Med **11:**185–197.
6. **Green BJ, Schmechel D, Summerbell R.** 2011. Aerosolized fungal fragments, p 211–243. *In* Adan OCG, Samson RA (ed), Fundamentals of mold growth in indoor environments and strategies for healthy living. Wageningen Academic Publishers, Wageningen, Netherlands.
7. **Méheust D, Le Cann P, Reboux G, Millon L, Gangneux J-P.** 2014. Indoor fungal contamination: health risks and measurement methods in hospitals, homes and workplaces. Crit Rev Microbiol **40:**248–260. http://dx.doi.org/10.3109/1040841X.2013.777687.
8. **Cho S-H, Seo S-C, Schmechel D, Grinshpun SA, Reponen T.** 2005. Aerodynamic characteristics and respiratory deposition of fungal fragments. Atmos Environ **39:**5454–5465. http://dx.doi.org/10.1016/j.atmosenv.2005.05.042.
9. **Seo S-C, Reponen T, Levin L, Grinshpun SA.** 2009. Size-fractionated (1→3)-β-D-glucans concentrations aerosolized from different moldy building materials. Sci Total Environ **407:**806–814. http://dx.doi.org/10.1016/j.scitotenv.2008.10.018.
10. **Douwes J, Zuidhof A, Doekes G, van der Zee SC, Wouters I, Boezen MH, Brunekreef B.** 2000. (1→3)-β-D-Glucans and endotoxin in house dust and peak flow variability in children. Am J Respir Crit Care Med **162:**1348–1354. http://dx.doi.org/10.1164/ajrccm.162.4.9909118.
11. **Reeslev M, Miller M, Nielsen KF.** 2003. Quantifying mold biomass on gypsum board: comparison of ergosterol and beta-*N*-acetylhexosaminidase as mold biomass parameters. Appl Environ Microbiol **69:**3996–3998. http://dx.doi.org/10.1128/AEM.69.7.3996-3998.2003.
12. **Lau APS, Lee AKY, Chan CK, Fang M.** 2006. Ergosterol as a biomarker for the quantification of the fungal biomass in atmospheric aerosols. Atmos Environ **40:**249–259. http://dx.doi.org/10.1016/j.atmosenv.2005.09.048.
13. **Womiloju TO, Miller JD, Mayer PM, Brook JR.** 2003. Methods to determine the biological composition of particulate matter collected from outdoor air. Atmos Environ **37:**4335–4344. http://dx.doi.org/10.1016/S1352-2310(03)00577-6.
14. **Reponen T, Seo S-C, Grimsley LF, Lee T, Crawford C, Grinshpun SA.** 2007. Fungal fragments in moldy houses: a field study in homes in New Orleans and Southern Ohio. Atmos Environ **41:**8140–8149. http://dx.doi.org/10.1016/j.atmosenv.2007.06.027.
15. **Seo S-C, Reponen T, Levin L, Borchelt T, Grinshpun SA.** 2008. Aerosolization of particulate (1→3)-β-D-glucans m moldy materials. Appl Environ Microbiol **74:**585–593. http://dx.doi.org/10.1128/AEM.01791-07.
16. **Seo S-C, Choung JT, Cehn BT, Lindsley WG, Kim KY.** 2014. The level of submicron fungal fragments in homes with asthmatic children. Environ Res **131:**71–76. http://dx.doi.org/10.1016/j.envres.2014.02.015.
17. **Bauer H, Claeys M, Vermeylen R, Schueller E, Weinke G, Berger A, Puxbaum H.** 2008. Arabitol and mannitol as tracers for the quantification of airborne fungal spores. Atmos Environ **42:**588–593. http://dx.doi.org/10.1016/j.atmosenv.2007.10.013.
18. **Madsen AM, Schlünssen V, Olsen T, Sigsgaard T, Avci H.** 2009. Airborne fungal and bacterial components in PM1 dust from biofuel plants. Ann Occup Hyg **53:**749–757. http://dx.doi.org/10.1093/annhyg/mep045.
19. **Rylander R, Salobir B, Tercelj M.** 2014. Measurements of NAHA to assess indoor fungal exposure. Chest **145**(3 Suppl)**:**251A. http://dx.doi.org/10.1378/chest.1813834.
20. **Rylander R, Reeslev M, Hulander T.** 2010. Airborne enzyme measurements to detect indoor mould exposure. J Environ Monit **12:**2161–2164. http://dx.doi.org/10.1039/c0em00336k.
21. **Adhikari A, Reponen T, Rylander R.** 2013. Airborne fungal cell fragments in homes in relation to total fungal biomass. Indoor Air **23:**1–6. http://dx.doi.org/10.1111/ina.12014.
22. **Tercelj M, Salobir B, Narancsik Z, Kriznar K, Grzetic-Romcevic T, Matos T, Rylander R.** 2013. Nocturnal asthma and domestic exposure to fungi. Indoor Built Environ **22:**876—880. http://dx.doi.org/10.1177/1420326X12460255.
23. **Popp W, Zwick H, Rauscher H.** 1988. Indirect immunofluorescent test on spore sampling preparations: a technique for diagnosis of individual mold allergies. Stain Technol **63:**249–253.
24. **Horner WE, Helbling A, Salvaggio JE, Lehrer SB.** 1995. Fungal allergens. Clin Microbiol Rev **8:**161–179.
25. **Kurup VP.** 2003. Fungal allergens. Curr Allergy Asthma Rep **3:**416–423. http://dx.doi.org/10.1007/s11882-003-0078-6.
26. **Green BJ, Tovey ER, Sercombe JK, Blachere FM, Beezhold DH, Schmechel D.** 2006. Airborne fungal fragments and allergenicity. Med Mycol **44**(Suppl 1)**:**S245–S255.
27. **Williams RH, Ward E, McCartney HA.** 2001. Methods for integrated air sampling and DNA analysis for detection of airborne fungal spores. Appl Environ Microbiol **67:**2453–2459. http://dx.doi.org/10.1128/AEM.67.6.2453-2459.2001.
28. **Vesper S.** 2011. Traditional mould analysis compared to a DNA-based method of mould analysis. Crit Rev Microbiol **37:**15–24. http://dx.doi.org/10.3109/1040841X.2010.506177.
29. **Reponen T, Vesper S, Levin L, Johansson E, Ryan P, Burkle J, Grinshpun SA, Zheng S, Bernstein DI, Lockey J, Villareal M, Khurana Hershey GK, LeMasters G.** 2011. High environmental relative moldiness index during infancy as a predictor of asthma at 7 years of age. Ann Allergy Asthma Immunol **107:**120–126. http://dx.doi.org/10.1016/j.anai.2011.04.018.
30. **Nygaard UC, Samuelsen M, Aase A, Løvik M.** 2004. The capacity of particles to increase allergic sensitization is predicted by particle number and surface area, not by particle mass. Toxicol Sci **82:**515–524. http://dx.doi.org/10.1093/toxsci/kfh287.
31. **Tovey ER, Green BJ.** 2005. Measuring environmental fungal exposure. Med Mycol **43**(Suppl 1)**:**S67–S70.
32. **Green BJ, Schmechel D, Sercombe JK, Tovey ER.** 2005. Enumeration and detection of aerosolized Aspergillus fumigatus and Penicillium chrysogenum conidia and hyphae using a novel double immunostaining technique. J Immunol Methods **307:**127–134. http://dx.doi.org/10.1016/j.jim.2005.10.001.
33. **Green BJ, Sercombe JK, Tovey ER.** 2005. Fungal fragments and undocumented conidia function as new aeroallergen sources. J Allergy Clin Immunol **115:**1043–1048. http://dx.doi.org/10.1016/j.jaci.2005.02.009.
34. **Green BJ, Millecchia LL, Blachere FM, Tovey ER, Beezhold DH, Schmechel D.** 2006. Dual fluorescent halogen immunoassay for bioaerosols using confocal microscopy. Anal Biochem **354:**151–153. http://dx.doi.org/10.1016/j.ab.2006.03.035.
35. **Sercombe JK, Eduard W, Romeo TC, Green BJ, Tovey ER.** 2006. Detection of allergens from Alternaria alternata by gold-conjugated anti-human IgE and field emission scanning electron microscopy. J Immunol Methods **316:**167–170. http://dx.doi.org/10.1016/j.jim.2006.08.016.
36. **Flannigan B, Miller JD.** 2011. Microbial growth in indoor environments, p 57–107. *In* Flannigan B, Samson RA, Miller JD (ed), Microorganisms in home and work environments: diversity, health impacts, investigation and control, 2nd ed. Taylor & Francis, Abingdon, England.
37. **Fischer ER, Hansen BT, Nair V, Hoyt FH, Dorward DW.** 2012. Scanning electron microscopy. Curr Protoc Microbiol **Chapter 2:**Unit 2B.2. http://dx.doi.org/10.1002/9780471729259.mc02b02s25.
38. **Samson RA, Frisvad JC, Hoekstra E.** 2004. Introduction to food and airborne fungi. Centraalbureau voor Schimmelcultures, Utrecht, Netherlands.
39. **Grote M.** 1991. Immunogold electron microscopy of soluble proteins: localization of Bet v I major allergen in ultra-thin sections of birch pollen after anhydrous fixation techniques. J Histochem Cytochem **39:**1395–1401. http://dx.doi.org/10.1177/39.10.1940310.
40. **Griffiths G, Hoppeler H.** 1986. Quantitation in immunocytochemistry: correlation of immunogold labeling to absolute number of membrane antigens. J Histochem Cytochem **34:**1389–1398. http://dx.doi.org/10.1177/34.11.3534077.
41. **Bridge P.** 1996. Protein extraction from fungi, p 39–48. *In* Doonan S (ed), Methods in molecular biology, 2nd ed. Humana Press, Totowa, NJ.
42. **Eduard W, Aalen O.** 1988. The effect of aggregation on the counting

precision of mould spores on filters. Ann Occup Hyg **32:**471–479. http://dx.doi.org/10.1093/annhyg/32.4.471.
43. **Mendell JR, Whitaker JN.** 1978. Immunocytochemical localization studies of myelin basic proteins. J Cell Virol **76:**502–511.
44. **Pady S, Kramer C.** 1960. Kansas aeromycology VI: hyphal fragments. Mycologia **52:**681–687. http://dx.doi.org/10.2307/3755867.
45. **Pady S, Gregory P.** 1963. Numbers and viability of airborne hyphal fragments in England. Trans Br Mycol Soc **46:**609–613. http://dx.doi.org/10.1016/S0007-1536(63)80063-7.
46. **Li D-W, Kendrick B.** 1995. A year-round comparison of fungal spores in indoor and outdoor air. Mycologia **87:**190–195. http://dx.doi.org/10.2307/3760904.
47. **Singh U, Reponen T, Cho KJ, Grinshpun SA, Adhikari A, Levin L, Indugula R, Green BJ.** 2011. Airborne endotoxin and β-D-glucan in PM1 in agricultural and home environments. Aerosol Air Qual Res **11:**376–386.
48. **Lindsley WG, Schmechel D, Chen BT.** 2006. A two-stage cyclone using microcentrifuge tubes for personal bioaerosol sampling. J Environ Monit **8:**1136–1142. http://dx.doi.org/10.1039/b609083d.
49. **Schmechel D, Simpson JP, Lewis DM.** 2005. The production and characterization of monoclonal antibodies to the fungus Aspergillus versicolor. Indoor Air **15**(Suppl 9)**:**S11–S19.
50. **Shi C, Belisle D, Miller JD.** 2011. Quantification of the Aspergillus versicolor allergen in house dust. J Immunol Methods **372:**89–94. http://dx.doi.org/10.1016/j.jim.2011.06.034.
51. **Liang Y, Zhao W, Xu J, Miller JD.** 2011. Characterization of two related exoantigens from the biodeteriogenic fungus Aspergillus versicolor. Int Biodeterior Biodegradation **65:**217–226. http://dx.doi.org/10.1016/j.ibiod.2010.11.005.
52. **Van Emon JM, Reed AW, Yike I, Vesper SJ.** 2003. ELISA measurement of stachylysin in serum to quantify human exposures to the indoor mold Stachybotrys chartarum. J Occup Environ Med **45:**582–591. http://dx.doi.org/10.1097/01.jom.0000071503.96740.65.
53. **Benndorf D, Müller A, Bock K, Manuwald O, Herbarth O, von Bergen M.** 2008. Identification of spore allergens from the indoor mould Aspergillus versicolor. Allergy **63:**454–460. http://dx.doi.org/10.1111/j.1398-9995.2007.01603.x.