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ORIGINAL ARTICLE/ARTICLE ORIGINAL

# Surveillance of fungal allergic sensitization using the fluorescent halogen immunoassay

*Surveillance de la sensibilisation aux allergènes fongiques par la technique d'immunofluorescence*

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Received 4 August 2009; accepted 8 October 2009

## KEYWORDS

Allergen;  
Fungi;  
Mold;  
*Alternaria*;  
*Cladosporium*;  
Conidia;  
Hyphae;  
Immunoassay

## Summary

**Objective.** – Conidia derived from a small number of common fungal genera are widely accepted as the etiological agents responsible for fungal allergic sensitization. The contribution of fungal conidia, spores, airborne hyphae, and subcellular fragments from other uncharacterized fungal genera remains unclear. In this proof-of-concept study, we examined the composition of myco-aerosols that atopic women were exposed and sensitized to in their own indoor environment using the fluorescent halogen immunoassay (fHIA).

**Patients and methods.** – Myco-aerosols were collected onto mixed cellulose ester protein binding membranes (PBMs) for 30 min with volumetric air sampling pumps. The PBMs were laminated with an adhesive cover slip and indirectly immunostained with individual patient serum IgE using the fHIA. Samples were examined using confocal laser scanning microscopy and immunostained particles were expressed as a percentage of total particles.

**Results.** – All air samples contained a broad spectrum of fungal spores, conidia, hyphae, and other fungal particulates. Airborne concentrations varied between individual study participant environments. Positively immunostained conidia belonging to moniliaceous amero-spores, *Cladosporium*, *Alternaria*, and many unknown species were observed in the majority of air samples. Other fungal genera including *Bipolaris*, *Curvularia*, *Pithomyces*, and *Stachybotrys*, in addition to, ascospore genera and dematiaceous hyphal fragments released detectable allergen. Twelve

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**MOTS CLÉS**

Allergènes ;  
 Champignons ;  
 Moisissures ;  
*Alternaria* ;  
*Cladosporium* ;  
 Conidies ;  
 Hyphes ;  
 Immunoessai

percent of all fHIA haloes quantified in the analysis were directed towards fungal particles. No immunostaining was detected to conidia belonging to *Epicoccum*, *Fusarium*, and *Spiegazzinia* species.

**Conclusion.** — In addition to characterized fungal aeroallergens, we observed a wider composition of fungi that bound human IgE. Field surveillance studies that utilize immunodiagnostic techniques such as the fHIA will provide further insight into the diversity of fungi that function as aeroallergen sources in individual study participant environments.

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**Résumé**

**Objectif.** — Les conidies provenant d'un petit nombre de genres fongiques communs sont généralement connues comme des agents étiologiques responsables de sensibilisation allergique. La contribution de conidies, de spores, d'hyphes aéroportées et de fragments subcellulaires provenant de genres fongiques non identifiés reste obscur. Dans cette étude, nous avons examiné la composition d'aérosols fongiques auxquels des femmes atopiques sont exposées et sensibilisées dans leur propre environnement domestique en utilisant une technique d'immunofluorescence indirecte (fHIA).

**Patients et méthodes.** — Les aérosols fongiques sont recueillis sur membrane de cellulose (PBMA) pendant 30 minutes avec une pompe volumétrique. Les membranes sont alors recouvertes d'une feuille adhésive et traitées par les IgE sériques de chaque patientes selon la technique fHIA. Les échantillons sont examinés au microscope à balayage laser confocal et les particules immunofluorescentes sont dénombrées en pourcent des particules totales.

**Résultats.** — Tous les échantillons d'air contiennent un large spectre de spores fongiques, de conidies, d'hyphes et autres particules fongiques. Les concentrations de matériel fongique aéroporté varient selon l'environnement individuel. Des conidies fluorescentes appartenant aux genres *Cladosporium* et *Alternaria* et à de nombreuses espèces inconnues sont observées dans la majorité des échantillons. D'autres genres fongiques comme *Bipolaris*, *Curvularia*, *Pithomyces* et *Stachybotrys*, des champignons ascospores et des dématiées génèrent des allergènes détectables. Douze pour cent des particules fluorescentes quantifiées dans cette analyse sont d'origine fongique. Aucune conidie appartenant aux genres *Epicoccum*, *Fusarium* et *Spiegazzinia* n'a été détectée.

**Conclusion.** — En plus des allergènes fongiques aéroportés détectés, nous avons observé une large gamme de champignons qui fixent les IgE. Les études de surveillance de terrain qui utilisent les techniques d'immunodiagnostic telles que la fHIA fourniront davantage d'informations sur la diversité des champignons générateurs d'allergènes aéroportés dans l'environnement domestique.

Publié par Elsevier Masson SAS.

**Introduction**

Exposure assessment scientists characterize personal fungal exposure as the inhalation of fungal conidia derived from environmentally abundant and morphologically discernible fungal genera, such as *Alternaria*, *Aspergillus*, *Cladosporium*, and *Penicillium*. These fungal genera are the most widely recognized etiological agents associated with fungal allergic sensitization [11], and respiratory morbidity [5,29,49]. However, the contribution of fungal conidia, hyphae, and subcellular fragments from uncharacterized fungal genera remains unclear because of the methodological challenges associated with immunodiagnosis, as well as identifying fungi in environmental samples [19].

Compared to other perennial and seasonal allergens, fungal bioaerosols are viable heterotrophic microorganisms. Some fungal conidia possess unique virulence factors that facilitate colonization by reducing respiratory cilia beat frequency [4,6,10,50]. Depending on the site of deposition, some fungi may also germinate and release a complex assortment of mycotoxins, antigens, and other immunostimulatory macromolecules [15]. Germination is required for

fungal colonization (chronic rhinosinusitis and invasive aspergillosis), and may result in further individual exposure to fungal allergens [15,32,48]. Because of these aspects, the characterization of fungal allergens has lagged behind other aeroallergen sources. Complex life cycles, rich biodiversity, and variability of fungal allergens have all hindered understanding fungal allergic sensitization. These confounding factors have also influenced the standardization of commercial extracts available for diagnosis and have limited the availability of diagnostic reagents for many uncharacterized fungi [51].

New developments in immunodiagnostic methodologies, such as the Halogen Immunoassay (HIA), have enhanced the ability to collect, and detect allergic sensitization in patients exposed to myco-aerosols [32,20,21,38,37,52]. The uniqueness of this method is that it provides a method to match the spectrum of an individual's allergic responses with the inherent diversity of bioaerosols collected from the patient's own environment. Compared to other commercially available immunodiagnostic tests, the HIA correlates with Phadia ImmunoCap technologies but less well to skin prick testing [21]. Specifically, eluted surface antigens bind in

close proximity to the particle and are immunostained with the patient's serum immunoglobulin E (IgE). IgE from sensitized patients binds to the immobilized bioaerosol antigens and following secondary detection, a halo of immunostaining will illuminate the particle.

Studies utilizing HIA have provided insight into the contribution of fungal particulates to the aeroallergen load, particularly discrete dematiaceous hyphal fragments lesser than 100  $\mu\text{m}$  long, partial multicellular conidia, and subcellular fragments [19,16]. However, several limitations have hindered the performance of the HIA as a field surveillance methodology. Alkaline phosphatase-based enzymatic methodologies can detect nanogram quantities of antigen. Given the picogram quantities of antigen released from the smaller fungal spores and fragments, this had limited the ability to immunostain these particles in air samples [19]. Recent improvements utilizing Alexa Fluor<sup>®</sup> (Molecular Probes, Inc., Eugene, OR) secondary detection and confocal laser scanning microscopy have mitigated these limitations and enabled the detection of moniliaceous amerspores (colorless unicellular conidia) and fungal fragments as small as 0.5  $\mu\text{m}$  [19,20]. The development of the fluorescent HIA (fHIA) is an important immunodiagnostic development given the recent observations of elevated concentrations of smaller fungal particulates in indoor and occupational environments [14,22,30].

In this observational study, we explored the feasibility of the fHIA as a field surveillance methodology to detect individual fungal allergic sensitization profiles in a cohort of atopic women. Compared to traditional immunodiagnostic methods, it is possible using the fHIA to understand the complete composition of mycoaerosols to the aeroallergen load.

## Materials and methods

### Human subjects

Serum samples were collected from 24 women living in New York City. Each subject was enrolled in a prospective longitudinal study of an existing birth cohort of Puerto Rican ethnicity, where the subject (mother of newborn infant of Puerto Rican ethnicity) had a clinical history of asthma, allergic rhinitis, and/or inhalant allergy [1]. Following serum collection, all samples were stored in aliquots for future use at  $-70^{\circ}\text{C}$ . The Columbia University Medical Center Institutional Review Board approved the study (AAAA-9880), and the subjects gave written informed consent following a full explanation of the study.

### Personal air sampling

The sampling train consisted of a button sampling head (SKC Ltd, Eighty Four, PA) connected to a SKC Airchek 2000 sampling pump (SKC Ltd, Eighty Four, PA) that provided a constant 3.0 liters per minute (lpm) air flow through a mixed cellulose ester protein binding membrane (PBM). The button sampling head was sterilized prior to use and fitted with a 0.8  $\mu\text{m}$  pore size mixed cellulose ester PBM (Millipore Corp., Bedford, MA) for use in the fHIA. Compared to other samplers, the button sampling head allows

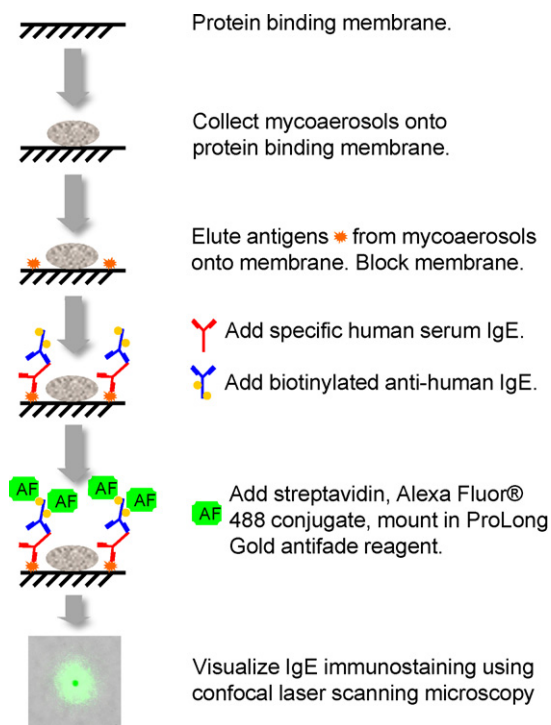
better dispersion of particles across the PBM especially when air currents are turbulent [2]. Air samples were collected from 24 homes located in New York City for a period of 30 minutes, in the room identified as the study participant's bedroom as previously described [1]. These were homes of participants in a birth cohort study designed to characterize the factors that contribute to allergen exposure and allergic sensitization. During the air sampling, dust samples were vacuumed (Eureka Mighty Mite, Bloomington, IL) from the subjects bed for a period of 3 minutes and from the surrounding floor also for 3 minutes; thus the air sample was not collected during quiescent conditions. At the completion of each sampling interval, the PBM was removed from the button sampler and stored at room temperature until fHIA analysis. The sampling pumps were post-calibrated, and measurements ranged from 2.8–3.02 lpm.

### Fluorescent Halogen Immunoassay

The PBM was removed from the button sampling head, placed in a humid chamber overnight to enable conidia germination and immunostained using the fHIA as described previously [16]. The germination of fungal conidia increases the detection thresholds of the immunoassay [15,32]. Impacted fungal particles were permanently laminated to the mixed cellulose ester PBM by overlaying the sample with an optically clear adhesive/glass coverslip [20]. The laminated samples were immersed in borate buffer (pH 8.2) at room temperature for three hours to enable antigens and other macromolecules to elute from the fungal particles and bind in close proximity to the PBM. Membranes were blocked in 5% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 90 minutes. PBMs were incubated overnight at  $4^{\circ}\text{C}$  with individual patient serum diluted 1:3 in 5% BSA/PBS/0.05% Tween 20 [20]. After the primary antibody incubation, the membranes were rinsed three times in PBS/0.05% Tween 20 and incubated for 1.5 h with biotinylated goat anti-human IgE (Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted 1:500. Following incubation, the membranes were rinsed three times in PBS/0.05% Tween 20 and then incubated for 3 h with streptavidin, Alexa Fluor<sup>®</sup> 488 conjugate (green fluorescence; Molecular Probes, Inc., Eugene, OR) diluted 1:500 in 5% BSA/PBS/0.05% Tween 20. This procedure was followed by rinsing the membranes three times in  $\text{dH}_2\text{O}$ . Each sample was then mounted on a microscope slide in ProLong<sup>®</sup> Gold (Molecular Probes, Inc., Eugene, OR) antifade reagent. The principle of the fHIA is outlined in Fig. 1.

Negative control treatments consisted of the collection of culture-derived *Alternaria alternata* (ATCC 11612), *Cladosporium herbarum* (ATCC 6506), *Epicoccum nigrum* (ATCC 34929), and *Paecilomyces variotii* (ATCC 66705) conidia and hyphae onto PBMs as previously described [20]. Each negative control PBM was processed in parallel by substituting human atopic IgE with:

- non-atopic human IgE, biotinylated goat anti-human IgE, and streptavidin, Alexa Fluor<sup>®</sup> 488 conjugate;
- biotinylated goat anti-human IgE, and streptavidin, Alexa Fluor<sup>®</sup> 488 conjugate;
- Streptavidin, Alexa Fluor<sup>®</sup> 488 conjugate.



**Figure 1** Experimental flow chart of the fHIA.  
Schéma du protocole de la technique fHIA.

### Confocal Laser Scanning Microscopy Images

Confocal laser scanning images were captured using a Zeiss LSM 510 laser scanning confocal system (Carl Zeiss Inc., Thornwood, NY) with an Axioplan 2 microscope, 40 × C-Apochromat water immersion objective lens, and Argon and HeNe lasers. The images of human IgE-immunostained fungal particles were captured using 488 nm excitation and a narrow emission filter bandwidth (505–550 nm). Fluorescent and differential interference contrast images (DIC) were captured using Zeiss software version 3.2 (Carl Zeiss Inc., Thornwood, NY). All settings on the confocal microscope remained constant in the analysis of positive samples and negative controls.

An unblinded examiner examined entire membranes and the number of positively immunostained samples were recorded and expressed as percentages of totals. Fungi were identified to genus level using morphologically discernible phenotypic features including, conidia shape, size, and septation. A mycologist confirmed the identification of several fungal conidia that were unable to be identified during the analysis. Fragments of dematiaceous fungi were classified as hyphal fragments if hyphal septation was present in the fragment.

### Results

Fungal conidia and spores were present in all collected air samples. The predominant fungal genera included, moniliaceous ameroconidia (unicellular colorless conidia belonging to *Aspergillus*, *Penicillium*, and other genera), *Cladosporium*, ascospores, *Fusarium*, *Epicoccum*, and

*Alternaria* species (Table 1). Airborne dematiaceous hyphal fragments were also present in all air samples. The quantities of airborne fungal conidia and hyphae collected onto individual PBMs varied substantially between study participants' environments (Table 1). The greatest variation in airborne counts was observed for moniliaceous ameroconidia, unknown conidia, *Cladosporium* conidia, as well as airborne hyphae (Table 1).

Individual study participants had total IgE concentrations that exceeded 0.35 International Units IgE (kU/L), however, no relationship was observed between the total number of quantifiable haloes and total IgE (results not shown). Interestingly, we found that 12% (range 0–61%) of all immunostaining were directed towards fungal conidia and hyphae. Positive immunostaining was observed in the study population to a wide diversity of fungal spores. Sixty-seven percent (16/24), 25% (6/24), and 17% (4/24) of study participants had positive immunostaining to moniliaceous ameroconidia, *Cladosporium*, and *Alternaria*, respectively. A smaller prevalence of positive immunostaining was also observed among the study population to other fungi in particular, *Pithomyces* (8%), *Curvularia* (4%), *Bipolaris* (4%), Ascospores (4%), and *Stachybotrys* (4%). Interestingly, 13% (3/24) of the study population had positive immunostaining to airborne hyphae (Table 1). No immunostaining was observed with collected *Epicoccum*, *Fusarium*, and *Spegazzinia* conidia, however, many other fungal spores that remained unidentified also released allergen that bound human serum IgE. Approximately 29% of the study subjects did not have detectable HIA immunostaining to any captured fungal spores or fragments.

fHIA patterns of immunostaining varied with each individual fungal genus. Moniliaceous ameroconidia immunostaining was localized around the entire conidia (Fig. 2A) or around the tips of emerged *Cladosporium* hyphae (Fig. 2B). Immunostaining of other well-documented fungal aeroallergens belonging to the family Pleosporales, such as *Alternaria* (Fig. 2C and D), *Pithomyces* (Fig. 2G), and *Bipolaris* (Fig. 2I) was observed at the beak of *Alternaria* conidia (Fig. 2C), septal junctions (Fig. 2G), basal regions (Fig. 2C and D), and the outer periphery of conidia (Fig. 2G). In contrast, the localization of IgE immunostaining in dematiaceous hyphal fragments and ascospore genera was localized to the site of germination (Fig. 2E), around ungerminated hyphal tips (Fig. 2F), or around the entire length of emerged ascospore hyphae (Fig. 2H). For unknown conidia and other amorphous particulates, resultant immunostaining was heterogeneous and restricted to the septal junctions or around the entire particle (Fig. 2K and L). However, for some fungal conidia such as *Stachybotrys*, immunostaining was not only localized on the PBM in close proximity to the spore, but was also concentrated on the surface ornamentation of the conidia (Fig. 2J). No immunostaining was observed in culture derived negative control samples (data not shown).

### Discussion

In order to better understand the etiology of fungal allergic sensitization, it is essential to collect precise information on the distribution of fungi in the ambient environment of the patient. Traditional methods of exposure assessment and immunodiagnosis currently fail to achieve this goal.

**Table 1** Halogen Immunoassay data for each individual study participant ( $n = 24$ ).  
*Résultats des immunoessais pour chacune des participantes à l'étude ( $n = 24$ ).*

Subject	Total IgE <sup>a</sup>	Total Halo <sup>b</sup>	Collected fungal genera											
			Monilaceous amerspore <sup>c</sup>		Unknown		<i>Cladosporium</i>		<i>Alternaria</i>		Hyphae		<i>Pithomyces</i>	
			+ <sup>d</sup>	- <sup>e</sup>	+	-	+	-	+	-	+	-	+	-
1	110	125	4	14	3	15	Nd	15	Nd	Nd	Nd	10	Nd	1
2	24.8	92	Nd	67	Nd	25	8	19	2	Nd	Nd	3	Nd	Nd
3	942	70	Nd	20	Nd	28	Nd	18	Nd	Nd	Nd	5	Nd	Nd
4	52.9	30	Nd	23	Nd	15	Nd	5	Nd	Nd	Nd	11	Nd	Nd
5	45.3	16	Nd	34	Nd	5	Nd	9	Nd	Nd	Nd	24	Nd	Nd
6	327	44	1	46	1	10	Nd	2	1	Nd	1	23	Nd	Nd
7	1147	130	6	21	1	12	Nd	4	Nd	Nd	Nd	4	Nd	Nd
8	1469	58	Nd	81	Nd	6	Nd	1	Nd	Nd	Nd	5	Nd	Nd
9	442	240	66	322	Nd	11	2	2	1	Nd	1	23	1	Nd
10	341	156	10	112	3	4	Nd	5	2	Nd	nd	28	Nd	Nd
11	232	201	5	90	2	1	1	1	Nd	Nd	1	53	Nd	Nd
12	201	167	5	102	Nd	2	Nd	1	Nd	1	Nd	45	Nd	Nd
13	221	18	Nd	76	Nd	1	Nd	4	Nd	Nd	Nd	28	Nd	Nd
14	41.4	650	60	340	2	28	Nd	58	Nd	Nd	Nd	116	Nd	Nd
15	34.8	115	4	132	1	4	1	16	Nd	Nd	Nd	19	1	1
16	185	39	4	262	Nd	3	1	4	Nd	Nd	Nd	26	Nd	Nd
17	10.7	46	28	100	Nd	4	Nd	10	Nd	Nd	Nd	20	Nd	Nd
18	311	49	9	140	Nd	2	1	3	Nd	Nd	Nd	31	Nd	Nd
19	45.4	25	Nd	46	Nd	1	Nd	Nd	Nd	Nd	Nd	25	Nd	Nd
20	244	515	120	285	1	2	Nd	7	3	Nd	Nd	12	Nd	Nd
21	4.15	125	34	130	Nd	1	Nd	4	Nd	Nd	Nd	16	Nd	Nd
22	12	30	Nd	34	Nd	1	Nd	Nd	Nd	Nd	Nd	18	Nd	Nd
23	806	134	79	2155	Nd	Nd	Nd	15	Nd	Nd	Nd	215	Nd	Nd
24	83.7	3320	3	108	Nd	1	Nd	Nd	Nd	Nd	Nd	42	Nd	Nd
Prevalence of HIA staining <sup>f</sup>			67%	100%	33%	96%	25%	88%	17%	100%	13%	21%	8%	13%

## Collected fungal genera

<i>Curvularia</i>		<i>Bipolaris</i>		Ascospore		<i>Fusarium</i>		<i>Epicoccum</i>		<i>Spegazzinia</i>		% <sup>g</sup> Total Halo %
+	-	+	-	+	-	+	-	+	-	+	-	
1	1	Nd	Nd	Nd	7	Nd	Nd	Nd	Nd	Nd	Nd	6
Nd	1	Nd	Nd	Nd	6	Nd	Nd	Nd	Nd	Nd	Nd	11
Nd	Nd	Nd	Nd	Nd	3	Nd	Nd	Nd	Nd	Nd	Nd	0
Nd	Nd	Nd	Nd	Nd	5	Nd	Nd	Nd	1	Nd	Nd	0
Nd	Nd	Nd	Nd	Nd	4	Nd	Nd	Nd	1	Nd	Nd	0
Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	1	Nd	Nd	7
Nd	Nd	Nd	Nd	Nd	3	Nd	1	Nd	Nd	Nd	Nd	5
Nd	Nd	Nd	Nd	Nd	Nd	Nd	1	Nd	Nd	Nd	Nd	0
Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	30
Nd	Nd	1	Nd	Nd	1	Nd	3	Nd	Nd	Nd	Nd	10
Nd	Nd	Nd	1	Nd	4	Nd	Nd	Nd	Nd	Nd	Nd	5
Nd	Nd	Nd	Nd	Nd	2	Nd	2	Nd	Nd	Nd	Nd	3
Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	0
Nd	Nd	Nd	Nd	Nd	8	Nd	18	Nd	Nd	Nd	Nd	10
Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	6
Nd	Nd	Nd	Nd	Nd	1	Nd	Nd	Nd	Nd	Nd	Nd	13
Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	61
Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	1	20
Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	0
Nd	Nd	Nd	Nd	Nd	1	Nd	Nd	Nd	1	Nd	Nd	24
Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	1	Nd	Nd	27

Table 1 (Continued)

Collected fungal genera													
<i>Curvularia</i>		<i>Bipolaris</i>		Ascospore		<i>Fusarium</i>		<i>Epicoccum</i>		<i>Spegazzinia</i>		% <sup>g</sup> Total Halo %	
+	–	+	–	+	–	+	–	+	–	+	–		
Nd	Nd	Nd	Nd	Nd	1	Nd	Nd	Nd	Nd	Nd	Nd	0	
Nd	Nd	Nd	1	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	59	
Nd	Nd	Nd	Nd	1	Nd	Nd	Nd	Nd	Nd	Nd	Nd	0.1	
4%	8%	4%	12%	4%	58%	0%	21%	0%	21%	0%	4%	12 <sup>h</sup>	

<sup>c</sup> 29% of the subject population did not have detectable HIA immunostaining to any collected fungal spores or hyphal fragments.

<sup>a</sup> Total IgE concentrations are expressed as a concentration of International Units IgE (kU/L).

<sup>b</sup> Total halo, represents the total number of positive haloes recorded in each sample.

<sup>c</sup> Moniliaceous amero-spores refers to unicellular colorless conidia belonging to *Aspergillus*, *Penicillium*, and other genera.

<sup>d</sup> Represents the number of particles belonging to the fungal group with positive HIA immunostaining.

<sup>e</sup> Represents the number of particles belonging to the fungal group with no HIA immunostaining.

<sup>f</sup> Percentage of the study participant population with (+) or without (–) HIA immunostaining for each collected fungal genera.

<sup>g</sup> Prevalence of haloes to fungal particulates for each individual study participant.

<sup>h</sup> Average percentage of haloes directed toward fungal conidia, spores, hyphae, and fragments (range 0–61%;  $n = 24$ ).

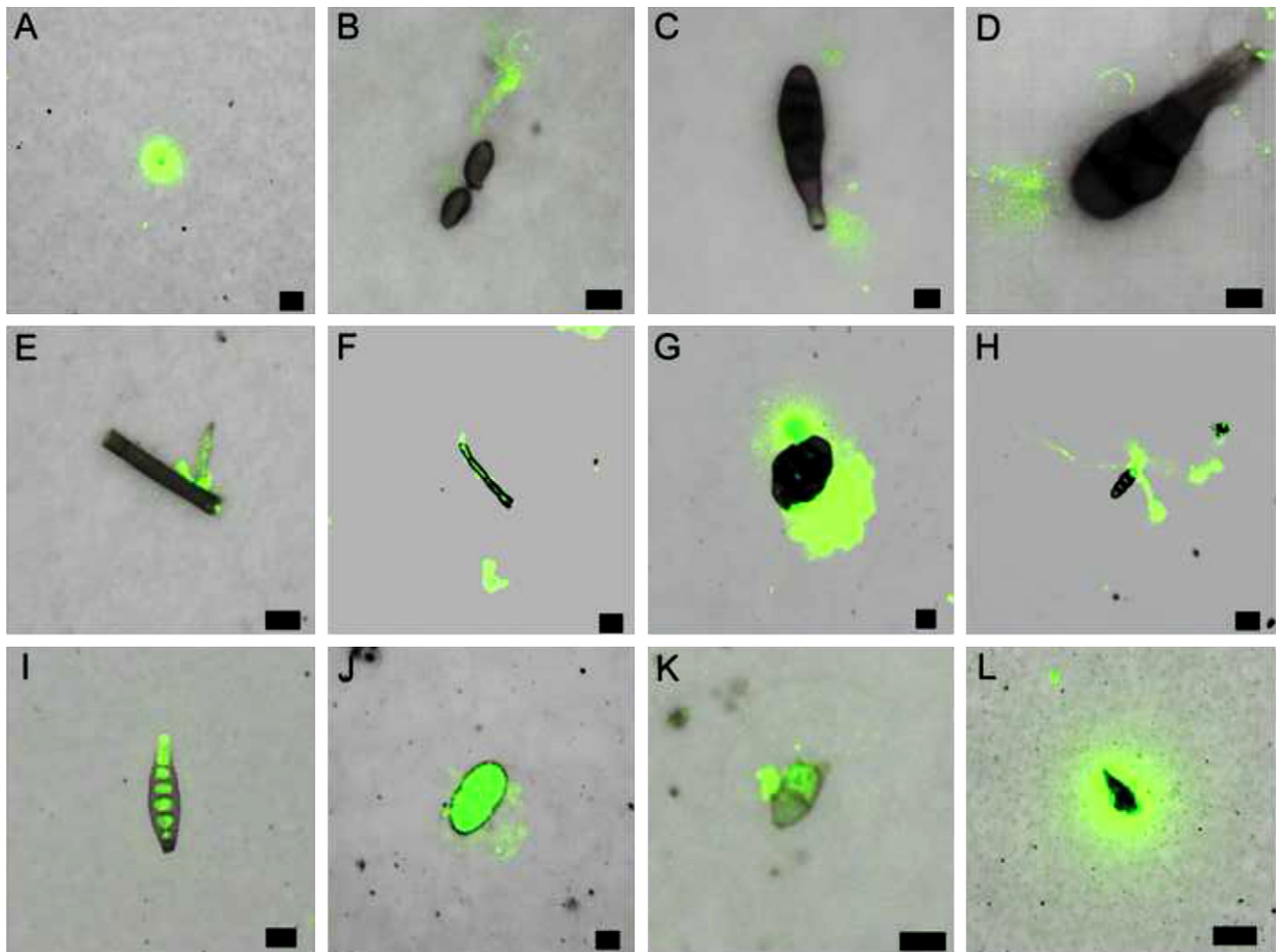
Typically, few fungal extracts are available for skin prick or *in vitro* testing and there is the inference that these represent the fungal species to which exposure occurs. In addition, positive results to these diagnostic tests can be confounded by the high degree of cross reactivity between fungi, providing a distorted view of the diversity and significance of exposure to individual fungal species. Utilizing innovative immunodiagnostic methodologies such as the fHIA mitigate a number of the limitations associated with traditional diagnostic platforms. This surveillance methodology enables the testing of native antigens derived from myco-aerosols collected from the patient's own environment.

The results of the present study further demonstrate the contribution of previously overlooked myco-aerosols to the aeroallergen load. Although IgE immunostaining to moniliaceous amero-spores, *Cladosporium*, and *Alternaria* was predominant, dematiaceous hyphal fragments and other clinically unrecognized fungal conidia also released detectable allergen. These findings confirm previous Australian indoor observations utilizing the HIA [16]. Moreover, the results of the study are in agreement with the observations of Pitkäranta and colleagues [36]. Using molecular techniques, the authors identified a diverse spectrum of fungi within the indoor environment. Basidiomycetous yeasts (*Cryptococcus*, *Malassezia*, and *Aureobasidium* species), ascomycetes (*Leptosphaerulina* species), and *Cladosporium* were predominant compared to traditionally cultivable *Aspergillus* and *Penicillium* species [36]. Many of these fungi contain allergens and have been associated with allergic sensitization [8,13,27,33]. To date, particulates derived from these myco-aerosols including amorphous fungal wall fragments; intracellular and extracellular structures of spores, conidia, hyphae, chlamydoconidia, and yeast-like cells have also remained uncharacterized as potential aeroallergen sources. In addition, many of the antigens associated with IgE binding in the present study remain uncharacterized and are derived from previously unrecognized aeroallergen sources.

The contribution of aerosolized dematiaceous hyphal fragments to the troposphere was first described following Charles A. Lindbergh's bioaerosol collection expeditions over the Arctic and Atlantic Ocean in 1933 [31]. Ensuing studies

identified hyphal fragments as common atmospheric bioaerosols in numerous locations throughout the world [24,34,35,46]. Hyphal fragments vary in size (7–100  $\mu\text{m}$ ) [34,35] and are characterized in terms of their wall thickness, melanization, septation, and conidiophore features [19,16,35]. In particular, fragments derived from dematiaceous fungi belong to allergenic species in the orders Capnodiales, Pleosporales, and Eurotiales [35]. Various immunostimulatory macromolecules including antigens [14], allergens [16], mycotoxins [7,47], and (1-3)- $\beta$ -D-glucans [40,44] have been detected in fungal fragments. Recently, aerosolized hyphal fragments have also been proposed as an indicator of fungal contamination [54] and a potential aeroallergen source in indoor environments [19,16]. In the present study, dematiaceous hyphal fragments were recovered in all indoor samples, but only a small proportion bound specific human serum IgE; this is comparable with previous observations in Australia [16]. To date, very little is known about the process of hyphal fragmentation, and the species that contribute the greatest concentrations of fungal fragments to the indoor environment. This continues to be the focus of future research.

Fungal conidia derived from Pleosporalean fungi were also frequently identified in the current study. Multi-celled *Leptosphaeria*-like ascospores as well as *Bipolaris*, *Pithomyces*, and *Curvularia* conidia released antigens that were capable of binding IgE. Clinically, this is an important advancement in the understanding of fungal allergic sensitization. Prior to the development of the fHIA, it was widely accepted that personal exposure to *Alternaria* conidia was one of the most important etiological agents responsible for fungal induced exacerbations of respiratory morbidity [5,29,9,39,43,42]. However, recent studies demonstrated that other closely related Pleosporalean fungi, such as *Stemphylium*, *Curvularia*, and *Helminthosporium* species share many *Alternaria* allergens, and often in greater concentrations [3,26,28,41]. Moreover, the teleomorphs of prevalent fungal anamorph genera including *Alternaria*, *Phoma*, *Stemphylium*, and *Pithomyces* species include *Didymella*, *Pleospora*, *Leptosphaerulina*, and *Leptosphaeria* species. Although ascospores are not clinically recognized aeroallergen sources, epidemiological studies have demonstrated



**Figure 2** High resolution images of the fHIA immunostaining (green fluorescence) of collected fungal conidia, spores, and hyphal fragments. Each image is a composite of two focal image planes, membrane level and fungus level (8  $\mu\text{m}$  interval) superimposed on the DIC image of the fungal particulates. Moniliaceous amero-spore (A); germinated *Cladosporium* conidia (B); *Alternaria* conidia (C and D); germinated hyphal fragment (E); hyphal fragment (F); *Pithomyces* conidia (G), germinated *Leptosphaeria*-like ascospore (H); *Bipolaris* conidia (I), *Stachybotrys* conidia (J); unknown conidia (K); and an amorphous particle (L) labelled with human serum IgE. The photomicrographs depicted represent typical immunostaining patterns of immobilized fungal particles on each membrane. Scale bar, 5  $\mu\text{m}$  (Fig. 2A–E, G, J, and K), 10  $\mu\text{m}$  (Fig. 2F, H, and I), and 20  $\mu\text{m}$  (Fig. 2L).

*Images en haute résolution de la technique d'immunofluorescence fHIA (fluorescence verte) des conidies fongiques, des spores et des fragments d'hyphes récoltés. Spore de moniliacée (A), conidie germée de Cladosporium (B), conidie d'Alternaria (C et D), fragment d'hyphe germé (E), fragment d'hyphe (F), conidie de Pithomyces (G), pseudo-ascospore germée de Leptosphaeria (H), conidie de Bipolaria (I), conidie de Stachybotrys (J), conidie inconnue (K) et particule amorphe (L) marqués par les IgE humaines. Ces microphotographies sont représentatives des particules fongiques fixées sur chaque membrane. Barre d'échelle : 5  $\mu\text{m}$  (A–E, G, J et K), 10  $\mu\text{m}$  (F, H et I), 20  $\mu\text{m}$  (L).*

associations between ascospore exposure and respiratory morbidity [8,12,23,25,53]. Moreover, Pleosporalean hyphal fragments are also abundant bioaerosols [46] that may contain greater concentrations of allergen compared to conidia [15,32]. Using the HIA, other Pleosporalean fungal conidia belonging to *Leptosphaeria*, *Leptosphaerulina*, *Sporidesmium*, *Pleospora*, *Curvularia*, *Exserohilum*, and *Pithomyces* species have been shown to release allergen [16]. These studies, in addition to the present findings, further demonstrate that Pleosporalean fungi may make significant contributions to the aeroallergen load of many environments.

In addition to the contribution of Pleosporalean conidia, a germinated *Leptosphaeria*-like ascospore and *Cladosporium*

conidia released hyphal allergen that bound specific human IgE. Conidia germination followed by the release of greater concentrations of hyphal allergens is another emerging paradigm for fungal allergic sensitization. Previous studies have demonstrated that significantly greater quantities of allergen are released following germination from hyphal tips and septal junctions of various fungal species [15,32,48]. The presence of germinated fungal conidia and yeast-like spores in the nasal cavity and turbinates of immunotolerant subjects has also been recently confirmed [45]. Using the enzymatic HIA, allergen released from other germinated conidia and ascospores such as *Pleospora*, *Leptosphaeria*, and two-celled ascospores have been demonstrated in Australia [16].

To our knowledge, this is the first reported observation of allergen release from environmentally derived ungerminated and germinated conidia, ascospores, and hyphal fragments in North America.

It was possible to detect IgE immunostained moniliaceous ameroconidia and other species using the fHIA. Compared to previous studies utilizing enzymatic HIAs [16–18], this methodology greatly enhanced the detection of picogram quantities of allergen derived from ameroconidia, subcellular particulates, and improved particle resolution and contrast. Recent improvements to the fHIA have allowed the enumeration of immunostained fungal fragments as small as 0.5  $\mu\text{m}$  [19,20]. It was also observed that not all fungal spores, conidia, and hyphal fragments released detectable allergen. Environmental parameters such as prolonged exposure to ultra violet radiation, solubilization of surface antigens, and specificity of individual human serum IgE have been proposed to account for the decreased immunostaining [16]. Future epidemiological studies should consider this observation when interpreting fungal exposure assessment data. Utilizing the fHIA in fungal allergic sensitization surveillance studies will enhance the detection of the complete spectrum of myco-aerosols in the environment.

In summary, the fHIA has several distinct advantages over conventional immunodiagnostic and fungal exposure surveillance methodologies. The fHIA provides unique insight into the diversity of myco-aerosols that patients are exposed and sensitized to in their own environment, in addition to localizing the ultra structural sites associated with native allergen release. Results derived from utilizing this methodology have challenged traditional paradigms of fungal allergic sensitization. Our surveillance approach mitigates many of the potential limitations associated with traditional exposure assessment methodologies. The combination of environmental and serological monitoring will provide patient-specific exposure and sensitization profiles that will help to elucidate adverse health effects associated with personal fungal exposure. Ultimately, these advances will contribute to better patient diagnosis and therapeutic management.

## Acknowledgments

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the National Institute for Occupational Safety and Health. This work was supported in part by the Inter-Agency Agreement NIEHS Y1-ES0001-06 and the National Institutes of Health grants: R01 ES 10922 and P30 ES 009089. The authors would like to thank Dr. Michael Muilenberg for his mycological assistance identifying several unknown fungal spores.

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