
Alternaria measures in inner-city, low-income housing by immunoassay and culture-based analysis

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Background: Sensitivity to *Alternaria* allergens has been associated with severe asthma and life-threatening exacerbations, and a high prevalence of *Alternaria* sensitivity has been reported among inner-city populations. Traditionally, epidemiologic studies have measured indoor *Alternaria* concentrations by cultural analyses; however, the number of viable spores may not be a good proxy for allergen levels. Furthermore, other genera share epitopes with *Alternaria* that may contribute to the allergenic effect.

Objective: To compare measures of *Alternaria* antigen by enzyme-linked immunosorbent assay with measures of *Alternaria* and cross-reactive genera (*Ulocladium*, *Curvularia*, *Epicoccum*, and *Stemphylium*) by cultural analysis.

Method: Antigen assays and cultural analyses were performed on vacuum-collected bed dust samples collected between June 18, 2002, and February 9, 2004, from 3 inner-city, low-income public housing developments.

Results: *Alternaria* antigen was found in all bed dust samples regardless of season. However, culturable *Alternaria*, *Ulocladium*, *Curvularia*, *Epicoccum*, and *Stemphylium* were only found in 50%, 35%, 6%, 11%, and 0% of bed samples, respectively. No correlations were found between *Alternaria* antigen and culturable concentrations of *Alternaria* or of its cross-reactive genera except for marginal correlation with *Ulocladium* culturable concentrations.

Conclusions: The results confirm that exposure to *Alternaria* antigens and allergens can occur even in the absence of culturable *Alternaria* or its cross-reactive genera, so further refinement and use of assays are essential for characterizing the distribution and determinants of indoor fungal allergen levels for sensitive populations.

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INTRODUCTION

Alternaria allergens are among the most important fungal allergens associated with respiratory allergic diseases, including asthma. Studies suggest that sensitivity to *Alternaria* allergens is a risk factor for asthma.^{1–3} Exposure to *Alternaria* has also been associated with severe asthma and life-threatening exacerbations.^{4–9} Sensitivity to *Alternaria* allergens is found in urban and rural populations¹⁰; a study that compared grouped rural and small city populations to the National Cooperative Inner-City Asthma Study participants found significantly greater sensitivity to *Alternaria* in the urban population.¹¹

Currently, fungal prevalence is primarily measured by spore count and cultural analysis. Even though these methods are appropriate in identifying the presence of fungi, they may be less than adequate as a measure of fungal allergen exposure. Nonviable and therefore nonculturable components, including mycelial fragments, may also contain allergenic material.^{12–14} In addition, viable recoveries could be affected by growth medium, incubation conditions, or ability to compete with other fungi on the plate. The amount of allergens produced by fungi often depends on environmental and nutritional conditions so that identifying the presence of a fungus may not be indicative of allergenic potential.¹⁵ There may also be variability in the antigenicity in different strains of the same fungal species.¹⁶ Furthermore, there are fungal genera with shared epitopes, which may contain similar allergenic components and could elicit a response in sensitized individuals.^{17–19}

Immunosorbent assays are useful exposure evaluation tools, and recent advancements have facilitated quantifying *Alternaria* antigens and allergens using this method.^{18,20,21} However, to date, neither *Alternaria* antigens nor allergens in residential environments have been evaluated to the same extent as other aeroallergens such as dust mites. Immunoassays may be monoclonal (derived from a single cell line) or polyclonal (derived from multiple cell lines). Unlike polyclonal assays, monoclonal assays are reproducible, but they

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do not reflect the mix of immune responses to an antigen or antigens and, therefore, may not adequately represent real or "correct exposure."²² Irreproducibility is also a problem for cultural analysis, which in addition to the drawbacks mentioned herein is affected by sample storage and analysis timing.

The objective of this study was to compare the *Alternaria* antigen concentrations by polyspecific polyclonal enzyme-linked immunosorbent assay (ELISA) with culturable concentrations of *Alternaria* and cross-reactive genera measured from the same dust sample. Genera with allergen components cross-reactive with *Alternaria* include *Ulocladium*, *Stemphylium*, *Curvularia*, and *Epicoccum*.^{17–19,23,24} An additional objective was to determine if the choice of growth medium (malt extract agar [MEA] or dichloran-18% glycerol agar [DG-18]) for cultural analysis influences the results comparing antigen and culturable concentrations. Seasonal effects were also evaluated.

METHODS

Samples were collected as part of the Boston Healthy Public Housing Initiative, a collaborative community-based study that seeks to understand and improve public housing conditions, particularly those that affect asthmatic children. Samples were obtained from a total of 78 apartment units of participants recruited from 3 family developments between June 18, 2002, and February 9, 2004. In 48 of the apartments, interventions aimed at cockroach allergen reduction, including mattress replacement, were implemented after the first sample was collected from the unit. The study was approved by the human subject committees of Boston University, Harvard University, and Tufts University, and required written informed consent was obtained from all study participants.

Dust Samples

Dust samples were collected from beds using the standard method for collecting dust as described by Chew et al.²⁵ In brief, all layers of the bedding, including the mattress and pillows, were vacuumed for a total of 5 minutes.

Sample Analysis

All samples were analyzed by immunosorbent assay for *Alternaria* antigen. A subset of 47 samples was available for analysis by culture. Dust samples were sieved before analysis.

For cultural analysis, 25 mg of the fine bed dust was suspended in 2 mL of 0.02% Tween 20 in distilled water. The suspension was 10× serially diluted, and 0.1 mL of the full-strength, 1:10, and 1:100 dilutions was plated in duplicate on MEA and on DG-18 (culture media made in the laboratory). Cultures were grown at 21°C to 23°C under fluorescent room lighting (approximately 12 hours on and 12 hours off). Fungi were identified to genus after 7 to 21 days of incubation using standard mycological criteria,^{26–28} and counts used were primarily from the dilution having closest to 10 to 50 colonies per Petri dish. Concentrations are reported

as colony-forming units per gram of dust. The lower limit of detection for the cultural analysis was 400 CFU/g.

For immunoassay analysis, dust was divided into 100-mg aliquots and stored at –20°C until extraction. Samples were extracted using 2 mL of borate-buffered saline per 100 mg of sample. Antigen concentrations were detected by a competitive inhibition ELISA, using commercially prepared rabbit polyclonal antibodies (lot ZA4–4L; Greer Laboratories, Lenoir, North Carolina) produced against whole mycelial extracts of *Alternaria alternata* (lot XPM1-X10; Greer Laboratories) as described by Salo et al.²¹ allowing the detection of multiple antigenic components of *A alternata*, including Alt a 1 allergen.³ The lower limit of detection for the *Alternaria* ELISA is 0.14 µg/g of dust.

In this study, seasons were defined as winter (December to February), spring (March to May), summer (June to August), and fall (September to November).

Statistical Analyses

The concentrations of *Alternaria* antigen from ELISA and *Alternaria*, *Ulocladium*, *Curvularia*, and *Epicoccum* colonies from cultural analysis were not normally distributed. Therefore, nonparametric statistical analyses were used. Spearman rank test was used to determine correlations between culturable fungi and antigen levels and Wilcoxon signed-rank for paired comparison between media. For regression analysis, generalized linear mixed models were used to accommodate multiple repeated measures. For these models, a log-normal distribution was used to address normality issues. $P < .05$ was considered statistically significant and $P < .10$ as marginally statistically significant. All analyses were performed using SAS statistical software (version 9.1; SAS Institute Inc, Cary, North Carolina).

RESULTS

Alternaria Antigen and Fungal Cultural Concentrations

Alternaria antigens were detected in all bed samples. Concentrations ranged from 0.48 to 12.23 µg/g, with a median concentration of 2.77 µg/g (Table 1). *Alternaria* was found in 50% of the cultured samples, with levels that ranged from less than 400 to 20,000 CFU/g. Since *Ulocladium*, *Stemphylium*, *Curvularia*, and *Epicoccum* are cross-reactive with *Alternaria*,^{17–19,24} the levels of these cross-reactive genera were also examined and combined with the *Alternaria* levels in some analyses. Like *Alternaria*, these genera are primarily saprobic, dematiaceous fungi that normally occur outdoors on dead or dying plant materials. *Stemphylium*, *Curvularia*, and *Epicoccum* tend to thrive under relatively dry conditions; indoors they favor damp but not wet building material. *Ulocladium* is a moist-loving mold that requires wet or near-saturated conditions.²⁹ *Stemphylium* was not found in any of the samples. *Ulocladium*, *Curvularia*, and *Epicoccum* were found in 35%, 6%, and 11% of the samples, respectively. In 34% of the bed samples, neither *Alternaria* nor any of the cross-reactive genera were found. In 34% of the samples at

Table 1. Concentrations of *Alternaria* Antigen (ELISA Analysis) and *Alternaria* and Its Cross-reactive Species (Cultural Analysis)^a

Variable	Samples with detectable concentration, %	No. of samples	Median concentration (range)		
			ELISA, $\mu\text{g/g}$	MEA, CFU/g	DG-18, CFU/g
<i>Alternaria</i> antigen	100	177	2.77 (2.93–12.23)		
<i>Alternaria</i>	50	47		<400 (1,262–16,000)	<400 (1,747–20,000)
<i>Ulocladium</i>	35	47		<400 (5,629–188,000)	<400 (400–128,000)
<i>Curvularia</i>	6	47		<400 (<400–800)	ND
<i>Epicoccum</i>	11	47		<400 (<400–1,200)	<400 (<400–400)
<i>Stemphylium</i>	0	47		ND	ND

Abbreviations: DG-18, dichloran-18% glycerol agar; ELISA, enzyme-linked immunosorbent assay; MEA, malt extract agar; ND, no detectable concentration.

^a All samples were taken from beds.

least 1 genus was found, in 24% of the samples 2 genera were found, and in 11% of the samples 3 genera were found.

Comparison of Fungal Culturable Concentrations Among Growth Media

Similar to results found in a previous study,³⁰ we did not find a significant difference in mean *Alternaria* concentrations compared between DG-18 and MEA. However, it is not well known how concentrations of *Ulocladium*, *Curvularia*, and *Epicoccum* differ by growth medium or how the choice of growth medium could alter the allergen to culturability comparison. Our study found no differences in mean *Ulocladium* or *Epicoccum* concentrations by media. *Curvularia* was detected only on MEA.

Positive correlations were observed between *Alternaria* on MEA and *Alternaria* on DG-18 ($r = 0.45$; $P = .002$) and between *Ulocladium* on MEA and *Ulocladium* on DG-18 ($r = 0.35$; $P = .02$). Correlations between *Alternaria* and its cross-reactive genera in the same media were also determined. Correlations between *Alternaria* and *Ulocladium* were found to be significant regardless of media (MEA: $r = 0.31$; $P = .03$; DG-18: $r = 0.33$; $P = .02$).

Comparison of Antigen Concentrations With Culturable Fungal Concentrations

No correlation was found between *Alternaria* antigen concentrations and *Alternaria* culturable concentrations. The correlation was still insignificant when only detects were compared ($n = 25$). In addition, no association was between bed dust antigen concentrations and culturable *Alternaria* plus its cross-reactive genera (Fig 1). However, significant correlations were found between *Alternaria* antigen concentrations and *Ulocladium* culturable concentrations for DG-18 ($r = 0.33$; $P = .03$) and MEA ($r = 0.31$; $P = .04$). The correlations seemed to have been influenced by the 2 valid, high values of *Ulocladium*. On average, those samples with *Ulocladium* culturable concentrations above the median had *Alternaria* antigen concentrations that were 1.33 to 1.54 $\mu\text{g/g}$ higher than those samples with *Ulocladium* below the median ($P = .07$ for DG-18 and $P = .03$ for MEA). However, the categorization of *Ulocladium* above or below the median only explains approximately 9% of the variation in *Alternaria* antigen concentration.

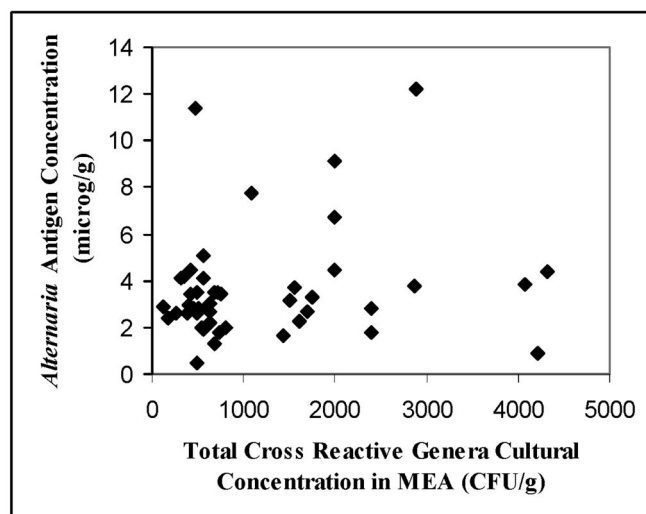


Figure 1. Comparison between *Alternaria* antigen levels and sum of cultural *Alternaria* plus cross-reactive genera counted from malt extract agar (MEA) ($r = 0.18$; $P = .24$). Graph excludes unusually large values of *Ulocladium*.

Seasonal Variations in Concentrations

Alternaria antigen concentrations showed marginally significant differences between geometric means of summer and spring ($P = .07$) (Fig 2a). After controlling for the introduction of new beds, seasonal differences between geometric means of winter and spring and between summer and spring became statistically significant ($P = .04$ and $P = .03$, respectively).

For analysis by culture, geometric mean *Alternaria* concentrations varied marginally by season between fall and winter ($P = .09$) and marginally between fall and spring ($P = .06$) in MEA (Fig 2b). In DG-18, geometric mean *Alternaria* concentrations varied between spring and summer ($P = .02$) and marginally between fall and spring ($P = .08$). For *Ulocladium* by culture, a significant difference was found between geometric means of winter and spring for both MEA ($P = .05$) and DG-18 ($P = .01$).

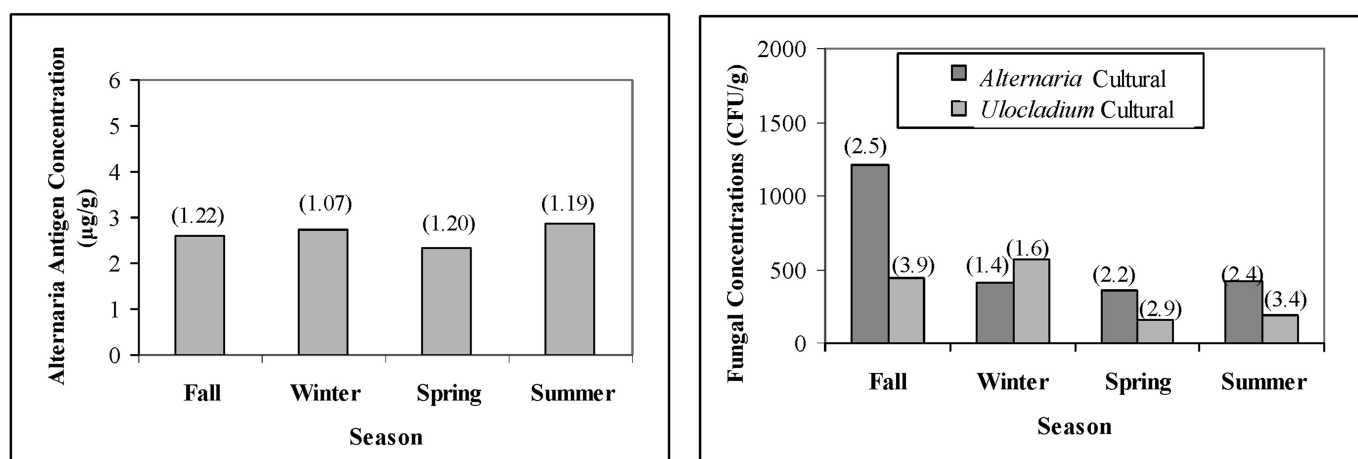


Figure 2. Left, Estimated geometric mean concentrations (geometric SD) of *Alternaria* antigen by season. Right, Estimated geometric mean concentrations (geometric SD) for culturable *Alternaria* and *Ulocladium* by season (counted from malt extract agar).

DISCUSSION

Most studies use cultural analysis or spore counts to estimate *Alternaria* exposure, and an association between *Alternaria* spore peaks and increases in asthma morbidity has been reported.⁵ Yet counts of spores or colony-forming units may not accurately represent *Alternaria* allergen levels for the following reasons: (1) nonviable components of *Alternaria* may still carry allergens; (2) recoveries of viable fungi are influenced by growth medium, incubation conditions, and competition among colonies; (3) closely related genera may also contain *Alternaria* allergens and be cross-reactive; and (4) environmental growth conditions can affect *Alternaria* allergen production. To date, the relationships between allergen levels with culturable levels of *Alternaria* and of cross-reactive genera, which may have the same or similar antigenic components,^{18,31} have not been adequately characterized. In addition, even though there is information on the effect of growth media on the comparison of levels of culturable fungi, little is known about the comparison among media with allergen levels.

A few other field studies have reported *Alternaria* allergen concentrations. A study by Flückiger et al³² compared airborne culturable *Alternaria* concentrations with airborne *Alternaria* antigen in 15 mixed-use buildings in Switzerland. Another study by Barnes et al³³ examined dust samples from residential homes, and a study by Salo et al²¹ evaluated dust-borne *Alternaria* antigen concentrations in homes as part of the US National Survey of Lead and Allergens in Housing. The study by Barnes et al³³ reported *Alternaria* concentrations found in samples taken from vacuum cleaner bags and canisters brought in by individuals associated with a pediatric allergy clinic in Kansas City, Missouri. *Alternaria* antigen was detected in 81% of the samples with a mean of 1.5 µg/g and an SD of 4.9 µg/g. The mean *Alternaria* antigen concentration found in beds in our study was nearly double this concentration at 2.9 µg/g with an SD of 1.6 µg/g. Barnes et

al³³ also found significantly more of 2 specific *Alternaria* allergens, Alt a 1 and Alt a 70 kD, in asthmatic homes than in control homes. Because this study used dust taken out of household vacuum cleaners rather than collected in a thimble from a specified place as was done in our study, their samples represented dust from various places in the house stored over varying amounts of time, depending on when the vacuum filter or bag was last changed, and may not be directly comparable to the standardized dust collection methods used in large-scale epidemiologic studies. The National Survey of Lead and Allergen in Housing found a geometric mean *Alternaria* antigen concentration in US homes of 4.88 µg/g, with an SE of 0.13 µg/g.

The percentage of samples with culturable *Alternaria* found in the dust in our study was comparable to that found in another study in the Boston area.³⁴ We found little correlation between culturable concentration and antigen content. Flückiger et al³² analyzed outdoor and indoor samples taken by slit-sampler and multistage liquid impingers in Switzerland in the summer using culture and ELISA methods and reported a correlation between *Alternaria* antigen content and *Alternaria* culturable concentration for outdoor samples but not for indoor samples. They found a higher ratio of antigen to culture concentration for indoor air, suggesting that cultural analysis may be underestimating the allergen load in indoor environments. The type of growth media was important in our comparison of antigen to culturable concentrations. The associations between antigen concentrations and culturable concentrations of *Alternaria* plus cross-reactive genera reached marginal significance only with culturable concentrations determined from MEA.

The lower concentrations of culturable *Alternaria* we observed in the winter and spring probably reflect the absence of an influx of new viable spores and a decrease in viability of existing spores. This may be due to the dry, heated conditions in Boston area apartments during the winter.³⁵ How-

ever, the bed antigen concentrations from this same dust did not significantly vary in the same way, pointing to the fact that nonviable spores can still carry allergens. Vailes et al²⁰ reported Alt a 1 concentrations of 64 ng/mL in dried, 20-year-old *Alternaria* spores and 11 to 73 ng/mL in actively growing *Alternaria* spores. Alt a 1 has been found to be the predominant allergen of crude *Alternaria* extracts and the most reactive with sera from *Alternaria*-sensitized patients.^{36,37}

Furthermore, *Ulocladium* and *Stemphylium* are closely related genera to *Alternaria*, show significant cross-reactivity with *A alternata*, and have been found to have higher concentrations of Alt a 1 than *A alternata*.^{5,17,18,23} There is also cross-reactivity between *Curvularia* and *Epicoccum nigrum* with *Alternaria*.^{24,38} We only found a significant correlation between *Ulocladium* culturable concentrations and *Alternaria* antigen concentrations, not between *Alternaria* culturable concentrations and *Alternaria* antigen content. *Ulocladium* concentrations were higher in the winter months, similar to what has been reported in other studies.^{30,39} It is possible that high *Ulocladium* concentrations in the winter are related to indoor sources and could be driving the unexpectedly high *Alternaria* antigen concentrations observed in some homes in the winter.

This study is limited by sample size, with only 47 samples with cultural analysis. This limitation may have affected the power to detect possible associations between cultural and antigen analysis. In addition, we did not collect outdoor fungal samples. However, fungal exposure measurements in indoor environments are collected for a various purposes, including (1) identifying indoor mold growth to inform building remediation and (2) assessing indoor fungal allergen exposure for potential respiratory impact. For assessing mold growth, source attribution is critical, requiring a measure of outdoor fungal contribution to apportion the contribution of sources indoors. Alternatively, it is recognized that indoor exposure is a combination of outdoor and indoor sources; therefore, it is important to characterize indoor allergen load from the combined sources.

In conclusion, in our inner-city public housing apartments, *Alternaria* antigen but not culturable spores was ubiquitous in bed dust regardless of season. We found no association between *Alternaria* antigen levels and cultural concentrations of *Alternaria* or its cross-reactive genera. Since *Alternaria* allergens are associated with allergic diseases and have been linked to asthma morbidity and mortality,^{4,9,40} characterization of exposure to fungal allergens by antigen assay is important in increasing the understanding of the relationship of allergen exposure to sensitization and asthma exacerbation and in recommending exposure reduction and asthma management strategies.

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