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Comparison of enzyme immunoassay–based assays for environmental *Alternaria alternata*

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Abstract

Background—*Alternaria alternata*–derived allergenic materials are causes of human disease. Several immunoassays exist to quantify these materials.

Objective—To compare methods for evaluating *Alternaria* content.

Methods—Four methods, including 1 monoclonal antibody (MAb)–based assay specific for recombinant Alt a 1, 1 MAb-based assay for chromatographically purified Alt a 1, 1 polyclonal antibody (PAb)–based assay for chromatographically purified Alt a 1, and 1 PAb-based assay for whole *Alternaria* extract, were evaluated. Environmental samples collected as part of the National Survey of Lead and Allergens in Housing were examined. *Alternaria* spore counts were determined in dust by observation.

Results—The MAb-based assay for recombinant Alt a 1 detected *Alternaria* in few samples (25%); the PAb-based assay for whole *Alternaria* proteins detected antigen in 97% of the samples. The PAb- and MAb-based assays for purified Alt a 1 detected antigen in 100% of the samples. There was a significant positive correlation between the 2 assays directed against purified Alt a 1. There was a positive correlation between the PAb-based assay for whole *Alternaria* and the PAb-based assay for Alt a 1. Nearly all the dust samples contained *Alternaria* spores, and there was a strong positive correlation between counts and all assays.

Conclusion—Because of the multifaceted nature of *Alternaria*, the disparities between methods for quantifying *Alternaria*, the cross-reactivity between fungal allergens, and the documented genetic promiscuity of this fungus, enzyme immunoassays using PABs against a range of *Alternaria* proteins will probably produce the most reliable estimation of overall *Alternaria* exposure in house dust.

INTRODUCTION

Alternaria alternata is ubiquitous in nature. In large multi-center studies,^{1–3} 7% to 12.9% of the general public and 40% of atopic individuals respond positively to *Alternaria* skin testing. Sensitivity to *Alternaria* has been shown to be associated with asthma and sudden death from asthma.^{4,5} Previous studies using quantitative methods for dust mites have led to the development of environmental interventions that have formed the basis for recommended guidelines used to educate mite-sensitive asthmatic patients. It would, therefore, be

advantageous for researchers attempting to relate asthma symptoms to environmental allergens to be able to quantify environmental *Alternaria* allergens.

Although spores of *Alternaria* are known to be widely distributed in the indoor and outdoor environments,^{6,7} the distribution of its allergens is poorly understood. The most commonly recognized allergen from *Alternaria* is the Alt a 1 protein. It is identified as a 28- to 31-kD band on nonreduced immunoblots that reduces to smaller fragments (14–16 kD) with β -mercaptoethanol treatment.^{8,9} Alt a 1 purified from allergen extracts by means of affinity chromatography and ion exchange chromatography^{10,11} has been recognized by more than 90% of persons allergic to *Alternaria*.¹⁰ The N-terminal sequence of this purified material has been reported,¹² and the deduced amino acid sequence for this material is known.^{13–16} Recombinant protein has been produced, and this material is available commercially.^{15,16}

It is widely appreciated that the allergen content of *Alternaria* extracts is variable, yet assays for their measurement are only beginning to be used. Allergen composition differences between spores and mycelia have been documented,^{17,18} and variability in biological activity has been observed.^{19,20} Four antibody-based assays for Alt a 1 have been reported, and 1 assay for *Alternaria* antigenic material has been described.^{14,21–25} In this study, we compare the performance characteristics of 4 different assays for *Alternaria* and Alt a 1 in house dust. Our hypothesis is that 1 assay for *Alternaria* can be identified that will best quantify exposure to *Alternaria* allergen and presumably correlate with clinical symptoms for *Alternaria*-allergic individuals. One of the monoclonal antibody (MAb)-based assays is available commercially and 1 was constructed using affinity-purified Alt a 1; 1 of the polyclonal antibody (PAb)-based assays was constructed from PABs produced against affinity-purified Alt a 1, and 1 was constructed using PABs directed against whole *Alternaria* extract. We compare these assays with *Alternaria* spore counts derived from house dust samples.

MATERIALS AND METHODS

Source Materials

The material used to standardize these assays was either purified from laboratory-grown *Alternaria* using chromatographic methods^{10,11,24} or purchased commercially as recombinant protein.^{25,26} Extracts assayed for protein content and antibody containing rabbit sera produced using these extracts were purchased from Greer Laboratories (Lenoir, NC). Polyclonal rabbit sera directed against purified Alt a 1 was provided by Hari Vijay, PhD (Health Canada, Ottawa, Ontario). The MAb-based assay specific for Alt a 1 was purchased from Indoor Biotechnologies Ltd (Manchester, England), as was recombinant Alt a 1 protein.

Forty selected dust samples collected in the National Survey of Lead and Allergens in Housing were obtained from the National Institute of Environmental Health Sciences. The samples were selected in a statistically random manner to represent low, medium, and high levels of *Alternaria*. The survey, which was representative of the US housing stock, was conducted between July 1998 and August 1999.²⁷ In 831 homes, dust samples were collected from bedroom beds and floors, living room floors and sofas, and the kitchen floor using a Eureka Mighty-Mite 7.0-amp vacuum cleaner (Eureka Co, Bloomington, IL). A 19 × 90-mm cellulose extraction thimble (Whatman International Ltd, Maidstone, England) was fitted into the distal end of the vacuum's extension tube, sealed with a rubber o-ring, and covered with a clean crevice tool. In the laboratory, dust samples were sieved through a 425- μ m pore grating. Aliquots were extracted at room temperature for 1 hour on a wrist-action shaker in borate-buffered saline (pH 8.5) at a concentration of 50 mg of dust per 1 mL of buffer. Extracts were clarified by means of centrifugation at 6,000 rpm. Supernatants were decanted and stored at -20°C .²⁷

MAB-Based Alt a 1 Assays

An assay kit specific for Alt a 1 constructed from MABs developed against recombinant Alt a 1 was purchased from Indoor Biotechnologies Inc (Charlottesville, VA). Assays were performed according to instructions provided by the supplier.^{21,25} This assay was standardized using recombinant Alt a 1. The level of detection was set at 0.02 ng/mL for recombinant Alt a 1. An assay was also performed using MABs developed against chromatographically purified Alt a 1. This assay, described previously,²⁴ was standardized using chromatographically purified Alt a 1. The level of detection was set at 15 U/mL for affinity-purified Alt a 1.

PAB-Based Alt a 1 Assay

An assay specific for affinity-purified Alt a 1 was developed from rabbit PABs supplied by Hari Vijay, PhD.¹⁰ These PABs were used to construct a sandwich-style enzyme immunoassay (EIA). For this assay, concentrations of antibodies required to give optimal results were determined empirically. Microtiter plates were coated overnight, and coated plates were washed and blocked with 0.5% gelatin (Bio-Rad Laboratories, Hercules, CA) for 1 hour at 37°C to reduce background. Serial dilutions of *Alternaria* or purified allergen protein dissolved in phosphate-buffered saline/Tween-20/azide buffer were added to each well and incubated for 2 hours. After washing, the plates were incubated with biotinylated PAB for 2 hours. The plates were again washed and incubated for 1 hour with avidin-conjugated alkaline phosphatase (1:1,000) (Sigma-Aldrich Corp, St Louis, MO). The enzyme activity was detected using alkaline phosphatase substrate tablets supplied by Sigma-Aldrich Corp. Antibodies directed against Alt a 1 used in the detection stage of the EIA were labeled with biotin. Two milligrams of 50% ammonium sulfate-concentrated rabbit serum containing PAB was dissolved in 200 μ L of 0.05M bicarbonate buffer (pH 7.5) and incubated at 4°C for 1 hour with 3 μ L of N-hydroxysuccinimide-biotin (Bio-Rad). Then, the biotinylated antibody was dialyzed at 4°C overnight against 0.1M Tris-buffered saline and stored at -20°C in 50% glycerol at a protein concentration of 5 mg/mL. The activities of biotinylated antibodies were confirmed by direct enzyme-linked immunosorbent assay using avidin-alkaline phosphatase (1:1,000) (Sigma-Aldrich Corp). This assay was standardized using chromatographically purified Alt a 1. The lower level of detection of this assay was 0.04 ng/mL for recombinant Alt a 1 and 0.8 U/mL for chromatographically purified Alt a 1.

Chromatographic Purification of Alt a 1

Alt a 1 protein used as standard was purified chromatographically using the method of Paris et al¹¹ described previously.²⁴ Briefly, *Alternaria* mycelial mats were ground using a mortar and pestle and were extracted overnight at 4°C in ammonium bicarbonate (1 g of mycelia to 10 g of liquid). The *Alternaria* extract was precipitated by the addition of 4 volumes of acetone. The acetone precipitate was dissolved in Tris-buffered saline at a concentration of 10 mg/mL. This was applied to a DEAE (diethylaminoethanol) ion exchange column (MemSep; Millipore, Bedford, MA) and was eluted with a 0 to 1M salt gradient. The material that eluted 0.3 to 0.4M sodium chloride was applied to a size exclusion column (Hydrobore; Rannin, Woburn, MA), and the 29- to 31-kD fraction was collected. The presence of the 29- to 31-kD allergen was monitored by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis immunoblots probed with sera from *Alternaria*-sensitive individuals. After the dry weight was measured, purified Alt a 1 was dissolved in Tris-buffered saline, and the protein concentration was measured by means of amino acid assay. Immunoassays were performed using serial dilutions of the purified allergens to generate a standard curve. The log of the known allergen concentrations was next compared with the optical density at 405 nm by means of linear regression.

Inhibition Assay for Whole *Alternaria*

For the inhibition immunoassays, *Alternaria* allergen preparations of known protein content and rabbit antiserum directed against this allergen preparation were obtained commercially (Greer Laboratories). Microtiter plates (Immulon 2; Dynatec, Chantilly, VA) were coated with 100 μ L of whole antigen (Greer Laboratories) at a concentration of 0.01 mg/mL of 0.1M bicarbonate buffer (pH 9.6). The plate was coated at 4°C overnight. The plate was blocked with 0.5% gelatin for 30 minutes at 37°C and then washed with phosphate-buffered saline/Tween-20/azide buffer. After blocking, specific unlabeled rabbit anti-allergen antibody was added to the antigen-coated wells along with serial dilutions of competitive antigen. The proper ratio of allergen to inhibitor was determined empirically for each assay. The concentrations were generally 1:1,000 dilution of antibody containing rabbit sera and 10 μ g/mL of *Alternaria* antigen titered 2-fold down the plate for 8 concentration points. After incubation, to allow free rabbit antibody to bind to the antigen-coated wells, unbound antibody was washed away. Next, alkaline phosphatase-labeled goat anti-rabbit antibody (Sigma-Aldrich Corp) was added and allowed to bind to the rabbit antibody-antigen complex. Unbound labeled antibody was washed away, and alkaline phosphatase substrate (Sigma-Aldrich Corp) was added. After suitable time for color development, the reaction was stopped, and the plate was read at 405 nM. The system must be optimized for each antigen and antibody lot obtained from the supplier (Greer Laboratories).²² The lower level of detection of this assay was previously reported to be 0.07 μ g/mL for whole *Alternaria* protein extract.²²

Methods for Visual Enumeration of Fungal Spores

For visual enumeration of fungal spores in house dust samples, circular patches approximately 5 mm in diameter were cut from clear plastic tape. These circles were held by the edge using forceps, and the surface of the tape was saturated with dust from each particular sample. The amount of dust in the sample vastly exceeded the capacity of the tape surface. The mean amount of house dust clinging to the tape samples was estimated by weighing the circles before and after the application of house dust to the surface. The tapes were then mounted onto microscope slides using Calberlas stain in glycerin jelly, and the spores present were counted microscopically at $\times 400$ magnification. The entire surface of the tape was examined. At this magnification, only large spores, including *Alternaria*, *Cladosporium*, *Curvularia*, *Dreschleria*/bipolaris, *Epicoccum*, *Pithomyces*, rust/smud/myxomycetes/*Periconia* (as a group), and *Ulocladium*, were enumerated. The person performing the counts is certified by the National Allergy Bureau of the American Academy of Allergy, Asthma, and Immunology.²⁸

Statistical Analyses

Correlations between the assays were computed using Spearman rank correlation coefficients to produce a nonparametric statistic measuring the strength of the associations.²⁹ Values lower than the lower level of detection for the assay were assigned a value of 0.5 times the lower level of detection. Analyses and graphical representations were performed using SAS version 8.02 (SAS Institute Inc, Cary, NC).

RESULTS

Results of Visual Counting

Visual enumeration of the *Alternaria* spores in selected dust samples indicated that 90% of the samples contained identifiable *Alternaria* spores. The mean number of *Alternaria* spores in these samples was 78,541 per gram of dust, and the variation covered nearly 3 orders of magnitude, ranging from 0 to 580,645 spores per gram of dust. Other spore types seen on these

collections at $\times 400$ magnification included *Cladosporium*, *Curvularia*, *Epicoccum*, and *Pithomyces*.

Results of EIA Determinations

Comparison of the results of the 4 types of assays for *Alternaria* indicates that the PAb assay 1, constructed with antibodies specific for native Alt a 1 (using as standard chromatographically purified Alt a 1), and the MAb assay 2, designed with MAbs screened against purified native Alt a 1, had the highest frequency of detection in environmental dust samples (100%) (Table 1). The PAb assay 2, constructed with PABs produced using whole *Alternaria* extract, had a similar frequency of detection of environmental dust samples (92.5%). The MAb assay 1, designed with MAbs constructed and screened against recombinant Alt a 1 protein, had a lower frequency of detection of antigen in environmental dust samples (25%).

Correlation of EIA Values With Each Other

The relationships of the 4 assays indicated a significant correlation between the 2 assays developed against chromatographically purified Alt a 1 ($r = 0.85$; $P < .001$) (Fig 1). Also, there was a positive but not significant correlation between these assays and the whole *Alternaria* assay developed using PABs ($r = 0.14$; $P = .38$; and $r = 0.10$; $P = .55$). Generally, all the assays developed to detect Alt a 1 were in agreement with each other, and the assay developed to detect whole *Alternaria* antigen was in strong agreement only with the MAb assay 1 developed against the recombinant Alt a 1 protein.

Correlation of EIA Values With Visual Counts

There was a significant positive correlation between visual counts of *Alternaria* spores in randomly selected dust samples and the 4 assays tested (Table 2). However, the MAb assay based on antibodies directed against recombinant Alt a 1 protein produced too few positive responses to be properly evaluated.

Cross-reactivity

To gain insight into possible cross-reacting antigens contained in the various fungal spores, an analysis of correlations between the occurrences of individual spore types was performed as it related to the allergen values determined for each immunoassay (Table 2). As anticipated, there was a significant positive correlation between the presence of *Alternaria* spores in a sample and the presence of spores from *Cladosporium*, *Epicoccum*, *Pithomyces*, and the rust/smut/myxomycetes group. The only significant positive correlations between the assays and the presence of other spores were that of the PAb assay 1 and spores of the rust/smut/myxomycetes group ($R = 0.44$; $P < .01$) and that of the MAb assay 1 and *Cladosporium* ($R = 0.47$; $P < .01$), *Pithomyces* ($R = 0.32$; $P < .05$), and the rust/smut/myxomycetes group ($R = 0.58$; $P < .001$).

DISCUSSION

Alternaria alternata is the most easily distinguished spore type among the Deuteromycetes. With its overall clublike shape, prominent beak, and vertical and horizontal septations, it can easily be distinguished at $\times 400$ magnification. Despite its overall morphologic similarity, *A. alternata* is a complex species that contains numerous differences between different unique strains isolated from the wild. These differences are also seen in the same strain when grown under varying laboratory conditions.²⁰ The variability of *Alternaria* allergen content has often been observed by means of electrophoresis and immunoblotting experiments.^{20,30–32} A gene for the *Alternaria* major allergen, Alt a 1, has been amplified from 52 species of *Alternaria*

and related genera, and sequence information has been used for a phylogenetic study.³³ This work supported several phylogenetic groupings of *Alternaria*, yet most researchers in the field of allergy tend to treat all *Alternaria* as *alternata*. The task of quantifying environmental allergenic material from this fungus will, therefore, be complex but not impossible. It is recognized that the Alt a 1 protein is one of if not the most important of the allergenic proteins from this fungus.³⁴ It has been recognized by more than 90% of *Alternaria*-allergic individuals in skin testing.^{21,24,35}

Previous studies^{14,21–25} beginning in 1998 have used MAbs and PABs to detect Alt a 1. Several studies^{24,36,37} have investigated the presence of *Alternaria* and Alt a 1 in environmental samples. These studies usually reported measurable amounts of the allergen in most samples. An Alt a 1-specific 2-site MAb enzyme-linked immunosorbent assay has been used to follow allergen expression and check optimal culture conditions for *A. alternata* extract production.¹⁴ In another study,²³ the content of Alt a 1 in actively growing *Alternaria* spores and dust samples was evaluated using PABs and MAbs produced against recombinant Alt a 1. The Alt a 1 was detected at concentrations of 3.5 ng/mL in 6×10^3 spores/mL but was not detected in any of the 12 dust samples tested.²³ Similarly, in the present study, there was a lower-than-anticipated response to Alt a 1 in environmental dust samples when using an assay built from MAbs raised against recombinant Alt a 1 protein.

Herein, we report considerable overall disagreement in the results produced by several assays tested. There are 2 possible reasons for the lack of correlation between the Alt a 1-specific assay and the whole *Alternaria* assay. First is the possibility of genetic heterogeneity in this protein. The strength of the recombinant protein technique provides for the ability to freeze the genetic drift in a given *Alternaria* strain and more completely characterize the Alt a 1 protein in that strain. However, at that point the genetic drift is so controlled that this removal from the diversity of the natural state may be a detriment to environmental measurement. For many years, *Alternaria* has been suspected to be genetically unstable. It has recently been reported that a gene for the *Alternaria* major allergen, Alt a 1, amplified from 52 species of *Alternaria* and related genera, evolved 3.8 times faster than glyceraldehyde-3-phosphate dehydrogenase sequences.³³

The second possible reason that recombinant protein and antibodies derived from it may not be suitable for environmental measurements is the variability in expression due to differing growth conditions. A sequence with homology to the Alt a 1 subunit from *A. alternata* has been identified in *Alternaria brassicicola*. The sequence homology is 90% at the DNA level, and this homolog was highly up-regulated during the infection process of *A. brassicicola* on *Arabidopsis*.³⁸

When choosing the best assay to use for determining *Alternaria* in environmental samples, 4 factors must be considered. The first is that *Alternaria* spores are found in most dust samples, so an appropriate assay must be able to detect *Alternaria* even when the spores are present in low concentrations. Second, *Alternaria* takes different forms, so the antibody should be able to recognize all the forms. Third, the assay should be concordant with natural human (IgE) response and should be able to recognize the varied epitopes contained not only in Alt a 1 but also in other *Alternaria* allergens. Finally, the assay should be able to distinguish *Alternaria* from other fungal genera with immunologic cross-reacting proteins.

Just as for the dust mite assays, where method validation depended on visual enumeration of mite bodies in house dust,^{39,40} environmental evaluation of *Alternaria* allergen in dust samples should have some relation to the presence of visually enumerated spores in dust. It is widely appreciated that *Alternaria* spores are ubiquitous in the environment. Studies in the arid

climate of Australia indicated that *Alternaria* was found nearly everywhere. This observation was confirmed in that a very high percentage of allergic subjects in these environments were sensitized to *Alternaria*.² In this study, visual examination of the house dust indicated that nearly 90% of all the samples contained numerous spores of *Alternaria*. Although these methods do not allow for the ability to distinguish between differing species of *Alternaria*, it seems that *Alternaria* allergens should be frequently found in environmental samples.

Alternaria takes different forms; therefore, the assay used for environmental monitoring should be able to recognize all the forms. One of the problems with generating sequence from proteins derived from small organisms is interspecies variability. One can be reasonably sure that all the myoglobin protein molecules purified from a sperm whale have the same amino acid sequence, but the same assumption should not be made for Alt a 1 from thousands of individual spores and hyphal fragments. In 1 report of the N-terminal sequence of the Alt a 1 protein, there was disagreement in at least 2 and at most 4 of the first 15 amino acids when comparing sequence from only 4 sources. A useful assay should be robust enough to accommodate numerous amino acid variations in significant epitopes.²¹

Cross-reactivity to fungal allergens is a well-documented phenomenon.^{41–44} The assay of choice for environmental monitoring of specific genera, therefore, would minimize cross-reactivity between genera as much as possible. In addition, because the human IgE response is polyclonal, assays that respond to multiple proteins in a genus are more likely to correlate with IgE response and visual counts.

CONCLUSION

We conclude, therefore, that the most logical assay to use for environmental monitoring is the assay constructed using PABs developed against whole *Alternaria* (PAB assay 2). It is chosen over the MAb assay 1 because it recognizes the *Alternaria* antigenic material in a higher percentage of samples. It is chosen over MAb assay 2 and PAB assay 1 because it exhibits lower cross-reactivity and correlates better with visual spore counts in house dust samples. Tests such as the ones we evaluated should impact the understanding of asthma development. Fungal allergens in homes are commonly associated with skin sensitivity and respiratory allergy. The ability to test materials taken from the homes of individuals with asthma could increase understanding of these disease processes.

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Figure 1.

Correlation of the assays for *Alternaria* antigen and Alt a 1. The polyclonal antibody (PAb) assay 2 correlated positively but not strongly with the other 2 assays for Alt a 1 (A and B) (PAb2 – PAb1: $r = 0.14$; $P = .38$; and PAb2 – monoclonal antibody [MAb] assay 2: $r = 0.10$; $P = .55$). The PAb assay 2 for whole antigen correlated strongly with the MAb assay 1 for Alt a 1 (C) ($r = 0.39$; $P < .05$). The MAb assays for Alt a 1 (F) correlate well with each other ($r = 0.45$; $P < .01$) and with the PAb assay for Alt a 1 (D and E) (MAb2 – PAb1: $r = 0.85$; $P < .001$; and MAb1 – PAb1: $r = 0.46$; $P < .01$).

Table 1

Comparison of the Specificity, Nature, and Nomenclature of the 4 Assays

Assay specificity	Designation	Reference	Antibody type	Material for antibody production	Standard material
Alt a 1	MAb assay 1	21, 25	Monoclonal	Recombinant Alt a 1	Recombinant Alt a 1
Alt a 1	MAb assay 2	24	Monoclonal	Chromatographically purified Alt a 1	Chromatographically purified Alt a 1
Alt a 1	PAb assay 1	10	Polyclonal	Chromatographically purified Alt a 1	Chromatographically purified Alt a 1
Whole <i>Alternaria</i>	PAb assay 2	22	Polyclonal	Commercial <i>Alternaria</i> extract	Commercial <i>Alternaria</i> extract

Abbreviations: MAb, monoclonal antibody; PAb, polyclonal antibody.

Table 2

Spearman Correlation Coefficients Comparing the Allergen Concentrations for the Different Assays and the Results of Visual *Alternaria* Spore Counts in Selected Dust Samples

	<i>Alternaria</i> spores	<i>Cladosporium</i> spores	<i>Pithomyces</i> spores	Rust/smud/ myxomycete spores
<i>Alternaria</i> spores	1.00	0.51 ($P < .001$)	0.47 ($P < .01$)	0.67 ($P < .001$)
MAB assay 1	0.66 ($P < .001$)	0.47 ($P < .01$)	0.32 ($P < .05$)	0.58 ($P < .001$)
MAB assay 2	0.38 ($P < .05$)	0.24 ($P = .15$)	0.16 ($P = .93$)	0.29 ($P = .07$)
PAB assay 1	0.38 ($P < .05$)	0.22 ($P = .17$)	0.17 ($P = .76$)	0.44 ($P < .01$)
PAB assay 2	0.51 ($P < .001$)	0.13 ($P = .13$)	0.27 ($P = .10$)	0.29 ($P = .06$)

Abbreviations: MAB, monoclonal antibody; PAB, polyclonal antibody.