ORIGINAL ARTICLE

Correlation Between Alt a 1 Levels and Clinical Symptoms in *Alternaria alternata*–Monosensitized Patients

F Feo Brito,[1] AM Alonso,[1] J Carnés,[2] R Martín-Martín,[3] E Fernández-Caldas,[2] PA Galindo,[1] T Alfaya,[1] M Amo-Salas[4]

[1]Allergy Section, General University Hospital, Ciudad Real, Spain
[2]Research & Development Department, Laboratory LETI, S.L., Madrid, Spain
[3]School of Architecture, Department of Mathematics, University of Castilla-La Mancha, Toledo, Spain
[4]Faculty of Medicine, Department of Mathematics, University of Castilla-La Mancha, Ciudad Real, Spain

■ **Abstract**

Background: *Alternaria alternata* is a risk factor for developing asthma. Alt a 1, which has been described as the major allergen in *A alternata*, shows a good correlation with *A alternata* spores only when they have germinated.

Objectives: The objective of this study was to determine the correlation between spore counts and clinical symptoms in patients with allergic asthma and/or rhinitis monosensitized to *A alternata*.

Methods: Two types of samplers were used to determine exposure: a Burkard spore trap to collect *A alternata* spores and a high-volume air sampler to collect airborne particles. A total of 366 air filters were collected. Alt a 1 levels were measured by monoclonal antibody-based enzyme-linked immunosorbent assay. Eighteen monosensitized patients were asked to record their daily symptoms throughout the year.

Results: *A alternata* spores were detected throughout the year, whereas Alt a 1 was detected only between March and December. Symptoms showed positive and significant correlations with spore counts (r=0.459, *P*<.001), and Alt a 1 levels (r=0.294, *P*<.001). The correlation between spores and Alt a 1 was low. The negative binomial model proved that an increase of 10 pg/m^3 in Alt a 1 levels increased the number of symptoms at a 3-day lag by 5%.

Conclusions: In patients who are allergic to *A alternata*, Alt a 1 levels can be considered an important marker for predicting the risk of respiratory symptoms.

Key words: *Alternaria alternata*. Alt a 1. Allergens. Rhinitis. Asthma.

■ **Resumen**

Antecedentes: *Alternaria alternata* es un factor de riesgo para desarrollar asma bronquial. El alérgeno mayoritario de *A. alternata*, Alt a 1, muestra una buena correlación con las esporas de A. alternata sólo cuando éstas se encuentran en estado germinativo.

Objetivos: Nuestros objetivos han sido determinar la correlación entre los niveles de esporas con la cuantificación de Alt a 1, y establecer su asociación con los síntomas de pacientes con rinitis y/o asma por monosensibilización a *A. alternata*.

Métodos: Se utilizaron dos tipos diferentes de captadores: un colector Burkard para cuantificar los granos de polen y un captador de partículas Air Sentinel para la recogida de aeroalergenos. Se recogieron un total de 366 filtros. Los niveles de Alt a 1 se determinaron mediante un anticuerpo monoclonal-ELISA. Se incluyeron 18 pacientesmonosensibles a *A. alternata*, que diariamente registraron síntomas y fármacos consumidos.

Resultados: A. alternata se recolectó durante todo el año, mientras que Alt a 1 se detectó durante el periodo de marzo a diciembre. Los síntomas mostraron una positiva correlación con las esporas (r=0.459, *P*<0.001), y Alt a 1 (r=0.294, *P*<0.001). La correlación entre esporas y Alt a 1 resultó baja. La distribución binomial negativa mostró que un incremento de 10 pg/m^3 en los niveles de Alt a 1 incrementó un 5% el número de síntomas con tres días de decalaje.

Conclusiones: En pacientes alérgicos a *A. alternata* la cuantificación de Alt a 1 puede ser considerada como un importante marcador para predecir el riesgo de síntomas respiratorios.

Palabras clave: *Alternaria alternata*. Alt a 1. Alérgenos. Rinitis. Asma.

Introduction

It has been clearly established that sensitization to *Alternaria alternata* is a risk factor for developing asthma [1]. Furthermore, asthma caused by *A alternata* allergy is characterized by more persistent symptoms, greater disease severity, and a higher risk of fatal outcome [2,3].

A alternata is a widespread saprophyte that is usually found on the ground and on plants. It is therefore considered an aeroallergen, although it can also be found in indoor environments. *A alternata* spores are considered to be the primary source of allergens, although other biological molecules derived from the hyphae as well as small fragments and dust particles can increase the allergenic activity of these spores [4,5]. The major allergen is Alt a 1, which has a molecular mass of approximately 30 kDa and an as yet unknown biological function. Recognition of Alt a 1 is the primary marker of sensitization to *A alternata*. Fungi from the Pleosporaceae family (*Stemphylium botryosum, Ulocladium botrytis, Curvularia lunata*) have a common major allergen that shows a high level of cross-reactivity with Alt a 1 [6]. Consequently, these fungi, despite their lower atmospheric concentrations, may cause an increase in allergen load.

Respiratory symptoms caused by allergy to pollen or fungi have typically been related to grain or spore counts. However, it is not always possible to establish this correlation, as allergens may be derived not only from pollen grains but also from other plant parts (leaves, stalks, orbicules) [7]. It has been demonstrated that grass allergens are present in the atmosphere before and after the pollen season, when pollen grains are absent [8]. Furthermore, in patients with rhinitis and/or asthma who are monosensitized to grasses or *Olea europaea*, symptoms show a much closer relationship with allergen levels than with pollen grain counts [9-10]. Agarwal et al [11] performed similar studies with *A alternata* and ragweed, and demonstrated a significant correlation between allergen activity and spore and pollen grain counts.

The objective of our study was to compare *A alternata* spore counts with daily levels of the major allergen Alt a 1 over the course of a year, and to determine their correlation with symptoms of rhinitis and/or asthma in patients monosensitized to *A alternata* in Ciudad Real, a province situated in central Spain, 190 km south of Madrid.

Methods

Patients

Patients with rhinitis and/or asthma caused by *A alternata* who were screened in the outpatient clinics of the allergy departments of 2 hospitals in the province of Ciudad Real—Hospital Universitario General de Ciudad Real in Ciudad Real and Hospital Santa Bárbara in Puertollano—were included in the study. The inclusion criteria were *a)* rhinitis and/or asthma of at least 2 years' duration based on the Allergic Rhinitis and its Impact on Asthma [12] and the Global Initiative for Asthma [13] criteria, *b)* monosensitization to *A alternata* as described below, *c)* residency in either of the cities chosen for the study

for at least 5 years, and *d)* an age of between 14 and 49 years. The following exclusion criteria were applied: *a)* sensitization to aeroallergens other than *A alternata*, *b)* nasal polyposis, and *c)* immunotherapy with aeroallergens.

Skin prick tests were performed with a panel of common aeroallergens, consisting of standardized extracts of mites, pollens, molds, and cat and dog dander (Laboratories ALK-Abelló). The inclusion criterion was defined as a positive skin prick test (>3 mm wheal diameter) exclusively to *A alternata* extract. All the participants gave their oral informed consent and the study was approved by the scientific ethics committee at Hospital Universitario General de Ciudad Real.

Symptoms and Medication Diary Cards

All the patients were given diary cards on which to record their daily use of antiallergy medication and their conjunctival, nasal, and bronchial symptom scores according to the following scale [10]: 0 = no symptoms; 1 = mild symptoms (slight nasal obstruction, slightly runny nose, or occasional sneeze or itching of the eyes); 2 = moderate symptoms (moderate nasal obstruction, moderately runny nose, some sneezing and congestion, some ocular itching, or mild asthma); and 3 = severe symptoms (complete nasal obstruction, almost continuously runny nose, frequent sneezing or ocular symptoms, or asthma attacks). Graded symptoms were summarized with a weighted score for the drugs used, as follows: 0 = no drugs; 1 = oral antihistamines and/or ß-agonists; 2 = nasal or bronchial corticosteroids; and 3 = systemic corticosteroids.

Of the 418 patients screened, 18, all of whom were monosensitized to *A alternata*, were selected for enrolment in the 1-year study.

Spore Counts

A alternata spore counts were measured from January 1 to December 31, 2004 using a Burkard spore trap (Burkard Manufacturing Co.) situated at a height of 15 m above street level in the city of Ciudad Real. The spores were counted and analyzed by trained personnel using the methodology proposed by the Spanish Aerobiology Network members [14]. The results were expressed in total number of spores/m^3 of air per day.

Air Sampling

A volumetric air sampler adapted for outdoor use (Air Sentinel, Quan-Tec-Air Inc.) [15] was used for aeroallergen collection and run continuously during 2004. The sampler was placed 15 m above street level in Ciudad Real. The air flow was 10 m^3/h. Airborne particles were collected using polytetrafluoroethylene (PTFE) filters (Quan-Tec-Air Inc.) composed of stretched Teflon with a polyester support backing and rated with a 99.9% efficiency at 0.3 μm. The sampling time for each filter was 24 hours, which corresponds to 240 m^3 of air per sample. Filters were replaced at approximately the same time each day. Removed filters were sealed in plastic bags and stored at 4ºC until extraction.

Filter Extraction and Allergen Quantification

A total of 366 PTFE filter membranes were placed in individual tubes containing 0.5 mL of 0.01 M phosphate buffered saline (PBS). The tubes were stirred until the filter was completely soaked and then left for overnight extraction at 4ºC in a rotary mixer (Labinco B.V.). The samples were centrifuged at 3500 rpm for 5 minutes; the supernatant was then collected and processed immediately to prevent loss of allergenic activity. All the samples were extracted and analyzed at the same time in order to avoid differences in results or variations due to handling.

Alt a 1 content was measured by monoclonal antibody-based enzyme-linked immunosorbent assay (Indoor Biotech). Briefly, flat-bottomed microtiter plates (Immulon II) were coated with 100 µL of anti Alt a 1 monoclonal antibody (mAb) at 0.1 µg/mL overnight at 4ºC. After blocking with 1% bovine serum albumin PBS-Tween (BSA-PBS-T), 100 µL of serially diluted recombinant Alt a 1 or samples in BSA-PBS-T were added and incubated. After washing, 100 µL of biotinylated anti Alt a 1 mAb was added to each well and incubated. The wells were washed and 100 µL of streptavidin-peroxidase was added and incubated. The color reaction was developed and analyzed on a plate reader at 405 to 450 nm.

Alt a 1 levels were extrapolated using the standard curve and final results were expressed in picograms of allergen per milliliter.

The threshold levels for *A alternata* counts and Alt a 1 levels were defined as the levels required to cause symptoms in all individuals who were clinically sensitive to *A alternata* (minimum symptom score = 1) [10].

Meteorological Data

Detailed daily records of temperature, humidity, and wind speed and direction were obtained from the meteorological observatory in Ciudad Real (39º N, 41º E; 630 m above sea level).

Statistical Analysis

Most of the statistical analyses were carried out using the SPSS statistical package, with statistical significance set at $P<.05$. A descriptive analysis was performed of number of symptoms, independent variables (spore counts and Alt a 1 levels), and confounding variables (meteorological variables). The correlations between variables were studied using the Pearson coefficient correlation or the Spearman rank in the case of nonnormally distributed data.

To evaluate the effects of the independent variables on the number of symptoms we developed a log-linear Poisson model in which linear and quadratic terms were included to control for the confounding seasonal effect. Finally, delayed effects up to 5 days were taken into account. A systematic strategy was used to construct the model. Overdispersion was encountered and a negative binomial distribution was therefore used.

Results

Patient Population

Eighteen patients (10 women and 8 men) were included in the study. The mean age was 21.2 years (range, 15-41 years). All the patients were monosensitized to *A alternata* and had allergic respiratory tract symptoms. They all had rhinoconjunctivitis (mild in 2 cases), and 12 (66.7%) had asthma (uncontrolled in 3 patients, partially controlled in 7, and controlled in 2) (Table 1).

Table 1. Clinical Characteristics of the Patient Population

Patient	Age	Sex	Symptoms	Classification
1	27	M	R,C,A	PCA
2	16	F	R,C,A	UA
3	24	F	R,C	SPR
4	41	M	R,C,A	UA
5	17	M	R,C,A	PCA
6	15	F	R,C	MPR
7	19	M	R,C,A	CA
8	20	F	R,C	SPR
9	15	M	R,C,A	PCA
10	29	F	R,C,A	PCA
11	18	M	R,C	SIR
12	14	F	R,C,A	PCA
13	21	F	R,C,A	UA
14	20	M	R,C	SPR
15	18	M	R,C,A	CA
16	33	F	R,C,A	PCA
17	19	F	R,C,A	PCA
18	16	F	R,C	MPR

Abbreviations: A, asthma; C, conjunctivitis; CA, controlled asthma; F, female; M, male; MPR, mild persistent rhinitis; PCA, partially controlled asthma; R, Rhinitis; SIR, moderate to severe intermittent rhinitis; SPR, moderate to severe persistent rhinitis; UA, uncontrolled asthma.

Spore Counts

A alternata spores were collected throughout 2004. The highest spore count, 626 spores/m³, was detected on June 8. The total number of spores documented for the whole year was 10 476. The threshold level for *A Alternata* spores was 24 spores/m³.

Immunochemical Quantification of Airborne Alt a 1

Variable amounts of airborne Alt a 1 were detected over the course of the year. The maximum values of Alt a 1 (110.25 pg/m³) were detected on November 16. The threshold level for Alt a 1 was 20.7 pg/m³.

Correlation Between Spores Counts and Alt a 1 Levels

A mean symptom score of 16.14 was registered over the course of the year. Figures 1 and 2 show the time series for the symptom scores versus spore counts and Alt a 1 levels, respectively. Figure 3 shows the correlation between spore counts and Alt a 1 levels. Results showed low correlation coefficients between both variables.

Significant correlations were found between daily symptom scores and spore counts (r=0.46, $P<.001$), Alt a 1

Alternaria alternata
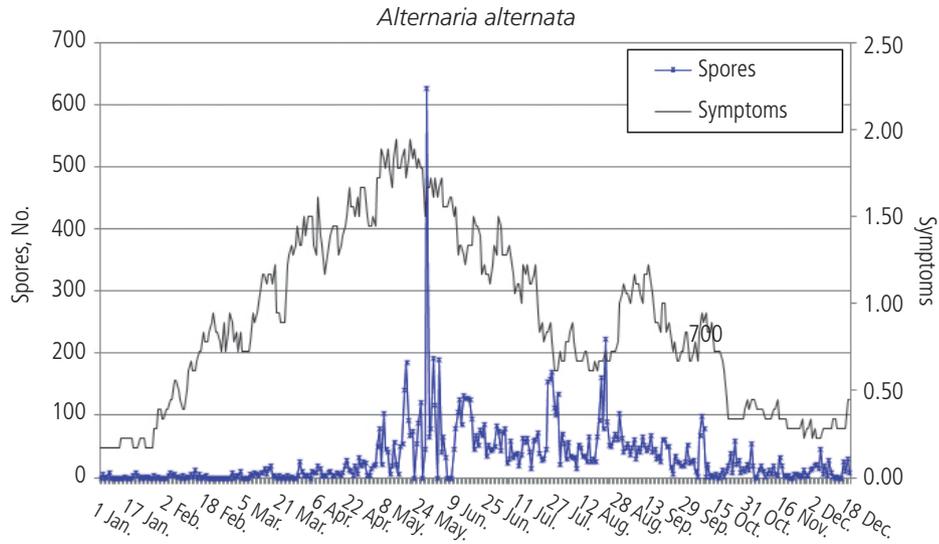
Figure 1. Correlation between symptoms and number of spores detected during the study period.

Alternaria alternata
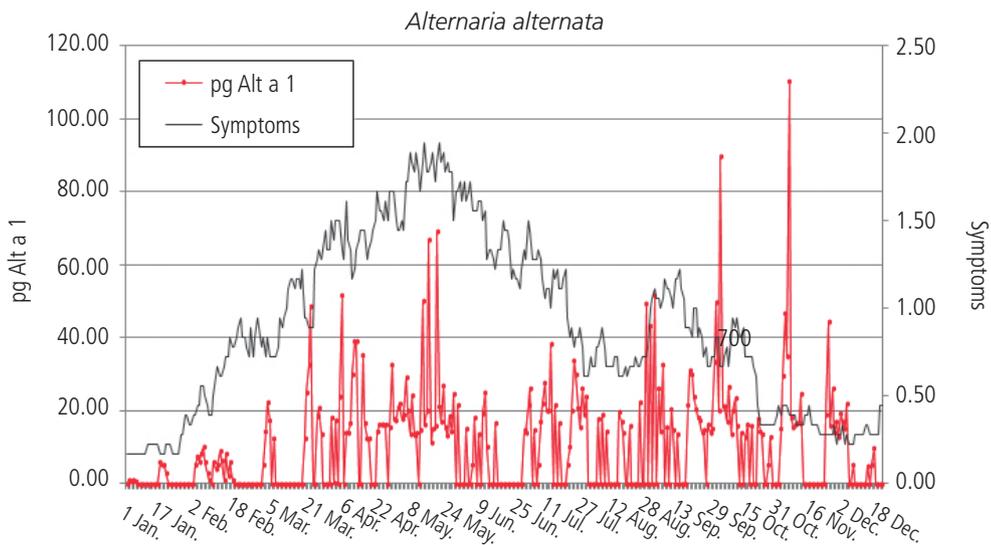
Figure 2. Correlation between symptoms and Alt a 1 levels during the study period.

$$y=0.1316x + 30.357$$
$$R^2=0.0014$$
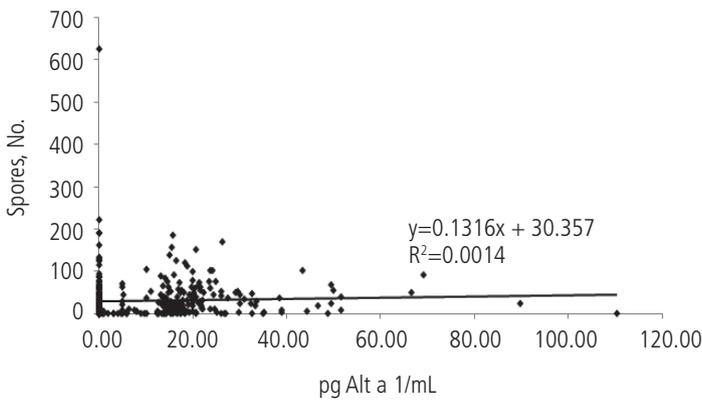
Figure 3. Correlation between Alt a 1 levels and spore counts (r=0.04).

Table 2. Results from Negative Binomial Model Analysis

No. of Symptoms	Coefficient	Standard Error	IRR
T	0.136261	.0181648	1.145981
Lag1(RH)	0.042339	.0124217	1.043249
T^2	-0.002816	.0004689	.997188
Lag3 (Alt)	0.004805	.0020787	1.004803
Lag1(RH^2)	-0.000366	.0000921	.999633
WS	0.026697	.0064552	1.02705
Lag3(WS^2)	0.001494	.0003369	1.00149

Abbreviations: Alt, Alt a 1 concentration; RH, relative humidity; IRR, incident rate ratio; T, temperature; WS, wind speed.

levels (r=0.30, *P*<.001), and meteorological variables and their quadratic terms. The results of the negative binomial model are presented in Table 2. This table summarizes the coefficients and the standard error of the variables determined in our study. Incident rate ratios show the relative changes in the variables analyzed. The model predicted that an increment of 1 pg/m3 of Alt a 1 would increase the number of symptoms by 0.48% at a 3-day lag. The Pearson chi-square of 350.38 for 332 degrees of freedom was not significant, indicating that our model fit the data.

Discussion

The complexity of the allergenic composition of *A alternata* spores makes it difficult to establish a relationship between exposure to spores and rhinitis and/or asthma symptoms in allergic individuals sensitized to *A alternata*. Aerobiologic analyses show marked variability in concentrations of *A alternata* in different geographical regions, with seasonal variations depending on climatic conditions and meteorological variables. In addition, high levels of Alt a 1, the major allergen of *A alternata*, are detected in the atmosphere even when spores are absent [16,17]. Furthermore, patients who are allergic to *A alternata* are frequently sensitized to other allergens such as grass and olive pollen, which are present in the atmosphere at the same time of the year as Alt a 1. Thus, despite the significant prevalence and recognized severity of asthma due to *A alternata* allergy, no studies to date have related symptoms in sensitized patients to spore counts. However, determining levels of Alt a 1 is a good option for establishing the risk of symptoms in patients allergic to *A alternata* despite the confounding effect caused by the expression of Alt a 1-like allergens by other fungi of the Pleosporaceae family [6]. In our study, allergy to *A alternata* was confirmed in 54 (13%) of the 418 patients diagnosed with allergic rhinitis and/or asthma in our area. Although this prevalence is similar to that reported for other European countries [18] and the United States [19], the number of monosensitized patients was extremely low (only 18 individuals [4%]). The main reason is probably high exposure to pollens, mainly grasses, olive, and weeds. Exclusive sensitization to *A alternata* is unlikely in Spain considering the allergenic profile of Spanish patients and the high exposure to other relevant allergens.

Patients allergic to *A alternata* had a more persistent and more severe clinical profile than those sensitized to other aeroallergens, and symptoms were present from April to November. Furthermore, 67% of our patients had asthma, contrasting with a rate of 40% detected in grass-allergic patients [9].

Symptoms in patients monosensitized to *A alternata* showed an acceptable and significant association with spore counts and Alt a 1 levels. However, in the predictive model we constructed on the basis of clinical, meteorological, and aerobiological data, the clinical course of patients with rhinitis and/or asthma caused by allergy to *A alternata* was related to Alt a 1 levels, without the participation of spores in the model. The symptom threshold was reached in May, with a concentration of 20.7 pg/m³ of Alt a 1. Furthermore, the negative binomial model showed that an increase in Alt a 1 levels of 10 pg/m³ caused a 5% increase in the number of symptoms at a 3-day lag. According to our results, it seems that the determination of Alt a 1 levels in the atmosphere can predict the risk of respiratory symptoms in patients sensitized to *A alternata*. While the presence of Alt a 1 could be due to other fungi of the Pleosporaceae family that also express this allergen [6], spores from these fungi were absent in the aerobiological analyses. Previous studies of grass [9] and ragweed [11] pollen have also shown pollen allergen activity in the absence of pollen grains. Furthermore, in the case of grasses, although there was an acceptable correlation between symptoms and pollen counts, the correlation with allergen levels was stronger.

Meteorological variables, particularly temperature and relative humidity, have a significant effect on the presence of spores and Alt a 1 in the atmosphere. The optimal conditions for high spore counts are temperatures of 20°C to 30°C and high humidity, explaining the seasonal pattern of *A alternata* allergy [16,20]. While spores were more common in our study area between June and September (peak count on June 8, with 626 spores/m³ air), Alt a 1 showed 2 peaks, with maximum levels during the months of April and May and October and November (peak concentration November 16, with 110 pg/m³). Symptoms showed a positive correlation with other meteorological variables such as temperature, relative humidity, and wind speed.

The main limitation of our study is that because of the low frequency of monosensitization to *A alternata*, we were only able to study a small number of patients. Ciudad Real has a dry continental climate, meaning that pollens are the main allergens in the area; monosensitized patients, therefore, account for only a very small proportion of the overall allergic population [9,10]. Another possible limitation is that we did not measure exposure to Alt a 1 or *A alternata* in homes, where the presence of allergens could be responsible for allergic symptoms. However, we consider that the climatic conditions in Ciudad Real are not propitious for the growth of *A alternata* in indoor environments. This consideration is based on a previous study conducted by our group in which we ruled out the presence of Alt a 1 in homes (data not published).

In our predictive model, symptoms of allergic rhinitis and/or asthma caused by sensitization to *A alternata* were significantly correlated with Alt a 1 levels (r=0.30) and total *A alternata* levels (r=0.46). Further studies in other regions with larger numbers of patients with respiratory symptoms caused by *A alternata* should investigate whether determination of Alt a 1 levels has greater clinical relevance than the traditional spore count method.

Acknowledgements

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▌ **Francisco Feo Brito**

Sección de Alergología
Hospital General Universitario
C/ Obispo Rafael Torija, s/n
13005 Ciudad Real, Spain
E-mail: feobrito1@gmail.com