Original Article

Variability of Alt a 1 Expression by Different Strains of *Alternaria alternata*

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Abstract. *Background*: While it is well known that there is significant intraspecific variation in the content and potency of *Alternaria alternata* allergens, little data has been published on intraspecific variability for individual allergens from moulds.
Objective: To assess the variability of Alt a 1 expression in different strains of *A alternata*.
Methods: Eleven strains of *A alternata* were cultured in a Czapek broth medium and culture filtrate extracts were obtained. A sensitive two-site enzyme-linked immunosorbent assay was used to measure Alt a 1 concentrations in medium following 3 weeks of culture and in culture filtrate extracts.
Results: Expression of Alt a 1 was highly variable in different strains of *A alternata* (coefficient of variation >135%). A good correlation was found between Alt a 1 concentrations at the beginning of the process and measurements at the end of extract production ($r = 0.940$).
Conclusions: The high variability of Alt a 1 expression in different *A alternata* strains makes it necessary to measure Alt a 1 concentrations during the first stage of allergenic extract production in order to be able to choose a suitable strain for producing extracts or purifying Alt a 1.

Key words: *Alternaria alternata*. Alt a 1. Major allergen. Variability.

Resumen. *Antecedentes*: Existen datos suficientes en la literatura publicada al respecto, que demuestran de forma inequívoca la variación tanto cualitativa como cuantitativa de los alérgenos que expresan las diferentes cepas de *Alternaria alternata.* No obstante, hasta la fecha no existen apenas datos que se refieran al fenómeno de variabilidad intra-específica de componentes alérgenos individualizados de origen fúngico.
Objetivo: Evaluar la variabilidad de la expresión de Alt a 1 a partir de diferentes cepas de *Alternaria alternata*
Métodos: Se obtuvieron extractos extracelulares a partir de 11 cepas de *Alternaria alternata* cultivadas en medio líquido de Czapek. La concentración de Alt a 1 se evaluó mediante un enzimoinmunoensayo con anticuerpos monoclonales, directamente a partir del medio de cultivo tras 3 semanas de incubación a 25°C, así como a partir de extractos de filtrados de cultivo.
Resultados: Los resultados mostraron una variabilidad significativa en la expresión de Alt a 1 dependiente de cada una de las cepas estudiadas (coeficiente de variación >135%). Se apreció una buena correlación entre los datos de concentración de Alt a 1 medidos al inicio y al final del proceso de producción de los extractos alergénicos ($r = 0.940$).
Conclusión: Estos resultados muestran la necesidad de medir la concentración de Alt a 1 durante las fases iniciales de la producción de extractos alergénicos de *Alternaria alternata* a fin de poder discriminar la productividad de las cepas elegidas así como el rendimiento de las mismas tanto a nivel de potencia alergénica como en procesos de purificación de dicho alergeno.

Palabras clave: *Alternaria alternata*. Alt a 1. Alergeno mayor. Variabilidad alergénica.

Introduction

Currently available extracts of allergenic moulds are not reliable tools for diagnostic purposes due to the difficulty of identifying related species based on morphological criteria, genetic variation and strain variabilities, the lack of consensus on production and quality control procedures, and the phenomenon of batch-to-batch variation [1-4]. The intraspecific variability of *Alternaria alternata* allergenic extracts has been recognized for a number of years and strain variability has also been extensively described for this species in terms of allergologic or immunologic [5-10] and biochemical [5-7, 9-11] data, as well as the yield of production processes [5, 9, 11]. However, less data is available on the intraspecific variability of individual allergens from *A alternata* [8, 12]. Rosenthal et al [12] found an Alt a 1-related allergen sequence in many strains of *A alternata*, with calculated concentrations for Alt a 1 mRNA similar for all strains. Those authors suggested that variability in allergen content is the result of posttranslational events.

Alt a 1 is continuously released into the medium of *A alternata* cultures, where it accumulates [13], and culture filtrates constitute the optimum source of raw material for the production of allergenic extracts [9, 14, 15].

To evaluate the variability of Alt a 1 expression in different strains of *A alternata*, we analyzed culture filtrates from 3-week cultures and extracts of excretion–secretion products obtained from 11 different strains of *A alternata* at the beginning of maximum growth in culture, when Alt a 1 reaches its highest concentrations [13].

Material and Methods

Strains

The following *A alternata* strains were used: CBS 106.24, CBS 105.24, CBS 105.49, CBS 104.26, CBS 247.85, CBS 154.31, and CBS 113.41 (Centraalbureau voor Schimmelcultures, Delft, The Netherlands); strains IMIM 282, IMIM 93039 and MR 455-2 (Laboratory of Research in Infectious Diseases and Mycology, Municipal Institute for Medical Research [IMIM], Barcelona, Spain); and strain FMR 3292 (Laboratory of Mycology, Department of Medical Sciences, Rovira Virgili University, Tarragona, Spain). Strain PC4 of the *Penicillium chrysogenum* complex (IMIM) was used as a control.

Extracts

Culture filtrate extracts were obtained from fungal cultures in a Czapek broth medium. All strains were incubated for 3 weeks at 25°C after introducing a standardized inoculum (10^3 spores). Culture filtrate extracts were obtained according to the method described by Martinez et al [9]. Briefly, culture filtrates were obtained by successive filtration using Whatman No. 1, AP, and sterilizing filters. The filtrate was dialyzed by tangential ultrafiltration with a 10 kilodalton cutoff point. Finally, the retained material was freeze dried. Each strain was cultured in triplicate.

Quantification of Alt a 1

Enzyme-linked immunosorbent assay using the 1D6 anti-Alt a 1 monoclonal antibody in the solid phase and biotinylated polyclonal anti-Alta a 1 antibodies as a second antibody reagent (Bial Laboratorios, Bilbao, Spain) was used to measure Alt a 1 concentrations from the original fungal culture filtrates after 3 weeks of incubation and from freeze-dried culture filtrate extracts, as described by Asturias et al [16].

Alt a 1 concentrations for freeze-dried culture filtrate extracts were calculated taking into account the extract yield obtained from each cultured strain: (mg of allergen/mg of freeze-dried material) × (mg of freeze dried material/L of culture medium) = mg of allergen per liter of culture medium. The results are expressed as the arithmetic mean of triplicates.

Statistical Analysis

All statistical calculations were performed with GraphPad Prism version 4.0 for Microsoft Windows (GraphPad Prism software. San Diego, CA, USA). Alt a 1 content of culture filtrates from 3 weeks incubation and from culture filtrate extracts was compared by linear regression analysis.

Results

All *A alternata* strains achieved maximum growth 16 days after inoculation of the cultures. Sporulation occurred after 2 weeks (mean ± SD, 16.64 ± 2.73 days).

Table 1 shows the yield values for the culture filtrate extracts obtained from the different *A alternata* strains. Results showed a high yield (421.4 mg of freeze-dried culture filtrate extract per liter of culture medium; range, 73.7-1104.5 mg/L) and a high coefficient of variation (81.1%).

Table 2 and the figure show Alt a 1 expression in terms of the concentrations of secreted *A alternata* major allergens from 11 strains of *A alternata* cultured for 3 weeks at 25°C. The data show very significant dispersion, with coefficients of variation of 133% for concentrations of Alt a 1 obtained directly from culture filtrates and 190% for measurements obtained from culture filtrate extracts.

Secreted material from *P chrysogenum* cultured for 3 weeks at 25 °C and culture filtrate extracts obtained from this mould did not contain detectable concentrations of Alt a 1.

Table 1. Variability of the Yield of *Alternaria alternata* Culture Filtrate Extracts.

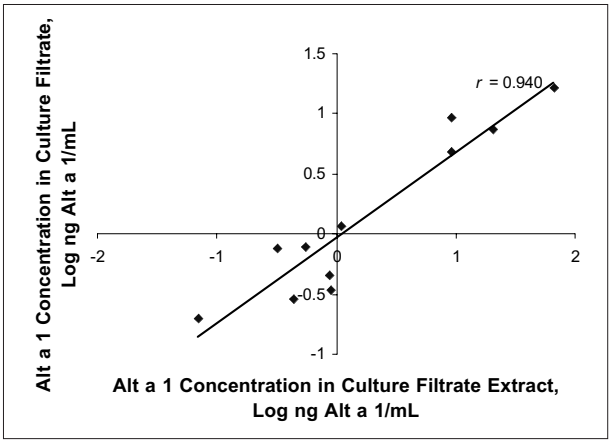
Strain	Yield, mg Freeze-Dried Culture Filtrate Extract Per Liter Of Medium
CBS 105.24	73.7
MR 455-2	122.4
CBS 113.41	240.9
CBS 154.31	316.7
CBS 106.24	758.5
IMIM 93039	839.7
FMR 3292	1104.5
CBS 105.49	912.2
IMIM 282	307.7
CBS 104.26	176.0
CBS 247.85	107.5
Arithmetic mean	421.4
SD	341.9
SEM	103.3
Coefficient of variation	81.1%

Table 2. Variability of Alt a 1 Expression: Quantification of Alt a 1 in Excretion–Secretion Material From 11 Strains of *Alternaria alternata*.

Strain	Alt a 1 Concentration in Culture Filtrate Extract, ng/mL	Alt a 1 Concentration in Culture Filtrate, ng/mL
CBS 154.31	0.5	0.8
CBS 113.41	0.6	0.8
FMR 3292	0.9	0.5
IMIM 93039	0.9	0.5
IMIM 282	0.5	0.5
CBS 105.49	0.5	0.5
CBS 105.24	9.2	4.8
CBS 106.24	67.8	16.1
CBS 104.26	20.8	7.3
CBS 247.85	9.4	9.3
MR 455-2	1.1	1.2
Arithmetic mean	11.2	4.2
SD	21.0	5.3
SEM	6.3	1.6
Coefficient of variation	190.0%	132.5%

Discussion

Allergen extracts produced from fungi show a clear lack of homogeneity for a range of reasons, including strain identification, culture conditions, production methods, genetic variation, and strain variability [1-11]. This situation makes their standardization for diagnostic and therapeutic purposes difficult.



Correlation between Alt a 1 concentrations from 11 strains of *Alternaria alternata*. Linear regression analysis of data from culture medium after 3 weeks of incubation and culture filtrate extracts.

In recent years, recombinant technology has made it possible to analyze a large number of allergens for use as diagnostic tools [17]. The major *A alternata* allergen, Alt a 1, is an excellent marker for measuring sensitization to *Alternaria* in allergic patients [2, 18]. Moreover, Unger et al [2] have suggested that these pure allergens can be used for therapeutic purposes.

The development of monoclonal antibodies against Alt a 1 has made it possible to improve the methodology for quantifying Alt a 1. These methods have been demonstrated to be essential for standardization and quality control of *A alternata* extracts. Current assays offer very high sensitivity (0.5-3.1 ng/mL) and specificity [16, 19]. Only members of the Pleosporaceae family have Alta a 1-like proteins that can be detected using these methods [16, 20].

Despite the fact that Rosenthal et al [12] demonstrated that mRNA encoding Alt a 1 is present in 8 different strains of *A alternata*, with similar concentrations in 7 of the 8 strains, to the best of our knowledge no data have been published on variability in Alt a 1 expression in different strains of *A alternata.* Measurement of Alt a 1 concentrations during the first step in the production process for allergenic extracts may enable suitable strains to be selected in order to produce extracts or obtain high-yield purified Alt a 1.

Our results revealed a high variability of Alt a 1 expression in different strains of *A alternata*, with coefficients of variation of more than 130%. Coefficients of variation for Alt a 1 between the different batches (triplicates) for each simple strain did not exceed 20%. Comparison of these data with those obtained by other authors showed 50% less variability [16]. Two main factors could account for this difference: the use of a small number of batches and the use of only 1 experiment for all different lots under the same environmental conditions.

Although Ibarrola et al [13] demonstrated a maximum expression of Alt a 1 in spent culture medium after 4 weeks of incubation, we decided to measure expression of the major *A alternata* allergen after 3 weeks of incubation, in accordance with previous reports [11, 20] and taking into account that all strains achieved maximum growth and sporulation.

Linear regression analysis revealed a good correlation (r=0.940) between concentrations of Alt a 1 at the beginning of the process (culture filtrate extracts from 3-week fungal cultures) and measurements at the end of extract production (freeze-dried allergenic extract). Although it is not easy to explain the underlying discrepancies between the Alt a 1 content in the medium and in the extract, it may be due to the extra manipulation and subsequent enrichment of the protein fraction. However, it is interesting to note that the significant discrepancies, only in terms of absolute values, were associated with strains that produce higher concentrations of Alt a 1. These data indicate the importance of strain in terms of quality and yield of fungal allergenic extracts and highlight the value of Alt a 1 as a marker for optimal culture conditions [13] and the importance of choosing a suitable *Alternaria* strain.

Acknowledgments

This study was funded by the Department of Education, Universities and Research of the Regional Government of the Basque Country, projects PI9305 and UE 96/6 and Sweden Diagnostics (Spain) S.L.

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