UNIVERSIDADE DA BEIRA INTERIOR
Ciências

Evaluation of Alt a 1 as a specific marker of exposure to fungal allergenic sources and clinical relevance of a manganese-dependent superoxide dismutase and a serine protease as new *Alternaria alternata* allergens

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To my parents and brother

Everyone can rise above their circumstances and achieve success
if they are dedicated to and passionate about what they do.
Nelson Mandela

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List of publications

Papers included in the thesis:

I. ***Alternaria alternata* allergens: Markers of exposure, phylogeny and risk of fungi-induced respiratory allergy**

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Medical Mycology (2015) 53:636-642.

III. **Characterisation of *Alternaria alternata* manganese-dependent superoxide dismutase, a cross-reactive allergen homologue to Asp f 6**

M. F. Gabriel, I. Postigo, A. Gutiérrez-Rodríguez, E. Suñen, J. Guisantes, C. T. Tomaz, J. Martínez

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IV. **Alt a 15 is a new cross-reactive minor allergen of *Alternaria alternata***

M. F. Gabriel, I. Postigo, A. Gutiérrez-Rodríguez, E. Suñen, J. Guisantes,J. Fernández, C. T. Tomaz, J. Martínez

Immunobiology (2015).In Press

V. **From respiratory sensitization to food allergy: Anaphylactic reaction after ingestion of mushrooms (*Agaricus bisporus*)**

M. F. Gabriel, P. González-Delgado, I. Postigo, J. Fernández, V. Soriano, B. Cueva, J. Martínez

Medical Mycology Case Reports (2015) 8: 14-16.

Chapter in a book included in the thesis:

I. **Fungal allergens: Recent Trends and Future Prospects**
Marta Gabriel, Jorge Martínez and Idoia Postigo
In: Mehdi Razzaghi-Abyaneh, Masoomeh Shams-Ghahfarokhi, Mahendra Rai, editors. Medical Mycology: Current Trends and Future Prospects. CRC Press. Taylor & Francis Group; 2015. 315-333.

Papers from scientific conferences:

I. ***Alternaria alternata*: cloning and sequence analysis of the cDNA sequence of a Asp f 6- like allergen**
M. F. Gabriel, I. Postigo, A. Gutiérrez-Rodríguez, E. Suñen, J. Guisantes, C. T. Tomaz, J. Martínez
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II. **A subtilisin like serine protease of *Alternaria alternata*: a potentially cross-reactive allergen**
M. F. Gabriel, I. Postigo, A. Gutiérrez-Rodríguez, E. Suñen, J. Guisantes, C. T. Tomaz, J. Martínez
Allergy 68, Suppl. 97 (2013), 189–336 p 659 (pag 265).
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III. **Is the sensitisation to *Alternaria alternata* manganese-dependent superoxide dismutase a risk factor for ABPA?**
M. F. Gabriel, I. Postigo, E. Suñen, A. Gutiérrez-Rodríguez, C. T. Tomaz, J. Martínez
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IV. **Development of a based-gene encoding allergen tool for detecting Alt a 1 and Alt a 1 homologues**
M. F. Gabriel, I. Postigo, A. Gutiérrez-Rodríguez, E. Suñen, C.T. Tomaz, J. Martínez

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V. **Cloning and characterisation of Alt a 15, a novel cross-reactive allergen from *Alternaria alternata***
M. F. Gabriel, I. Postigo, A. Gutiérrez-Rodríguez, E. Suñen, J. Guisantes,J. Fernández, C. T. Tomaz, J. Martínez
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List of scientific communications

Oral scientific communications resultant of the Doctoral work:

I. **Bases de datos de proteínas alergénicas/ *Databases of Allergenic proteins***
Marta Da Fonseca Gabriel and Ignacio García.
XLII Reunión AlergoSur, April 16, Reina Sofía Hospital, Córdoba, Spain, 2014.

Poster presentations resultant of the Doctoral work:

I. **Subtilisin like serine protease of *Alternaria alternata*: a potentially cross-reactive allergen**
M. F. Gabriel, I. Postigo, A. Gutiérrez-Rodríguez, E. Suñen, J. Guisantes, C. T. Tomaz, J. Martínez
European Academy of Allergy and Immunology & World Allergy Organization World Allergy & Asthma Congress, June 22-26, Milan, Italy, 2013.

II. ***Alternaria alternata*: cloning and sequence analysis of the cDNA sequence of an Asp f 6- like allergen**

M. F. Gabriel, I. Postigo, A. Gutiérrez-Rodríguez, E. Suñen, J. Guisantes, C. T. Tomaz, J. Martínez
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III. Is the sensitisation to *Alternaria alternata* manganese-dependent superoxide dismutase a risk factor for ABPA?

M. F. Gabriel, I. Postigo, E. Suñen, A. Gutiérrez-Rodríguez, C. T. Tomaz, J. Martínez

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List of contributions to official databases

Contributions to GenBank from the National Center for Biotechnology Information (NCBI) resultant of the Doctoral work:

I. ***Alternaria alternata*** **strain CBS 104.26 manganese superoxide dismutase mRNA, complete cds**
M. F. Gabriel, I. Postigo, E. Suñen, A. Gutiérrez-Rodríguez, C. T. Tomaz, J. Martínez
GenBank Acession number: KC923297
http://www.ncbi.nlm.nih.gov/nuccore/KC923297

II. ***Alternaria alternata*** **strain CBS 104.26 subtilisin-like serine protease mRNA, partial cds**
M. F. Gabriel, I. Postigo, E. Suñen, A. Gutiérrez-Rodríguez, C. T. Tomaz, J. Martínez
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Contributions to the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Database resultant of the Doctoral work:

I. **Alt a 14 ,** ***Alternaria alternata*** **official allergen**
M. F. Gabriel
http://www.allergen.org/viewallergen.php?aid=790 (2014).

II. **Alt a 15 ,** ***Alternaria alternata*** **official allergen**
M. F. Gabriel
http://www.allergen.org/viewallergen.php?aid=820 (2014).

Contributions to the Protein Model Database (PMDB) resultant of the Doctoral work:

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Resumo alargado

As doenças alérgicas são consideradas uma das epidemias do século XXI, afectando aproximadamente um terço da população em geral. De entre as fontes alergénicas ambientais capazes de induzir reacções de hipersensibilidade de tipo I, os fungos têm sido uma das áreas de estudo menos favorecidas. No entanto, a sensibilização a aero-alergénios de origem fúngica tem sido apontada por vários autores como um factor de risco indiscutível para o desenvolvimento, persistência e severidade de doença alérgica respiratória, nomeadamente da asma. Estes dados sugerem que a realização de estudos visando progressos, nomeadamente, nos métodos de detecção de exposição ambiental, assim como nas metodologias de diagnóstico, são necessários e cruciais para a melhor compreensão, diagnóstico e tratamento das doenças alérgicas provocadas por espécies fúngicas.

Os métodos de avaliação de contaminação ambiental por fungos de importância alergológica, tradicionalmente usados em estudos epidemiológicos e aerobiológicos, têm como base a detecção e identificação de espécies por observação microscópica de características morfológicas e contagem de esporos. Na sua generalidade, a execução destes métodos é bastante demorada, complexa e requer a presença de pessoal treinado com elevados conhecimentos na área da micologia. Desta forma, a definição ou exclusão de contaminação ambiental por uma determinada espécie fúngica e a associação de exposição com um padrão de sintomatologia alérgica tem sido bastante problemática. Em termos de diagnóstico, a principal dificuldade atual resulta do grande número de doentes que estão aparentemente sensibilizados a mais do que uma espécie fúngica. Nestes casos clínicos, o diagnóstico da causa primária de sensibilização é bastante complexo.

Vários estudos epidemiológicos realizados, fundamentalmente na Europa, têm descrito *Alternaria alternata* como a fonte de aero-alergénios de origem fúngica mais importante e com maior alergenicidade. Tais relatos sugerem que o estudo do painel de alergénios produzidos por esta espécie pode ser um alvo de estudo que pode contribuir para o avanço significativo na área de conhecimento das alergias a fungos. Entre as várias proteínas alergénicas descritas até ao momento, Alt a 1, constitui o alergénio de *A. alternata* mais importante, sendo responsável pela sensibilização de, pelo menos, 80% dos doentes diagnosticados como alérgicos a *Alternaria*. Devido a esta elevada prevalência de sensibilização, este componente alergénico tem sido correntemente utilizado como um marcador de sensibilização genuína a *A. alternata*, sendo referido por muitos autores como um marcador de sensibilização à família das Pleosporaceae. Nos últimos anos, o diagnóstico molecular tem sido apresentado como uma ferramenta com resultados bastante promissores para definir o perfil de sensibilização individual de cada doente alérgico e sua associação com as manifestações clínicas observadas, assim como para discriminar entre casos de co-sensibilização e reatividade cruzada. Nestes estudos, a sensibilização a Alt a 1 e aos outros

alergénios descritos, parece não ser suficiente para justificar todo o espectro de sintomas clínicos observado em doentes sensibilizados a *A. alternata*.

Tendo por base estes conhecimentos, estabeleceram-se como objectivos principais deste trabalho: por um lado, desenvolver uma tecnologia por detecção específica de *A. alternata* e espécies taxonomicamente relacionadas, utilizando Alt a 1 como um marcador molecular espécie-específico. E por outro lado, ampliar o painel alergénico de *A. alternata*, por caracterização de uma superóxido dismutase dependente de manganês (MnSOD) e uma protease de serina (SP) como dois novos alergénios de *A alternata* e estudar o seu papel no diagnóstico e prognóstico das doenças alérgicas causadas por fungos.

As estratégias usadas para a concretização destes objectivos tiveram como princípio base o estudo das relações filogenéticas das espécies fúngicas que partilham proteínas homólogas aos alergénios de *A. alternata*. Assim, primeiramente, o estudo de conservação dos genes de expressão de Alt a 1 e homólogos possibilitou o desenho de um par de *primers* para o reconhecimento da região conservada e imunologicamente relevante, ou seja, da zona que codifica para os epítopos alergénicos da molécula de Alt a 1. Foram então realizados ensaios de PCR utilizando este par de primers e um segundo par de oligonucleótidos que permite a amplificação do gene completo de Alt a 1. Desta forma, foram desenvolvidas e optimizadas duas metodologias específicas, sensíveis e rápidas: a primeira com potencial para a detecção de Alt a 1 e homólogos, independentemente da fonte alergénica que o produz e uma segunda que permitiu detetar de forma restringida as espécies filogeneticamente próximas, *A. alternata* e *A. tenuissima*.

O estudo de homologia e conservação de genes que codificam para proteínas homólogas às de *A. alternata*, foi também aplicado no desenho de *primers* degenerados para a região não variável das sequências nucleotídicas codificantes de MnSODs e SPs de origem fúngica disponíveis nas bases de dados. Os cDNA completos que codificam estas proteínas foram isolados pela realização de ensaios RACE-PCR; as respectivas moléculas recombinantes foram produzidas em *Escherichia coli* e purificadas a partir dos corpos de inclusão. A capacidade de reconhecimento de IgE existente em soros de uma população representativa de doentes sensibilizados a *A. alternata* foi analisada mediante *immunoblotting*. Desta análise verificaram-se prevalências de sensibilização às MnSOD e SP de *A. alternata* recombinantes de 11.5% (n=61) e 10.2% (n=59), respetivamente. Estes resultados foram comunicados ao World *Health Organization and International Union of Immunological Societies* (WHO/IUIS) *Allergen Nomenclature Sub-Committee* e ambas proteínas foram registadas por esta entidade como alergénios clinicamente relevantes de *A. alternata*, oficialmente designados de Alt a 14 e Alt a 15. Pela realização de ensaios de inibição por ELISA pôde constatar-se que os alergénios recombinantes produzidos apresentavam características imunoquímicas de reatividade para a IgE, similares às respetivas moléculas nativas existentes num extracto crude de *A. alternata*. Adicionalmente, Alt a 14 e Alt a 15 demonstraram um alto potencial para mediar reações de reatividade cruzada entre *A. alternata* e outras espécies fúngicas de importância alergológica, nomeadamente *Aspergillus fumigatus* e *Curvularia lunata*. A associação de

dados clínicos com as evidências de immunorreatividade e reatividade cruzada permitiu demonstrar que a sensibilização a Alt a 14 parece ser um indicador de desenvolvimento de aspergilose broncopulmonar alérgica (ABPA). Em relação a Alt a 15, a sensibilização a esta proteína alergénica pareceu ser restrita a doentes aparentemente sensibilizados a múltiplos fungos. Além disso, esta molécula parece justificar a sensibilização a *A. alternata* em alguns casos nos quais a sensibilização ao marcador de sensibilização genuína a *Alternaria* (Alt a 1) não foi verificada. Neste trabalho de investigação foi ainda apresentado um caso clínico de um doente com história clínica prévia de sintomas respiratórios associados a alergia diagnosticada a fungos ambientais, entre os quais a *A. alternata*, que recentemente sofreu um choque anafilático após a ingestão de cogumelos. Os resultados obtidos pela análise da reatividade da IgE sérica da doente identificaram uma MnSOD (homóloga da Alt a 14) e uma manitol desidrogenase (MtDH) existentes num extrato da espécie de cogumelo comestível *Agaricus bisporus,* como as moléculas responsáveis pelo desencadeamento da reação sistémica observada. Estas demonstrações sustentam a teoria de que uma anterior sensibilização a alergénios fúngicos ambientais de reatividade cruzada, tais como os que são apresentados neste trabalho, em particular Alt a 14, pode suscitar o desenvolvimento de reações sistémicas a alimentos que partilham alergénios homólogos aos sensibilizantes primários.

Em suma, neste trabalho doutoral, foi desenvolvida uma técnica de PCR específica e bastante sensível para aplicação no controlo de contaminação por espécies fúngicas produtoras de Alt a 1. Além disso, dois novos alergénios de *A. alternata*, Alt a 14 e Alt a 15, foram caracterizados como alergénios *minor* capazes de mediar fenómenos de reatividade cruzada com várias espécies fúngicas, resultando em quadros clínicos de resposta alérgica complexos. A disponibilidade destes alergénios como moléculas recombinantes com aptidão para serem aplicadas em diagnóstico molecular pode constituir uma mais valia para melhorar a precisão do diagnóstico das doenças alérgicas provocadas por fungos, através da definição de perfis de sensibilização individuais. Pelas evidências clínicas demonstradas ao longo deste trabalho pode também concluir-se que a correta identificação de sensibilização a Alt a 14 e Alt a 15, pode orientar o alergologista e o doente para a tomada de medidas de prevenção de potenciais quadros clínicos que podem ocorrer, nomeadamente, em consequência de reatividade cruzada.

Este trabalho fornece ferramentas e informações úteis e valiosas que podem contribuir para um avanço significativo na avaliação de exposição a alergénios de origem fúngica, assim como no diagnóstico, prognóstico e tratamento das doenças alérgicas provocadas por espécies fúngicas.

Palavras-chave

Alergénios, alergia a fungos, *Alternaria alternata,* anafilaxia, asma, aspergilose broncopulmonar alérgica, exposição ambiental, filogenética, protease de serina, proteínas homólogas, reatividade cruzada, recombinantes, sensibilização múltipla, superóxido dismutase dependente de manganês

Abstract

Allergic diseases are considered to be one of the epidemics of the century, affecting approximately one-third of the general population. Classically, among the allergenic sources able to induce IgE-mediated reactions, fungi have been one of the less favored areas of study. It has been demonstrated that sensitization to fungal aeroallergens, particularly from *Alternaria alternata,* represents an unequivocal risk factor for the development, persistence and severity of asthma. Thus, the better understanding and management of fungal allergy, namely by improvements in the actual assessment and diagnostic approaches are needed. Regarding the assessment of fungal exposure, actual methodologies are generally laborious and time-consuming making difficult to establish or exclude a fungal contamination and potential associations with allergic disease. In terms of diagnosis, the main difficulty arises from the high number of patients that are apparently sensitized to multiple fungi in which the identification of primary cause of sensitization is complex. Because *A. alternata* is one of the most abundant and potent source of airborne allergens, the panel of allergenic components produced by this fungal specie, seems to be a very relevant target of study. Among the several allergens described in this mold, Alt a 1 has been demonstrated to be the most important, sensitizing approximately 80% of *A. alternata* allergic patients. For this reason, Alt a 1 has been used as the diagnostic marker of genuine sensitization to *A. alternata*. However, given the complex *A. alternata* sensitization data, which shows a significant level of poly-sensitization, due to co-sensitization and/or cross-reactivity to several other phylogenetically related and non-related molds, other allergenic *A. alternata* components should be studied to explain the whole broad range of reported clinical observations. In the last years, component-resolved diagnosis (CRD) has been shown to be a valuable tool to elucidate clinical observations and to differentiate between cases of co-sensitization and cross-reactivity. Nevertheless, the actual available panel of *A. alternata* allergens appears to not be enough for achieving an accurate diagnosis and prognosis of sensitization to this mold.

Considering the above mentioned facts, the major aims of this work were, on one hand to develop an approach to specifically detect *Alternaria* and related species by using the *A. alternata* major allergen, Alt a 1, as a specie-specific molecular marker. And, on other hand, to characterize two new cross-reactive *A. alternata* allergens belonging to the manganese-dependent superoxide dismutase (MnSOD) and serine protease (SP) protein families and to study their role in *A. alternata* sensitization.

The strategies used to accomplish both aims made use of phylogenetic relationships among fungal species that share *A. alternata* allergen homologues. First, investigating conservation of the genes encoding for Alt a 1 and its homologues which allowed the design of a set of primers in the conserved immunologically relevant Alt a 1 coding sequence region. This primer set, together with a pair of primers to amplify the complete Alt a 1 encoding gene,

was applied in a polymerase chain reaction (PCR)-based system. This approach yielded two rapid, sensitive and specific methodologies with high potential to be applied both for the detection of Alt a 1 and Alt a 1 homologues and for specific identification of the existence of contamination by the very close taxonomically related species *A. alternata* and *A. tenuissima*. The investigation of conservation of the genes encoding for *A. alternata* protein homologues, was also employed to design primers in the invariant region of fungal MnSOD and SP nucleotide sequences available in public databases. The aforementioned primers along with rapid amplification of cDNA ends (RACE) and sequencing assays allowed for the isolation of the full-length cDNA encoding for *A. alternata* MnSOD and SP. Both proteins were then produced as recombinant proteins in *E. coli* and evaluated for IgE immunoreactivity using a comprehensive panel of sera from patients clinically labeled as to be sensitized to *A. alternata*. Immunoblotting analysis showed that IgE antibodies from *A. alternata*-sensitized patients bound to recombinant *A. alternata* MnSOD and SP with prevalence of 11.5% (n=61) and 10.2% (n=59), respectively. These results were reported to the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-Committee, and both proteins, respectively named Alt a 14 and Alt a 15, are currently included in the official *A. alternata* allergen list. By performing immunoblotting inhibition assays it was demonstrated that Alt a 14 and Alt a 15 are able to mediate IgE cross-reactivity with similar homologues allergens from other important allergenic fungal species, such as *A. fumigatus* and *C. lunata*, respectively. Furthermore, evidence of reactivity of Alt a 14 to IgE of Allergic Bronchopulmonary Aspergillosis (ABPA)-diagnosed patients was found, thus indicating that sensitization to this *A. alternata* allergen could play important implications in the development of ABPA. On the other hand, sensitization to Alt a 15 was shown to be restricted to apparently poly-sensitized patients and to justify some cases of sensitization to *A. alternata* in which there is no evidence of Alt a 1 sensitization. A homologue to Alt a 14, together with a manitol desidrogenase (MtDH), of the edible mushroom *A. bisporus* were identified to induce an anaphylactic shock reaction in a patient who presented a previous history of respiratory allergic symptoms associated to mold aeroallergens. These results were useful in proving that although minor allergens, *A. alternata* cross-reacting proteins may be the primary cause of strong allergic reactions to various other allergenic sources, such as mushrooms.

Overall, in this work we successfully developed a specific and sensitive PCR method based on the amplification of regions of the gene encoding for the allergenic Alt a 1 and Alt a 1 homologues which it is intended to be applied for environmental monitoring as well as for quality and biosecurity control of food stuffs. Moreover, cloning and characterization of Alt a 14 and Alt a 15 as minor allergens of *A. alternata* that can trigger cross-reactive IgE response with other important and prevalent allergenic fungal species were also accomplished. The availability of these allergens as recombinant molecules suitable for application in a molecule-based diagnostic approach to fungal allergy can improve the diagnostic process allowing for the definition of individual reactivity patterns as well as offering information of

prognosis of clinical manifestations and potential cross-reactivities. Furthermore, this can guide to a more effective specific immunotherapy using a single or a few allergenic molecules. Hence, this work provided valuable findings that can contribute to improving the accuracy of assessment of allergen exposure, diagnosis and management of IgE-mediated fungal diseases.

Keywords

Allergen homologues, Allergic bronchopulmonary aspergillosis, *Alternaria alternata*, anaphylaxis, asthma, cross-reactivity, exposure, fungal allergy, manganese-dependent superoxide dismutase, phylogeny, poly-sensitization, polimerase chain reaction, recombinant allergens, serine protease.

Thesis overview

This doctoral thesis is structured in four main chapters. The first chapter consists of two literature reviews: a book's chapter which revisits the actual trends in fungal allergens and their implications in diagnosis, management and environmental control of fungi-associated allergic diseases; and a review article (Paper I) specifically focused on allergens from *A. alternata* and their relevance as markers of phylogeny, exposure, diagnosis and risk of respiratory allergies. The chapter 1 provides the background that justifies the aims proposed for this doctoral work which are later explained in chapter 2 of the thesis. The third chapter includes the published original research articles reporting the findings obtained from the studies developed during the doctoral work and they are organized as follows:

Paper II- Development of a PCR-based tool for detecting immunologically relevant Alt a 1 and Alt a 1 homologue coding sequences

Paper III - Characterisation of *Alternaria alternata* manganese-dependent superoxide dismutase, a cross-reactive allergen homologue to Asp f 6

Paper IV - Alt a 15 is a new cross-reactive minor allergen of *Alternaria alternata*

Paper V – From respiratory sensitization to food allergy: Anaphylactic reaction after ingestion of mushrooms (*Agaricus bisporus*)

The fourth chapter summarizes all the concluding remarks obtained throughout the PhD work.

Index

Abbreviations

ABPA	Allergic bronchopulmonary aspergillosis
ABPM	Allergic bronchopulmonary mycosis
CRD	Component-resolved diagnosis
ELISA	Enzyme-linked immunosorbent assay
GA(2)LEN	Global Asthma and Allergy European Network
GST	Glutathione-S-transferase
ITS	Internal transcribed spaces
MnSOD	Manganese-dependent superoxide dismutase
MtDH	Mannitol dehydrogenase
PCR	Polymerase chain reaction
RACE	Rapid amplification of cDNA ends
SDAP	Structural Database of Allergenic Proteins
SP	Serine protease
WHO/IUIS	World Health Organization and International Union of Immunological Societies

Chapter 1

Book Chapter

Fungal allergens: Recent Trends and Future Prospects

Marta Gabriel, Jorge Martínez, Idoia Postigo

In: Mehdi Razzaghi-Abyaneh, Masoomeh Shams-Ghahfarokhi, Mahendra Rai, editors. Medical Mycology: Current Trends and Future Prospects. CRC Press. Taylor & Francis Group; 2015. 315-333.

CHAPTER 13

Fungal Allergens: Recent Trends and Future Prospects

Marta Gabriel,[a] *Jorge Martínez*[b] and *Idoia Postigo*[c,*]

Introduction

Allergic diseases affect millions of people and have shown a marked increase in recent years, particularly in industrialised nations (D'Amato et al. 2010). Allergens that cause disease include environmental allergens such as pollen, food, animal dander, and various fungi. Fungal spores and mycelial cells are two of the main factors causing several allergic diseases, including asthma, rhinitis, hypersensitivity pneumonitis, certain occupational lung diseases, fungal sinusitis, toxic pneumonia and allergic bronchopulmonary mycosis (ABPM) (Vijay et al. 2005). This broad panel of diseases results from the particular biology of each mould. Fungi are very common in the environment; therefore, the exposure to airborne spores is almost constant throughout the year. The inhalation and ingestion of fungal spores and vegetative cells (hyphae) may result in the colonization of the human body, and these particles may damage airways by producing toxins, proteases, enzymes (Kauffman et al. 2000) and volatile organic compounds (Fischer et al. 1999). Thus, moulds have a far greater impact on the immune systems of patients than other allergenic sources (Simon-Nobbe et al. 2008).

Although the exact prevalence of fungal allergies is not known, it is estimated that approximately 6 to 24% of the general population can be predicted to have allergic symptoms to moulds (Tariq et al. 1996; Pulimood et al. 2007). The prevalence of a respiratory allergy to fungi can increase to approximately 44% in atopic individuals

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(Corey et al. 1997) and up to 80% among asthmatics (Lopez and Salvaggio 1985). Various studies suggest that at least 3–10% of adults and children are affected by a fungal allergy (Kurup et al. 2000; Bush and Protnoy 2001); however, skin reactivities ranging from 3–91% have been reported depending upon the population studied, fungal extracts used and species tested (Lehrer et al. 1986; Sprenger et al. 1988; Horner et al. 1995).

Among the allergic diseases caused by fungi, asthma has been found to be more prevalent, and severe reactions are commonly associated with exposure and sensitisation to moulds (Bush et al. 2006). Recent studies regarding asthma included in the Global Initiative for Asthma (GINA) yielded a global figure of 197 million people suffering from the disease, and among these, 4.8 million adults were associated with allergic bronchopulmonary aspergillosis (ABPA) (Denning et al. 2013). This data, referring to one of several clinical manifestations in which fungi are implicated, indicates the relevance of this kingdom in the development of allergic diseases. We consider this data to be only an approximation. The exact prevalence of fungal allergy/asthma is difficult to assess because correlating fungal exposure with asthmatic symptoms remains problematic. One reason is that the majority of the extracts used in allergy testing are not standardised, which makes it difficult to ascertain the true prevalence of fungal sensitisation (Esch 2004). Furthermore, cutaneous responses do not necessarily indicate the presence of disease, although these types of responses are helpful in defining the frequency of sensitisation and could be considered as markers of the severity of asthma or rhinitis (Friedlander and Bush 2005).

Fungi are ubiquitous organisms, and over 80 genera have been shown to induce type I allergies in susceptible persons, whereas allergenic proteins have been only identified in 25 fungal genera (Simon-Nobbe et al. 2008).

The most important fungi involved in allergic diseases include the genera *Alternaria*, *Aspergillus*, *Aureobasidium*, *Bipolaris*, *Botrytis*, *Candida*, *Curvularia*, *Cladosporium*, *Drechslera*, *Epicoccum*, *Fusarium*, *Mucor*, *Penicillium*, *Phoma*, *Saccharomyces*, *Stemphylium*, *Trichophyton* and *Ustilago*. Among these, *Alternaria, Cladosporium*, *Penicillium* and *Aspergillus*, are classically considered the most relevant fungal genera that cause allergic diseases, including allergic asthma/rhinitis, ABPA or hypersensitivity pneumonitis.

From a traditional point of view, the diagnosis of fungal allergy has been based on a very well defined clinical history of the patient, on *in vivo* tests that include a skin prick test (SPT) and inhalation challenge, and on an *in vitro* test to detect and quantify the presence in serum of specific IgE or IgG antibodies directed against the fungal allergenic source. However, this practice involves certain unresolved issues. Many times, the accuracy and correlation of the results from the *in vivo* and *in vitro* assays are not in concordance due to the variability of the fungal extracts used. There is no standardisation in the production of these extracts (Esch 2004; Mari et al. 2003). The source of proteins could be the mycelia or the spores; therefore, the protein expression patterns will be different, which affects the allergenic composition. Moreover, the processes of growing the fungus and extracting the proteins dramatically affect the composition of each extract. The variability in the conditions results in a great variability of the content and proportion of each allergen in the extracts. All of the above may explain why there was no concordance between the *in vivo* and *in vitro* results in many cases.

Nevertheless, these problems with fungal extracts are being overcome by the use of recombinant allergens. The production of these proteins in the laboratory offers several advantages over the production of fungal extracts. On one hand, the recombinant protein preparations are more homogeneous, reproducible and easier to standardise for use as reagents in biological and immunological tests. On the other hand, the recombinant allergens used for diagnostic purposes may be used as clinical and prognostic markers; these allergens can be used as markers of clinical severity as well as to differentiate among co-sensitisation, co-exposure and cross-reactivity. This differentiation is important because the primary sensitising moulds must be known for successful immunotherapy.

Aerobiology: Fungal Allergenic Sources Associated with Allergy

Allergens are biomolecules, primarily proteins that can be found in very different substrates and often from biological origins. These substrates and/or biological material constitute the allergenic sources. The most important and common allergenic sources are the following: mites and mite by-products; pollens and pollen by-products; fungi and fungal by-products; epithelia and epithelial by-products; foods and food by-products; insects and their by-products; and drugs.

Among the above-mentioned allergenic sources, fungi are, without any doubt, one of the three main causes of respiratory allergy. The kingdom Fungi comprises a highly heterogeneous group of living organisms, including eukaryotic heterotrophic organisms that have either a unicellular (yeast-like) or pluricellular (a branched tubular structure of individual units, hyphae, that form the mycelium) vegetative structure and that reproduce via spores (from sexual and/or asexual origin). The most important roles of these organisms are the decomposition and removal of organic matter, parasitism (phyto- and zooparasitism), mutualism (e.g., lichens or mycorrhizae) and mycotoxin production.

Simon-Nobbe et al. (2008), in their excellent review, reported approximately eighty allergenic genera of a fungal nature. Studies of the allergic diseases caused by most of the species included in these genera mainly contain data concerning sensitisation in atopic patients, and only a limited number of genera and species have been studied in depth. *Alternaria*, *Aspergillus*, *Cladosporium* and *Penicillium* belonging to Ascomycota and *Malassezia* belonging to Basidiomycota are the genera included in this restricted group of allergenic fungi, for which not only the identification as an allergenic source and the prevalence of sensitisation but also the allergenic composition, the role in the clinical development of symptoms and the ability to interact with the immune system have been reported upon exhaustively (Breitenbach et al. 2002; Kurup 2005).

Moulds occur in both outdoor and indoor environments, and moulds grow on substrates with very different natures, including non-organic surfaces. The size of airborne fungal spores ranges from 2–3 μm (*Cladosporium, Aspergillus* and *Penicillium*) up to 160 μm (*Helminthosporium*), while some species such as *Alternaria longissima* contain larger spores (500 μm) (Ingold and Hudson 1993). The spore concentration in the atmosphere is high and ranges from 200 spores/m^3 to 10^6 spores/m^3. This concentration

represents between 100 to 1,000 times more than pollen concentrations (Lacey 1981; Burge 1989; D'Amato et al. 1995).

The daily analysis of airborne spores shows a quantifiable relationship between each fungal taxon in the atmosphere and allows the production of an airborne calendar of the different allergenic sources found at each place. Each location is associated with a particular panel of airborne spores, depending on the climate, flora, ecological conditions, etc., and this local calendar is an important tool that helps us in defining the panel of allergen extracts used to evaluate allergic sensitisation. With this in mind and as an example, the data obtained in Barcelona, Spain, during the last ten years shows the presence of fungal spores belonging at least to 26 different genera (*Agaricus, Agrocybe, Alternaria, Arthrinium; Aspergillus, Chaetomium, Cladosporium, Coprinus, Curvularia, Drechslera, Epicoccum, Fusarium, Ganoderma, Helminthosporium, Leptosphaeria, Nigrospora, Penicillium, Pithomyces, Pleospora, Polytricium, Puccinia, Stemphylium, Tilletia, Torula, Ustilago*, Venturiaceae and Xylariaceae). However, only a few genera have maximums of more than 100 spores/m^3 (*Agaricus*: 350 spores/m^3; *Alternaria*: 180 spores/m^3; *Aspergillus/Penicillium*: 450 spores/m^3; *Cladosporium*: 4,500 spores/m^3; *Coprinus*: 500 spores/m^3; and *Ustilago*: 750 spores/m^3). The periodic measurements reveal a high variability among the spore concentrations of each genera, and thus the genus *Alternaria* had a maximum airborne spore concentration of 180 spores/m^3 during the period 1994–2013, but the mean value in the same period was 60 spores/m^3 (http://www.lap.uab.cat/aerobiologia/es/). This data can be extended to other studies with similar objectives, which show the data associated with each location.

The composition of fungi growing inside homes depends on the outdoor fungal panel and the species that grow indoors, and the latter are influenced by humidity, ventilation, the content of biologically degradable material, and the presence of pets, plants and carpets (Dharmage et al. 1999). *Alternaria* spores are found in the atmosphere of many different locations as one of the predominant spore types (Newson et al. 2000; Sanchez and Bush 2001; Rizzi-Longo et al. 2009), and several studies performed worldwide indicate that sensitivity to moulds is a primary risk for asthma and that *Alternaria alternata* is the mould species responsible for the highest percentage of mould sensitivities (Gergen and Turkeltaub 1992; Peat et al. 1993; Halonen et al. 1997; Das and Gupta-Bhattacharya 2012), even though most individuals sensitised to moulds respond to several mould species (Postigo et al. 2011).

Although the number of spores in the environment is high, much more than the pollen grains, the occurrence of fungal sensitisation through the airborne counts is low, and the correlation between the number of spores and mould allergy is weak. Therefore, why would the fungi produce lower rates of sensitisation than pollens? Most likely, the availability of the fungal allergens released by the spores is significantly lower than that of the allergens released by the pollen grains. Alternatively, only a few main allergens have been described from fungi compared with pollens. Among these, Alt a 1 is the main marker of fungal sensitisation and the main risk factor for asthma among the fungal allergens (Lizaso et al. 2008; Postigo et al. 2011; Feo-Brito et al. 2012). Despite the fungal major allergens with no cross-reactivity as Alt a 1 or Asp f 1 are considered the main causes of fungal sensitization other fungal allergens

eliciting lower sensitization prevalence and limited cross-reactivity, also can play and important role not only in sensitization but also in the development of the allergy.

The prolonged intense exposure to fungal sources mimics the exposure to other perennial allergens, which likely contributes to both the chronicity and severity of asthma in mould- and especially *Alternaria*-sensitive subjects. The clinical development of an allergy from the components of a mycotic nature could be better understood by taking into account other minor allergens such as proteases that are common to a great variety of fungal species and that also induce inflammation and may thereby act as adjuvants in allergic sensitisation (Snelgrove et al. 2014).

Fungal Allergens

The World Allergy Organisation defines allergens as "antigens that trigger an allergy". Most of these allergens that bind IgE antibodies are proteins that are often linked to carbohydrates and are derived from a biological source. In recent decades, a large number of these allergens have been studied from a molecular point of view; the structural and functional characteristics of these allergens have been examined to answer the question "what makes an allergen an allergen?" (Stadler and Stadler 2003; Breiteneder and Radauer 2004; Ferreira et al. 2004; Jenkins et al. 2005; Radauer and Breiteneder 2006; Jenkins et al. 2007). Radauer et al. (2008) confirmed that allergens are distributed among two hundred protein families and that 16% of these families include 50% of the known allergens (Fig. 1). This data suggests that the narrow spectrum of biological functions of the allergens might explain the allergenic profile of these proteins.

There are more than 70,000 fungal species recorded in the literature, but only one hundred genera have been described as allergenic sources (Simon-Nobbe et al. 2008).

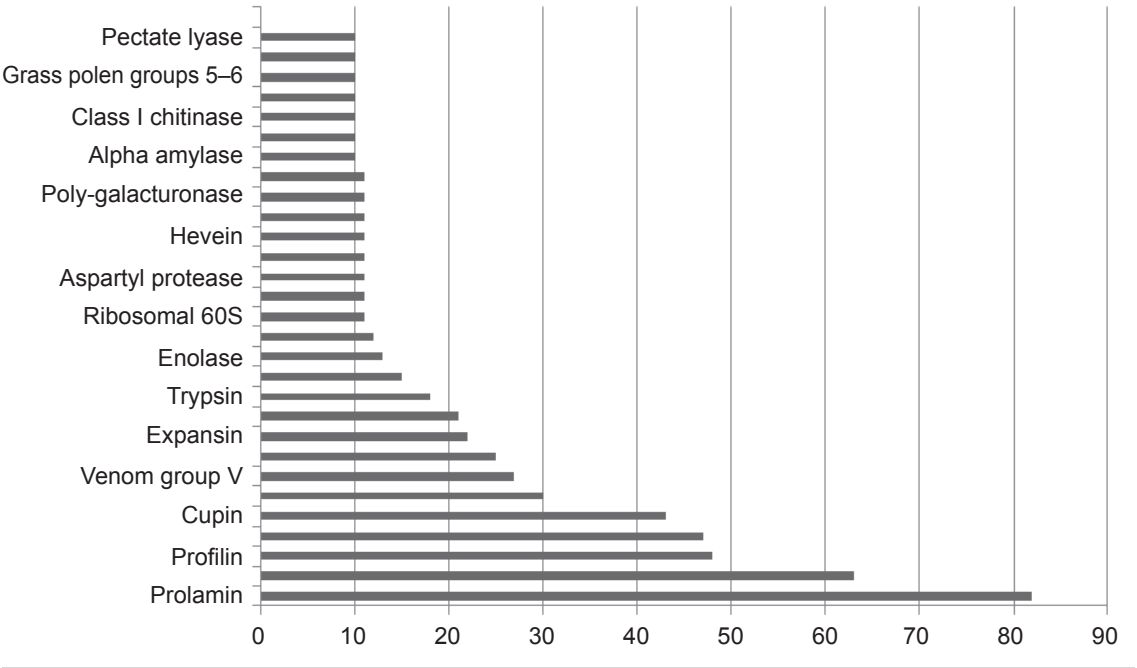

Figure 1. Number of Allergens Belonging to Each Family Protein.

Simon-Nobbe et al. (2008) listed approximately two hundred fungal allergens belonging to 24 fungal genera, and the Nomenclature Subcommittee of the International Union of Immunological Societies (IUIS; www.allergen.org) describes 108 allergens, of which 85 belong to the Ascomycota phylum and 23 belong to the Basidiomycota phylum.

Among the Ascomycota associated with allergic disorders, *Alternaria alternata* is one of the most frequently described species (D'Amato et al. 1997; Zureik et al. 2002; Mari et al. 2003). The major allergen of this species, Alt a 1, is recognised by up to 98% of *A. alternata*-sensitised patients (Asturias et al. 2005), and sensitivity to this allergen was shown to be a risk factor for life-threatening asthma (Lizaso et al. 2008; Postigo et al. 2011; Feo-Brito et al. 2012). The function of Alt a 1 has been recently studied, and this protein seems to act as a competitive inhibitor of the thaumatin-like proteins (Gómez-Casado et al. 2014). Taking into account that several members of the thaumatin protein family display significant *in vitro* inhibition of hyphal growth and sporulation by various fungi, the Alt a 1 could be a relevant fungal protein involved in the fitopathogenicity of *Alternaria* members (Gómez-Casado et al. 2014). Previously, the molecular structure of Alt a 1 was described (Chruszcz et al. 2012), and it was demonstrated that Alt a 1 conforms to a new type of protein family found exclusively in fungi. Sáenz-de-Santamaría et al. (2006) and Martínez et al. (2006) demonstrated that Alt a 1 is expressed by both *Alternaria* and other members of the Pleosporaceae family, including the allergenic species *Stemphylium botryosum* and *Ulocladium botrytis*. Their results showed that Alt a 1 must be considered a species- and family-specific allergen, and Alt a 1 must be used as the main marker for evaluating sensitisation to fungal allergenic species belonging to the Pleosporaceae family.

In addition to Alt a 1, the enolase Alt a 6 from the allergen array described for *Alternaria* also has a high diagnostic value. Several authors demonstrated that this allergen can play an important role in the phenomenon of cross-reactivity among several fungi and among species that belong to different phyla (Simon-Nobbe et al. 2000; Wagner et al. 2000). However, this protein can be considered to be a minor allergen because Alt a 6 is only able to sensitise approximately 15–22% of patients who are allergic to moulds (Simon-Nobbe et al. 2000; Wagner et al. 2000). Moreover, a high proportion of mould-allergic patients seem to be poly-sensitised to several mould species (Horst et al. 1990).

Postigo et al. (2011) evaluated the diagnostic value of Alt a 1 and enolase using the resolved-component diagnosis of allergy in a group of 30 patients who had been defined by a cutaneous and *in vitro* test as only allergic to *Alternaria*. The results revealed that the 23% of the analysed patients were indeed multi-sensitised and that enolase played an important role in the cross-reactivity phenomenon. However, the authors concluded that the panel of allergens used in the study was not sufficient for the accurate diagnosis of a fungal allergy. Among the allergens described for *Alternaria* (Table 1), at least nine have been identified in closely related moulds such as *Cladosporium herbarum*. Other *Alternaria* allergens, including heat shock protein 70, enolase, aldehyde dehydrogenase and glutathione-S-transferase, have been described in both fungal and non-fungal species (Simon-Nobbe et al. 2008). Recently, a new cross-reactive allergen from *Alternaria* has been included in the IUIS allergen list. The new allergen is a manganese-dependent superoxide dismutase (MnSOD) that is highly homologous to the MnSOD of *Aspergillus fumagitus*, Asp f 6, an allergen considered

Table 1. Fungal Allergens of Ascomycota According to their Biological Function.

Biological Function	Fungal species							
	Alternaria alternata	***Aspergillus fumigaus***	***A. niger***	***A. oryzae***	***Candida albicans***	***Cladosporium cladosporoides***	***Cl. herbrum***	***Curvularia lunata***
Unknown	Alt a 1 Alt a 2 Alt a 9	Asp f 4 Asp f 7	Asp n 4	Asp o 4 Asp o 7			Cla h 1 Cla h 2	
Heat shock protein 70	Alt a 3							
Disulfide isomerase	Alt a 4							
Ribosomal protein P2	Alt a 5	Asp f 8		Asp o 8			Cla h 5	
Enolase	Alt a 6	Asp f 22					Cla h 6	Cur l 2
YCP4 protein	Alt a 7						Cla h 7	
Mannitol-dehydrogenase	Alt a 8						Cla h 8	
Aldehyde-dehydrogenase	Alt a 10						Cla h 10	
Acid ribosomal protein P1	Alt a 12	Asp f 26	Asp n 26	Asp o 26			Cla h 12	
Glutathione-S-transferase	Alt a 13						Cla h 13	
Manganese superoxide-dismutase	Alt a 14	Asp f 6						Cur l 6
Alkaline serine protease		Asp f 13		Asp o 13				Cur l 1
Alternaria allergen	Alt a 15							
Mitogillin family		Asp f 1						
Peroxysomal protein		Asp f 3	Asp n 3		Can a 3			
Metalloprotease		Asp f 5		Asp o 5				
Aspartate protease		Asp f 10						
Peptidyl-prolyl isomerase		Asp f 11						
Heat shock protein P90		Asp f 12						
Vacuolar serine protease		Asp f 18	Asp n 18	Asp o 18		Cla c 9	Cla h 9	Cur l 4
L3 ribosomal protein		Asp f 23						
Cyclophilin		Asp f 27						
Thioredoxin		Asp f 29						

Table 1. contd....

Table 1. contd.

Biological Function	Fungal species							
	Alternaria alternata	***Aspergillus fumigaus***	***A. niger***	***A. oryzae***	***Candida albicans***	***Cladosporium cladosporoides***	***Cl. herbrum***	***Curvularia lunata***
PhiA cell wall protein		Asp f 30						
β-xylosidase			Asp n 14					
3-phytase B			Asp n 25					
TAKA-amylase A				Asp o 21				
Alcohol dehydrogenase					Cand a 1			
Peroxisomal membrane protein A					Cand a 2			
Transaldolase						Cla c 14		
N-acetyl-glucosaminidase								
Calreticulin								
Elongation factor 1 β								
Catalase								
Pectate-lyase								
Extracellular alkaline Mg-dependent exo-desoxy-ribonuclease								
Putative secreted alkaline protease Alp1								
Cerato-platanin		Asp f 15						
Glycosyl-hydrolase		Asp f 16 Asp f 9						
Galacto-mannoprotein		Asp f 17						
Fibrinogen binding protein		Asp f 2						
Cell wall protein		Asp f 34						
Catalase			Asp n 30					
Alpha-amylase				Asp o 21				
Cytochrome C								Cur l 3

Biological Function	Fungal species							
	Epicoccum purpurascens	***Fusarium culmorum***	***F. proliferatum***	***Penicillium brevicompactum***	***P. chrysogenum***	***P. citrinum***	***Stachybotrys chartarum***	***Trichophyton rubrum***
Unknown		Fus c 3						
Heat shock protein 70						Pen c 19		
Disulfide isomerase								
Ribosomal protein P2		Fus c 1						
Enolase						Pen c 22		
YCP4 protein								
Mannitol-dehydrogenase								
Aldehyde-dehydrogenase								
Acid ribosomal protein P1				Pen b 26				
Glutathione-S-transferase	Epi p 13							
Manganese superoxide- dismutase						Pen c 6		
Alkaline serine protease	Epi p 1			Pen b 13	Pen ch 13	Pen c 13		Tri r 4
Mitogillin family								
Peroxysomal protein						Pen c 3		
Metalloprotease								
Aspartate protease								
Peptidyl-prolyl isomerase								
Heat shock protein P90								
Vacuolar serine protease					Pen ch 18			
L3 ribosomal protein								
Cyclophilin								
Thioredoxin		Fus c 2						
PhiA cell wall protein								
β-xylosidase								
3-phytase B								

Table 1. contd....

Biological Function	***Fungal species***							
	Epicoccum purpurascens	***Fusarium culmorum***	***F. proliferatum***	***Penicillium brevicompactum***	***P. chrysogenum***	***P. citrinum***	***Stachybotrys chartarum***	***Trichophyton rubrum***
TAKA-amylase A								
Alcohol dehydrogenase								
Peroxisomal membrane protein A								
Transaldolase			Fus p 4		Pen ch 35			
N-acetyl-glucosaminidase					Pen ch 20			
Calreticulin					Pen ch 31			
Elongation factor 1 β						Pen c 24		
Catalase						Pen c 30		
Pectate-lyase						Pen c 32		
Extracellular alkaline Mg-dependent exo-desoxy-ribonuclease							Sta c 3	
Putative secreted alkaline protease Alp1								Tri r 2
Cerato-platanin								
Glycosyl-hydrolase								
Galacto-mannoprotein								
Fibrinogen binding protein								
Cell wall protein								
Catalase								
Alpha-amylase								
Cytochrome C								

Table 2. Fungal Allergens of Basidiomycota According to their Biological Function.

	Fungal species					
Biological Function	***Coprinus comatus***	***Malassezia furfur***	***Malassezia sympodialis***	***Psilocybe cubensis***	***Rhodotorula mucilaginosa***	***Schizophyllum commune***
Unknown	Cop c 3 Cop c 5 Cop c 7		Mala s 1 Mala s 5 Mala s 7 Mala s 8 Mala s 9	Psi c 1		
Heat shock protein 70			Mala s 10			
Enolase				Rho m 1		
Manganese superoxide- dismutase			Mala s 11			
Peroxysomal protein		Mala f 2 Mala f 3				
Vacuolar serine protease					Rho m 2	
Cyclophilin				Psi c 2		
Thioredoxin	Cop c 2		Mala s 13			
Leucine zipper protein	Cop c 1					
Mitochondrial malte dehydrogenase		Mala f 4				
Glucose-methanol-choline oxidoreductase			Mala s 12			
Glucoamylase						Sch c 1

to be a marker of ABPA (Postigo et al. 2011). The clinical relevance of this allergen must be studied, but this MnSOD could have important implications in *Alternaria* sensitisation as a risk factor for the development of ABPA (Jubin et al. 2010).

Aspergillus is an allergenic mould belonging to Ascomycota. This mould is frequently found growing in decaying vegetation or indoors (Kurup et al. 2000; Terr 2004) releasing large quantities of spores, which can reach the terminal airways via inhalation (Kurup and Banerjee 2000; Vijay and Kurup 2004). In some cases, large clusters of spores are deposited in the upper respiratory tract (Kurup 2003), causing disorders ranging from allergic rhinitis, sinusitis or asthma to hypersensitivity pneumonitis and ABPA (Terr 2004).

The IUIS lists 31 allergens belonging to five species of *Aspergillus*, including *A. flavus*, *A. versicolor*, *A. niger*, *A. oryzae* and *A. fumigatus*. Among these species, *A. fumigatus* is implicated in approximately 80% of *Aspergillus*-related infections (Simon-Nobbe et al. 2008). Due to its high prevalence, a large number of allergens from *A. fumigatus* have been cloned, characterised and purified as recombinant allergens (Crameri and Blaser 1996; Crameri et al. 2006).

The main major allergens of *A. fumigatus* are Asp f 1 and Asp f 3 (Table 1). The first, Asp f 1, is a non-glycosylated ribotoxin of 18 kDa that is recognised by 85% of the ABPA patients as well as asthmatic patients with a positive cutaneous test to this mould (Arruda et al. 1990). Arruda et al. (1992) demonstrated that Asp f 1 was abundantly released after germination of the spores and during the early phases of fungal growth; therefore, Asp f 1 was considered to be a virulence factor promoting the colonisation and infection of human tissue. Kurup et al. (1998) analysed the B- and T-cell epitopes of this allergen and demonstrated that the C-terminal region (aa 115–149) was involved in both humoral and cell-mediated immune responses in ABPA, whereas the T-cell epitopes were localised in aa 45–65 and aa 106–125. This allergen is unique and has not been found to share sequence homology with any other known allergen (Bowyer and Denning 2007).

The second main allergen, Asp f 3, is a 19 kDa protein that binds IgE in 72% of the ELISA-determined cases, and it is known that this allergen shares common IgE-binding epitopes with peroxisomal membrane proteins from *Candida boidini* (Hemmann et al. 1997). The clinical relevance of Asp f 3 in *Aspergillus* disorders was demonstrated long ago (Hemmann et al. 1998). The analysis of the B- and T-cell epitopes by Ramachandran et al. (2002) demonstrated the presence of 12 amino acids located at the N-terminus and 8 at the C-terminus that are critical for IgE binding.

Other allergens from *Aspergillus* have been described, including Asp f 2, Asp f 4 and Asp f 6 (Kurup and Banerjee 2000; Vijay and Kurup 2004), and are available as recombinant proteins (Crameri and Blaser 1996). Asp f 4 and Asp f 6 are intracellular proteins; thus, the availability of these proteins as aeroallergens is unlikely. It seems that the process of sensitisation to these allergens occurs when the mould is growing inside the lungs; therefore, some authors have concluded that the use of these two markers is sufficient to allow a precise diagnosis of ABPA (Hemmann et al. 1999).

As can be observed in the annexed tables, a large number of fungal allergens have been described and this number is expected to increase in the coming years. Currently, special attention is being given to several allergens that have protease activity. The biological function of these allergens seems to be closely related to the initiation of the

sensitisation process and the pathogenicity of fungal aeroallergens. These proteases act directly on the pulmonary epithelium and disrupt the cells, thereby eliciting robust and rapid inflammation, which allows the massive influx of other allergens and induces the exacerbation of asthma (Snelgrove et al. 2014). These proteases are founded in several fungal genera and it is thought that they are important cross-reactivity allergens.

Diagnosis of Fungal Allergy

Fungi are an important source of airborne allergens associated with asthma and allergic rhinitis. The specific diagnosis of fungal allergy is made by combining aerobiological studies, a clinical history, complementary tests (mainly the determination of specific and total IgE) and a careful analysis of the symptoms in relation to the causative agents, the allergens.

However, to date, the diagnosis of an allergy to fungi has been difficult in many cases and inefficient due to various reasons, such as the high variety of fungal species, the difficulty in species identification, the complexity and variability of the allergen extracts of fungal origin and the lack of quality and standardisation of the most available fungal extracts (Martínez et al. 1994).

Among the patients allergic to moulds, there are commonly a large proportion of individuals sensitised to several fungal species (Zureik et al. 2002; Simon-Nobbe et al. 2008; Postigo et al. 2011). This situation complicates the final diagnosis of these patients. The diagnosis of these cases by implementing individualised allergens has gained increased attention.

The application of genomics and proteomics to the study of allergenic proteins has allowed the development of new diagnostic tools that make the diagnosis of the allergy more effective. The new models are based on the identification of the individual molecules that are involved at the beginning and during the development of the allergenic phenomenon.

At the beginning of the 21st century, Valenta et al. (1999) described the basis of the molecular diagnosis of allergy, which involves using a classical technique to measure the specific IgE levels (ImmunoCAP ®), wherein the complete allergen extracts (allergen sources) coupled to the solid phases are replaced with individualised allergens, either in their native form or as recombinant proteins.

A few years later, the microarray technique was applied to the CRD concept. Small slides spotted with very small quantities of allergens allowed the molecular diagnosis of allergy against hundreds of allergens using a minimal quantity of serum (Jahn-Schmid et al. 2003). This new concept (CDR) has allowed the individualised profiles of sensitisation in the allergic patient to be precisely defined and subsequently associated with the clinical expression of the allergy.

Similarly, concepts such as cross-reactivity, multiple or primary sensitisation, the prediction of the severity of the allergic reactions and the association of the molecular profiles of sensitisation with the development of clinical symptoms are now easier to understand and evaluate, whereas these parameters were previously difficult to assess (Valenta and Dietrich 2002; Casquete et al. 2009; Knol and Knulst 2010).

Currently, there are no doubts regarding the diagnostic effectiveness of replacing allergenic sources with individual allergens. By applying a molecular diagnosis,

we can overcome several problems with the standardisation of allergenic extracts, including the variability of the fungal strains, the variability in the batch-to-batch production, the choice of the fungal structure to use as raw material, the type of culture and technology used to prepare the allergen extracts and the autodegradation of the extract once obtained.

A large number of fungal allergens have been identified (Table 1). However, the lack of availability of large quantities of these allergens for widespread use limits their diagnostic application.

The use of CRD and, more specifically, the miniaturised version of CRD, which can simultaneously evaluate a patient's sensitisation against hundreds of allergens belonging to different Phyla, allow us to associate the molecular sensitisation profiles with different stages of the allergic phenomenon and to find molecular markers that predict the severity of the allergic reactions, molecular markers of cross-reactivity and molecules able to differentiate between sensitisation and allergy (Kurup et al. 2005; Ebo et al. 2010a; Ebo et al. 2010b; Nicolaou et al. 2010).

Out of more than 70,000 fungal species described in the literature, Simon-Nobbe et al. (2008) only refer to somewhat less than a hundred genera that have been described as allergenic sources. The same authors report regarding the identification of somewhat more than 200 individualised allergens that belong to 24 genera of fungi (Simon-Nobbe et al. 2008).

In view of this data, we may ask ourselves whether all sources of fungal allergens are equally relevant in the diagnosis of a fungal allergy. Are these 200 fungal allergens enough for the efficient diagnosis of a fungal allergy?

Studies on aerobiology, the cutaneous reaction to fungal allergens and allergenic characterisation, suggest that the minimum number of species that will ensure an acceptable diagnosis of an allergy to fungi would include the following: *A. alternata, Aspergillus fumigatus, Cladosporium herbarum, Epicoccum nigrum, Fusarium roseum, Penicillium crisogenum, Candida* spp. and *Malassezia* spp. (Crameri et al. 2006). Recently Postigo et al. (2011) has stated that *Curvularia lunata* should be another species to be included in the panel of essential fungi used for diagnosis. Additionally, if we consider the results of studies conducted in different geographical areas, we should also include *Trichophyton* spp. (Mari et al. 2003), *Helminthosporium* spp., *Trichoderma* spp. and *Aureobasidium* spp. (Zureik et al. 2002). According to this data, if we look for more relevant species described in the literature or in new studies developed in other geographical areas, it would be very likely that we would have to add new fungal allergenic sources to the above-mentioned panel.

However, taking into account the ratio between the individualised allergens and their allergenic sources, we can assert that the 200 described fungal allergens belong to approximately 20 genera (Simon-Nobbe et al. 2008), and that most of these allergens are related to each other because the allergens belong to the same protein family. Therefore, these allergens could form an allergic unit which would belong to the same group. Thus, from the allergens referred to by Simon-Nobbe et al. (2008), the number of allergens homologous to Alt a 1 described in other species but phylogenetically related to *A. alternata* is approximately fifty.

Alt a 1 is most likely the best allergen to define the diagnosis of a respiratory allergy caused by moulds in our environment (Postigo et al. 2011; Portnoy et al. 2008;

Bartra et al. 2009) and is a very useful tool as a marker of asthma severity (Feo-Brito et al. 2012). An allergen such as fungal enolase is a panallergen and is associated with cross-reactivity. This allergen could explain part of the apparent multiple sensitisations commonly observed in this type of clinic (Postigo et al. 2011). In the same way, it can be observed in Table 1 that there are more allergens belonging to unrelated species, including serine proteases, peroxisomal proteins and the ribosomal proteins P1 and P2, which could be considered to be markers of cross-reactivity between non-phylogenetically related species. These proteins may be responsible for the apparent multi-sensitisations that are frequently observed in fungal allergies (Simon-Nobbe et al. 2008; Horst et al. 1990).

Conclusions

For a long time, the diagnosis of fungal allergy has been difficult due to the complexity of molds and the variability in the allergenic composition of the fungal extracts used. However, nowadays, using biological tools including the genomic and proteomic, we are able to identify new individual allergens belonging to different fungal sources that have been associated with a different diagnostic value in the diagnosis of fungal allergy. Some allergens have been identified as markers of the risk to suffer asthma such as Alt a 1 and the others are implicated in cross-reactivity and/or poly- and co-sensitivity reactions such as the enolase. Currently, it remains necessary to identify which proteins are implicated in the cross-reactivity phenomenon. Using the above-mentioned tools, the genomic and proteomic, we are able to obtain these allergens as individualised molecules. The availability of these allergens would make it possible to study the role of each molecule in the diagnosis and prognosis of a mould allergy, thereby providing a solution for poly-sensitisation and/or cross-reactivity.

The future of the molecular diagnosis of a fungal allergy is moving in two main directions: (1) the identification of new fungal allergens, their structure and bioloogical activities, including markers of both primary sensitisation and cross-reactivity; and (2) the establishment of the relationship between each allergen and the expression of the different clinical profiles that occurs in mold allergy.

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Paper I

Alternaria alternata allergens: Markers of exposure, phylogeny and risk of fungi-induced respiratory allergy

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Submitted

Alternaria alternata allergens: Markers of exposure, phylogeny and risk of fungi-induced respiratory allergy

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Abstract

Alternata alternata spores are considered a well-known biological contaminant and a very common potent aeroallergen source that is found in environmental samples. The most intense exposure to *A. alternata* allergens is likely to occur outdoors; however, *Alternaria* and other allergenic fungi can colonize in indoor environments and thereby increase the fungal aeroallergen exposure levels. A consequence of human exposure to fungal aeroallergens, sensitivity to *A. alternata*, has been unequivocally associated with increased asthma severity. Among allergenic proteins described in this fungal specie, the major allergen, Alt a 1, has been reported as the main elicitor of airborne allergies in patients affected by a mold allergy and considered a marker of primary sensitization to *A. alternata*. Moreover, *A. alternata* sensitization seems to be a triggering factor in the development of poly-sensitization, most likely because of the capability of *A. alternata* to produce, in addition to Alt a 1, a broad and complex array of cross-reactive allergens that present homologues in several other allergenic sources. The study and understanding of *A. alternata* allergen information may be the key to explaining why sensitization to *A. alternata* is a risk factor for asthma and also why the severity of asthma is associated to this mold but not to other aeroallergens that are commonly found in the environment. Indeed, compared to other common environmental allergenic sources, such as pollens and dust mites, fungi are reported to be neglected and underestimated. The rise of the *A. alternata* allergy has enabled more research into the role of this fungal specie and its allergenic components. Indeed, recent research on the identification and characterization of *A. alternata* allergens has allowed for the consideration of new perspectives in the categorization of allergenic molds, assessment of exposure and diagnosis of fungi-induced allergies.

Keywords

Alternaria alternata allergens, asthma, cross-reactivity, diagnosis of allergy, exposure to fungal allergens, poly-sensitization.

Abbreviations

ABPA, allergic bronchopulmonary aspergillosis; ABPM, allergic bronchopulmonary mycosis; CRD, component-resolved diagnosis; ELISA, enzyme-linked immunosorbent assays; GA(2)LEN, Global Asthma and Allergy European Network , GST, glutathione-S-transferase; ITS, internal transcribed spacer; MnSOD, manganese-dependent superoxide dismutase; MtDH, mannitol dehydrogenase; SDAP, Structural Database of Allergenic Proteins; SP, serine protease; WHO/IUIS, World Health Organization and International Union of Immunological Societies

1. Introduction

Alternaria alternata is one of the most common saprophytes found worldwide and has been clinically associated to asthma (Bush and Prochnau, 2004), allergic rhinosinusitis (Schell, 2000), hypersensitivity pneumonitis (Ogawa et al., 1997), occulomycosis (Ozbek et al., 2006), onychomycosis (Romano et al., 2001), skin infections (Mayser et al., 2002) and Allergic Bronchopulmonary Mycosis (ABPM) (Singh and Denning, 2012; Chowdhary et al., 2012). Although there is a wide range of clinical manifestations, *A. alternata* is rarely found to be a cause of invasive infections in humans. This fungal specie is mainly related to the induction of IgE-mediated respiratory diseases. *A. alternata* spores are considered one of the most abundant and potent sources of airborne sensitizer allergens. The most intense exposure to *A. alternata* allergens is likely to occur outdoors; however, *Alternaria* and other allergenic fungi can colonize in indoor environments and thereby increase exposure levels (Salo et al., 2005).

The true prevalence of sensitization to *A. alternata* has been difficult to estimate, and several epidemiological and diagnostic studies have reported a highly variable prevalence of human IgE reactivity to *Alternaria*. The European Community Respiratory Health Survey found that 4.4% of an adult population (n=11355) was sensitized to *Alternaria* (Bousquet et al., 2007). More recently, in a large skin test study funded by the Global Asthma and Allergy European Network (GA(2)LEN) that involved 17 collaborating centers from 14 European countries, *Alternaria* sensitization prevalence was approximately 9%, with sensitization rates ranging from 2% (Finland) to 23.8% (Greece) (Burbach et al., 2009). Among a fungal-sensitized population, more than 60% reacted to diagnostic *A. alternata* extracts (Mari et al., 2003).

There are some population groups in which the *A. alternata* sensitization rates are much higher. Mold sensitivity, particularly to *A. alternata*, seems to be much more prevalent in the asthmatic population (Neukirch et al., 1999). Younger respiratory allergic patients seem to be more at risk for *A. alternata* sensitization than older ones (Mari et al., 2003; Katotomichelakis et al., 2012). Farm and sawmill workers have also been reported to be at high risk for occupational airway disease caused by *A. alternata* because significant levels of *A. alternata* allergen have been monitored in farms (Prester and Macan, 2010).

It is well known that most individuals sensitized to *A. alternata* present associated allergic manifestations that require medical attention. In particular, in the GA(2)LEN study, clinically relevant symptoms were clearly noticed in 69% of the European patients who were sensitized to *Alternaria* (Burbach et al., 2009). The severity of typical allergic reactions varies from mild to potentially life-threatening, and it is dependent on several internal and external factors. The most severe IgE-mediated response to *A. alternata* aeroallergens is severe asthma that is characterized by impaired lung function and frequent exacerbations that may result in patient death. Indeed, sensitization to *A. alternata* has been reported to be unequivocally

associated with increased asthma severity, hospital admissions to intensive care for asthma and deaths related to asthma (Neukirch et al., 1999; Black et al, 2000; Zureik et al., 2002).

Because *A. alternata* has been increasingly recognized as a powerful respiratory allergic disease inducer, it has been proposed that at least some *A. alternata* allergens might play a role in the underlying mechanisms of allergic reaction severity. This may be the key to finding an explanation for why sensitization to *A. alternata* is a risk factor for asthma and why asthma severity is associated with this mold but not with other aeroallergens that are commonly found in the environment as pollens (Zureik et al., 2002). Similarly, allergenic protein components of this mold may be a clue for understanding the interesting recent finding that *Alternaria* activates the innate immune system and enhances lung inflammation induced by unrelated allergens such as grass pollen (Kim et al., 2014). However, compared with other common allergenic sources, fungi, particularly, *A. alternata*, are still neglected and underestimated source of allergens (Crameri et al., 2014).

Considering the above-mentioned facts, there is a clear need to identify the complete array of allergenic components from *A. alternata* and elucidate the role of these proteins in the development of respiratory allergies from biology, diagnosis, prognosis and disease management perspectives. At present, a total of 12 allergenic proteins, fulfilling the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee criteria, have been officially listed as *A. alternata* allergens. Another five *A. alternata* allergenic proteins are encompassed in other allergen platforms. These lists include proteins that are restricted to a small number of fungal species that are taxonomically related to *A. alternata* and ubiquitous proteins that are conserved throughout the evolutionary process; these proteins present their homologs in several fungal families. The existence of multiple homologue allergens across the fungal kingdom that carry cross-reactive epitopes that are structurally indistinguishable from IgE-binding epitopes may raise a considerable problem in the diagnosis and classification of fungal allergies (Bowyer et al., 2006). In agreement with this finding, compelling evidence shows that apparent sensitization to multiple fungi represents a frequent clinical observation in patients sensitized to *A. alternata* and may be a consequence of the existence of IgE cross-reactivity between fungal proteins (Gupta et al., 2002; Crameri et al., 2009).

Recent phylogenetic studies have increasingly clarified that clinical IgE reactivity data reflect the taxonomical distribution of the fungal allergenic sources in a majority of the cases (Soeria-Atmadja et al., 2010). Thus, individualized fungal allergens should be fully characterized and analyzed with respect to protein family membership, taxonomic distribution and interspecies variability. In this way, establishing relationships between *A. alternata* allergens and the homologues expressed by other allergologically important fungal species might provide valuable information for defining a taxonomic categorization of fungal allergenic sources and a molecular classification of fungal allergens. The definition of

allergens as molecular markers will contribute to an understanding of their potential role in fungal biology, improve diagnostic decisions by prediction of cross-reactivity and develop more robust detection tools to assess exposure allergen levels. Correctly assessing the exposure to aeroallergens in the environment is of major importance for predicting the risk of respiratory symptoms in an atopic population and informing the implementation of appropriate public health measures. The use of specific allergens as tools to accurately detect allergen contaminants and thereby enable the identification of the fungal origin source seems to be a promising strategy.

This review is intended to focus on relevant issues regarding *A. alternata* sensitization and its allergens, such as the immunological characterization and clinical relevance of the allergens identified to date, the phylogenetic relationships based on shared allergen homologues and advances in the assessment of *A. alternata* exposure and diagnosis of fungal sensitization.

2. Alternaria alternata allergens and their sensitization prevalence

Significant research has been conducted to identify and characterize the proteins that are involved in *A. alternata* allergy. *Alternaria* allergens are a diverse group of molecules that possess different chemical and biological properties. To date, 12 *A. alternata* allergens are included and officially recognized by the WHO-IUIS Allergen Nomenclature Sub-committee (Table 1).

Thus, the *A. alternata* allergens recognized to date, as well as their clinical relevance concerning an *A. alternata* allergy, are discussed extensively here.

2.1. Alt a 1

Alt a 1 is predominantly located in the cell wall of airborne *Alternaria* spores (Twaroch et al., 2012) that access the respiratory tract and elicit allergic reactions in patients affected by a mold allergy. It is considered to be the marker of primary sensitization to *A. alternata* and is undoubtedly the more extensively characterized *A. alternata* allergen. The population that is allergic to *A. alternata* presents approximately 80% of the sensitization prevalence for Alt a 1. This allergen is an abundant protein expressed by the *Alternaria* species, which is most likely why this allergen displays very high values of sensitization incidence.

Natural Alt a 1 is a 30 kDa dimer that dissociates into 16.4 and 15.3 subunits under reducing conditions, thus suggesting a disulfide bond linking the monomers (Deards and Montague, 1991). Investigations by means of molecular dynamics suggested that Alt a 1 possesses a greatly stable dimeric structure presenting epitopes with a proper orientation for IgE cross-linking compared with other major allergens such as Bet v 1 and Pru p 3 (Garrido-Arandia et

al., 2014). Two linear epitopes (K41-P50 and Y54-K63) showing consistent reactivity with serum IgE from an *Alternaria* allergic patient were identified in the Alt a 1 protein sequence (Kurup et al., 2003). Recently, the recombinant form of Alt a 1 has been used to analyze the three-dimensional structure of the molecule by X-ray crystallography (Chruszcz et al., 2012). In this work, the Alt a 1 protein presented a dimeric ß-barrel structure, a completely novel fold among allergens that potentially define a new fungal protein family with an unknown function (Chruszcz et al., 2012). Although the function of this major allergen remains unclear, evidence has raised some hypotheses for the role played by this protein in fungal biology:

a) Alt a 1 release was detected to be significantly higher in germinated *Alternaria* spores, which indicates that this allergen might be involved in spore germination (Mitakakis et al., 2001).

b) Its *A. brassicicola* homologue was found to be highly expressed during the infection process of *A. thaliana* (Cramer and Lawrence, 2004).

c) Alt a 1 was found to induce the expression of plant defense proteins belonging to the PR5-TLP family and interact with it as a competitive inhibitor (Gómez-Casado et al., 2014).

The last two findings suggest that the role of Alt a 1 can be related to virulence and fungal infection pathogenicity.

Alt a 1 is a conserved protein that is highly specific for *Alternaria* and its related taxa because homologs of Alt a 1 have been reported in other Pleosporaceae species (Hong et al., 2005). A numerous list of Alt a 1 and Alt a 1-like genes and proteins has been deposited into databases.

2.2. Alt a 3 and Alt a 5

The first report of Alt a 3 and Alt a 5 as minor *A. alternata* allergens occurred when De Vouge *et al.* (De Vouge et al., 1998) cloned two sequences encoding IgE-binding fragments of *A. alternata* allergens by screening the *A. alternata* cDNA library with *A. alternata*-sensitive patients' pooled sera. The IgE-binding fragment that presented high homology in comparison with a region near the C-terminus of a heat shock protein 70 kDa from *Cladosporium herbarum* was recognized on immunoblotting by 5% of the sera from *A. alternata*-sensitized patients, and it was officially named Alt a 3. Members of the HSP 70 protein family are highly conserved across both prokaryotic and eukaryotic organisms and play a major role protecting the cell during thermal and oxidative stress (Kiang and Tsokos, 1998).

The second *A. alternata* allergenic fragment cloned by De Vouge *et al.* (Alt a 5) was identified as belonging to the acidic ribosomal protein P2 family, and the recombinant protein had positive IgE-immunoblotting reactivity with 14% of the tested atopic patients' sera (De Vouge

et al., 1998). Biologically, these proteins are known to be ribosomal components that are involved in interactions with elongation factors during the course of protein synthesis (Tchórzewski, 2002).

2.3. Alt a 4, Alt a 7 and Alt a 10

Achatz and collaborators identified, cloned and characterized Alt a 4, Alt a 7 and Alt a 10 allergens (Achatz et al., 1995; Achatz et al., 1996).

Alt a 4 is a 57 kDa *A. alternata* (Alt a 4) that bound IgE from 42% of the *A. alternata* sensitized population. In turn, the 22 kDa *Alternaria* protein was homologous to YCP4 yeast (Alt a 7) and Alt a 10, an 11 kDa aldehyde dehydrogenase bound IgE in 7% and 2%, respectively, of the *Alternaria*-sensitive subjects (Achatz et al., 1995; Achatz et al., 1996).

2.4. Alt a 6

cDNA coding for *A. alternata* enolase was cloned by two research groups using a similar approach that consisted of screening the cDNA library using a *C. herbarum* DNA fragment as a probe (Unger et al., 1999, Simon-Nobbe et al., 2000). In one of the studies, a skin test with recombinant protein showed a positive response in two of the seven allergics to *Alternaria* (Unger et al., 1999). In the second study, blotted purified recombinant Alt a 6, was recognized by 22% of *A. alternata* sensitized patients' sera (n=23) (Simon-Nobbe et al., 2000). Recently, a similar Alt a 6 sensitization prevalence among an *A. alternata* allergic population (n=30) was found by the application of resolved-component diagnosis (Postigo et al., 2011). Native enolase purified from an *A. alternata* extract possesses high thermostability and presents a molecular weight of 47 kDa and an optimal pH of 6,8 (Kustrzeba-Wójcicka and Kmiecik, 2001). Enolases (also called 2-phospho-D-glycerate hydrolyases or phosphopyruvate hydratase) are highly expressed key enzymes of glycolysis and gluconeogenesis catalyzing the interconversion of 2-phospho-D-glycerate and phosphoenolpyruvate (Wagner et al., 2000). Because these enzymes are highly conserved, investigations of IgE reactivities of various fungal enolases by cross-inhibition experiments revealed that enolase is a highly cross-reactive fungal allergen (Simon-Nobbe et al., 2000), as described in detail below.

2.5. Alt a 8

The recombinant *A. alternata* NADP*-dependent mannitol dehydrogenase (MtDH), 28.6 kDa was recognized by 41% of A. alternata allergic patients (n=22), and its cross-reactivity with

MtDH of *C. herbarum* was confirmed by inhibition-ELISA experiments (Schneider et al., 2006). These enzymes mediate the reversible conversion of mannitol to fructose in the metabolism cycle of mannitol, which serves as a storage or translocated carbohydrate that seems to be needed for fungal phytopathogenesis (Vélëz et al., 2007; Vélëz et al., 2008).

2.6. Alt a 13

Alt a 13 is a 26KDa protein that belongs to the glutathione-S-transferase (GST) protein family. The biochemical function of these enzymes is that they take part in the detoxification of endogenous and xenobiotic compounds or their metabolites by catalyzing their biotransformation in glutathione conjugates that present lower toxicity and are more easily excreted outside the cell than their parental compounds (McGoldrick et al., 2005).

Shankar *et al.* produced the recombinant protein and referred to Alt a 13 as a major *A. alternata* allergen demonstrating that 14 of the 17 patients who presented a skin reaction to an *A. alternata* extract recognized rGST and nGST (Shankar et al., 2006). In a recent report, the same authors bioinformatically identified epitopes and residues of the Alt a 13 molecule that seemed to be involved in the IgE-reactivity (Shankar et al., 2009).

2.7. Alt a 14

Manganese-dependent superoxide dismutases (MnSODs) are phylogenetically conserved enzymes that are involved in defense against oxidative stress by preventing oxidative damage (Crameri et al., 1996). Our research group identified the natural *A. alternata* MnSOD, Alt a 14, as an IgE-reactive protein of 24 kDa (Postigo et al., 2011). More recently, the recombinant molecule was produced, and it was demonstrated that 11.5% of the sixty-one *A. alternata*-sensitized patients' sera reacted to the rAlt a 14 by IgE immunoblotting (Gabriel et al., 2015c). In the same work, we found that rAlt a 14 was also able to bind IgE from patients who were clinically diagnosed as suffering of Allergic Bronchopulmonary Aspergillosis (ABPA), which suggested that the sensitivity to this *A. alternata* allergen may be related to the pathogenesis of ABPM.

2.8. Alta 15

Serine proteases (SPs), particularly subtilisin SP, are recognized as one of the most important families of allergenic proteins (Radauer et al., 2008), and they have been extensively designed as pan-allergens (Lee et al., 2007; Shen et al., 2007). Recently, a subtilisin-like serine protease was cloned and characterized as an official allergen of *A. alternata*, named Alt a 15 (Gabriel et al., 2015a). Immunoblotting analyses revealed that IgE antibodies from a

population sensitized to *A. alternata* (n=59) bound to rAlt a 15 with a prevalence of 10.2%. It was also showed that IgE-mediated sensitization to this allergen is highly specific for patients who are poly-sensitized to molds, namely to *C. lunata* (Gabriel et al., 2015a).

It is well known that the proteases produced by various allergenic sources may directly impact respiratory epithelial biology, thus playing an important role in initiating an allergic response in the lung (Yike, 2011; Wills-Karp et al., 2010). Proteases, which act at the same time as allergen, may enhance bystander-type reactions, thereby facilitating antigen access to the subepithelium and potentially modifying other allergens in such a way that they become more potent antigens.

Furthermore, it has been demonstrated that intrinsic SP-specific activity of *Alternaria alternata* extracts plays an important role in the physiopathogenesis of asthma via the elicitation of an increase in the permeability of bronchial epithelial cells (Leino et al., 2013) and the promotion of a rapid and robust release of early innate mediators and prolonged Th2 inflammation (Kouzaki et al., 2009; Boitano et al., 2011; Snelgrove et al., 2014). The above-mentioned data support the need to further investigate the allergenic properties of *A. alternata* SPs.

2.9. Non-IUIS A. alternata allergenic proteins

Five other *A. alternata* proteins (Alt a TCTP, Alt a NTF2, Alt a 2, Alt a 9 and Alt a 70 kDa) that were reported to be able to bind IgE from sensitized patients are not encompassed in the referred official allergen list, but they were already comprised in the Allergome database (http://www.allergome.org/). The translationally controlled tumor protein from *A. alternata* (Alt a TCTP) was characterized as an allergenic protein with the demonstrated ability to react with 4% (n=16) of *A. alternata* patients' sera (Rid et al., 2009). *A. alternata* nuclear transport factor 2, Alt a NTF2, was described as a 13.7 kDa IgE-reactive protein that reacts with a low prevalence with sera proceeding from mold-sensitized individuals (Weichel et al., 2003). However, the sensitization prevalence in a population of patients sensitized to *Alternaria* was not studied. Reports respective to Alt a 2 allergen characterization are controversial. First, it was reported to be a major allergen with a sensitization rate of 61% among *Alternaria*-sensitive patients (Bush et al., 1999). In a study performed by Asturias *et al.* (Asturias et al., 2005), it was surprisingly found that none of the 42 *A. alternata*-sensitized patients' sera reacted with Alt a 2. Alt a 9 was identified as a 42 kDa protein that bound IgE in 5% of patients (Achatz et al., 1995). For Alt a 2, Alt a 9 and Alt a 70 kDa proteins, the structural and biochemical functions are unknown. The last one was reported to induce skin reactivity in 87% (n=16) of the *A. alternata*-sensitized patients (Portnoy et al., 1990).

3. *A. alternata* allergens and cross-reactivity

The allergen cross-reactivity phenomenon typically occurs when IgE antibodies that are originally raised against one allergen bind or recognize a similar protein from another allergenic source. Such recognition can trigger an allergic reaction or can be completely irrelevant for the patient. The clinical relevance of cross-reactivity depends on factors such as the host, exposure and implied allergen (Ferreira et al., 2004). Cross-reacting allergens are evolutionary conserved proteins that play vital functions and are therefore ubiquitously distributed in several biological sources (Hauser et al., 2010).

The cross-reactivity of *A. alternata* with other airborne fungal species has been extensively described (Gupta et al., 2002), and there is evidence that a significantly high percentage of patients sensitized to *A. alternata* are poly-sensitized to more than one other fungal species and might also be sensitized to other environmental aeroallergen sources such as pollens, mites or even to food allergens (Mari et al., 2003). It is well known that the existence of cross-reactivity, particularly among different molds, may have implications for the diagnosis and treatment of fungal allergies (Crameri et al., 2009). In terms of diagnosis, it is common to observe that if a patient is primarily sensitized to highly conserved allergenic proteins, the patient's serum could also give a positive result for a cross-reactive component from different allergenic mold species. This occurrence often complicates the identification of the primary sensitizer specie and results in defective management of the allergic disease. From both diagnostic and treatment perspectives, it is important that poly-sensitization resulting from allergen cross-reactivity be distinguished from co-sensitization to multiple allergenic sources. Immunologically, co-sensitization occurs when genuine sensitization to more than one allergen source is not due to cross-reactivity because it is not mediated by shared epitope-specific antibodies (Canonica et al., 2013). Therefore, for each allergological relevant allergen source, clarifying the cross-reactive structures and genuine markers among the whole allergen array is necessary.

Most *A. alternata* allergenic proteins are at least potentially cross-reactive across one or more species. As can be observed in Table 2, *A. alternata* possesses allergens that have their homologues in the 3 other genera that, together with *Alternaria,* are most commonly associated with the development of fungal allergy: *Cladosporium*, *Penicillium* and *Aspergillus*.

The high identity scores of the alignments are strong evidence of the potential for the existence of IgE cross-reactions (Boywer et al., 2006). A sequence identity of at least 50% has been accepted as required for allergenic cross-reactivity (Aalberse, 2005); however, it can also rarely be detected when lowest sequence homologies are noticed. Differences in the amino acid sequence or structure, localization and/or amount of allergenic proteins reflect differences of allergenic properties of homologues and are determinant factors of cross-reactivity.

In relation to the major *A. alternata* allergen, the Allergome database (http://www.allergome.org/) includes a list of 171 Alt a 1-related proteins from several species of *Alternaria* and related taxa; however, to date, no evidence of IgE reactivity has been assessed for most of these molecules. In fact, most *A. alternata*-related taxa have been described to be associated mainly with the pathogenicity of plants and spoilage and the biodeterioration of fruits, feedstuffs and foods (Thomma, 2003) instead of environmental allergenic sources. Analysis of the molecular diversity of indoor *Alternaria* isolates in the USA revealed that species of *Alternaria* section *Alternaria* represented 98% (153 isolates), of which 137 isolates were assigned to *A. alternata*, 15 to the *A. arborescens* specie and a single isolate to *A. burnsii* (Woudenberg et al., 2015). Gutiérrez-Rodríguez *et al*. demonstrated that fungal species from the Pleosporaceae family, such as *Stemphylium* and *Ulocladium*, have a common allergen that shows a high level of cross-reactivity with Alt a 1 from *A. alternata* (Gutiérrez-Rodríguez et al., 2011). Given the scarce but existing evidence of Alt a 1 homologue allergenicity and even with their expected low frequencies as environmental contaminants, evaluating the presence of other species that liberate Alt a 1-like proteins could contribute to the total allergenic Alt a 1 load to which atopic patients are exposed and may be an interesting task to consider further.

The remaining *A. alternata* allergens are mostly phylogenetically conserved proteins that are recognized as cross-reactive fungal allergen classes that may cross-react with homologous protein from other fungal species but also from organisms from different phyla. Numerous reports present the mediation of the cross-reaction between minor *A. alternata* allergens and other fungal allergenic species (Breitenbach et al., 1997; Rid et al., 2009; Shankar et al., 2005; Simon-Nobbe et al., 2000; Simon-Nobbe et al., 2008; Weichel et al., 2003). Among these, enolase (Alt a 6), for which cross-reactivity with *Cladosporium*, *Saccharomyces*, *Candida*, *Aspergillus* and *Hevea brasiliensis* was demonstrated (Breitenbach et al., 1997; Simon-Nobbe et al., 2000), is considered to be the *A. alternata* cross-reactive allergen with high allergological importance fundamentally in terms of diagnosis (Postigo et al., 2011).

Recently, we undertook efforts to identify and characterize two new cross-reacting allergens: Alt a 14, a MnSOD, and Alt a 15, a vacuolar SP. In our experiments, these two allergens seemed to play a role in the cross-reactivity phenomena between *A. alternata* and other important allergenic molds, namely *A. fumigatus* and *C. lunata*. Specifically, it was found that Alt a 14 showed cross-reactivity with Asp f 6 (Gabriel et al., 2015c), an *A. fumigatus* allergen that, together with Asp f 4, is regarded as a specific marker for ABPA (Fricker-Hidalgo et al., 2010; Hemmann et al., 1999). Some studies have referred to *A. alternata* as one of the potential etiologic agents of this hypersensitivity-mediated disease of the lower airways (Chowdhary et al., 2012; Singh and Denning, 2012; Chowdhary et al., 2014. Related to this, Jubin *et al.* observed a significant association of ABPA with a prior or concomitant sensitization to *Alternaria* and proposed that it may be the result of an allergenic cross-reactivity between products from *Alternaria* and *Aspergillus* species (Jubin et al., 2010).

Thus, because rAlt a 14 was also able to bind IgE from ABPA patient sera, it was suggested that this MnSOD could have important implications in *A. alternata* sensitization as a risk factor for the development of ABPA (Gabriel et al., 2015c). Moreover, it has been described that allergens belonging to the MnSOD protein family may cross-react also to homologous human MnSOD, which could contribute to the perpetuation of the inflammatory response in asthma (Crameri et al., 1996).

Associations between sensitivity to airborne molds (*A. alternata*, *C. herbarum* and *A. fumigatus*) and food allergies, namely to mushrooms and spinach, have also been reported (Herrera et al., 2006). In a recent case report, a prior sensitization to *A. alternata* was associated with a severe food reaction to cross-reacting homologue mushroom proteins. A MtDH and a MnSOD from *Agaricus bisporus* mushroom with significant homology to Alt a 8 and Alt a 14, respectively, were identified as patient-specific IgE-binding proteins (Gabriel et al., 2015b).

Although a high number of fungal species shares IgE-binding homologue molecules, not all of them possess the same clinical importance. In fact, as an example, the well-recognized fungal pan-allergens belonging to the serine protease protein family are major allergens in several fungi, namely *Aspergillus*, *Cladosporium*, *Curvularia*, *Penicillium*, *Rhodotorula* and *Trichophyton* species; however, the *A. alternata* homologue (Alt a 15) presents a relatively low prevalence of recognition among *A. alternata*-sensitive patients. Quantitative differences rather than qualitative ones, e.g., the amount of allergen released, most likely explain why some of these proteins are minor or major allergens depending on the producer's fungal species.

Because a significantly high number of *A. alternata*-sensitized patients are poly-sensitized to other aeroallergens that are simultaneously present in high frequencies such as grass, olive, cat epithelia, *Dermatophagoides* and cypress (Katotomichelakis et al., 2012), the existence of cross-reactive or adjuvant phenomena must be considered.

4. *Alternaria* allergens and their phylogenetic information

Traditionally, fungal species are systematically categorized on the basis of their morphologic characteristics. However, in recent years, promissory phylogenetic approaches mainly based on sequence comparison of ribosomal RNA genes have emerged and have allowed a more advanced classification of fungi (Schoch et al., 2012). Simultaneously, it has been increasingly proposed that the knowledge of fungal biology and how it is evoluting may explain the antigenic nature of allergologically important fungal species.

The evolutionary process seems to be responsible for the observation that a limited number of fungal species produce a specific allergen that is highly recognized by the human immune

system. In fact, the immune system of each atopic individual will recognize the foreign proteins existing in the surrounding environment, and the degree of the foreign nature of the recognized allergens is highly dependent on their evolutionary distance from human proteins. Accordingly, recently, Soeria-Atmadja *et al.* demonstrated that IgE sensitization patterns of individuals sensitized to fungi strikingly mirrors fungal phylogenetic relationships (Soeria-Atmadja et al., 2010). These observations reflect the premise that more closely phylogenetically related fungi will have greater shared antigens.

The existence of shared allergens across fungal species causing IgE cross-reactivity suggests that some genes coding for allergens recognized by IgE antibodies may be used as powerful phylogenetic markers that are useful to categorize fungi systematically and to identify proteins causing IgE cross-reactivity through the fungal kingdom. In this regard because *A. alternata* has been considered the main producer of fungal allergens, it is proposed that information on the evolution process of the gene coding for the allergen may be valuable in understanding the allergen importance in fungal biology and the stimulation of the allergic response. Generally, the molecular studies founded on fungal allergen proteins are limited but results are quite promising (Davolos and Pietrangeli, 2007). In one of their studies, Hong *et al.* found gene homologues of the major *A. alternata* allergen, Alt a 1, exclusively in *Alternaria* and taxonomically related species. In the same study, the authors used Alt a 1 as a molecular marker to produce phylogenetic trees of *Alternaria* and related taxa with high resolution and bootstrap supports (Hong et al., 2005). Indeed, the evolutionary distance of *Alternaria* and related other allergenic molds seems has led to the development of species-specific proteins, such as Alt a 1, and this process explains the reason why it is the main primary sensitizer in a fungi-sensitized population and is widely considered to be a marker of sensitization to Pleosporaceae members (Postigo et al., 2011). Given its high phylogenetic content, the Alt a 1 gene and protein may be useful to unequivocally identify and detect a restricted group of fungal species (Gabriel et al., 2015d); this issue will be more extensively addressed below. In contrast, as described above, most *A. alternata* allergenic proteins are minor allergens that possess homologs across diverse fungal species.

The taxonomic distribution of *Alternaria* allergens homologues may be highly correlated with sensitization and cross-reactivity data. Species phylogenetically related to *A. alternata* are likely to produce a similar allergen array and thus produce similar sensitization patterns whereas phylogenetically distant ones will only share ubiquitous cross-reactive molecules, thus explaining why the induced IgE sensitization data are less correlative. The IgE-based hierarchical tree proposed by Soeria-Atmadja *et al.* positioned *A. alternata* in the Pleosporales species cluster with the least similarity to other species of the same order (Soeria-Atmadja et al., 2010). Because this study included only a few Pleosporales members (*Curvularia lunata*, *Stemphylium herbarum*, *Epicoccum purpurascens*, *Phoma betae* and *Setomelanomma rostrata*) in which an Alt a 1-like protein is not described, the lower similarity of sensitization pattern reflects that, as expected, the patients are sensitized to *A.*

alternata mainly through Alt a 1. Future similar studies including a higher number of fungal species, namely those that are phylogenetically close to *A. alternata*, should be performed. This will allow obtaining the complete pattern of association between fungal molecular systematic and IgE sensitization data and ascertaining the value of Alt a 1 and other cross-reactive fungal components in the delineation of a hierarchical organization among allergy-related fungi.

To establish complete phylogenetic analyses conducted on gene coding for allergens recognized by IgE antibodies and fully correlate the data with the immune system's response displayed in fungi-sensitized individuals, research on allergenic protein homologues existing across fungal species is critical.

5. Exposure to *A. alternata* allergens and its assessment

Airborne measurement of mold exposure showed that increased atmospheric levels of fungal spores are linked to severe manifestations of respiratory allergic responses, occurrence of hospital admissions and deaths related to asthma (Targonski et al., 1995)

The threshold *Alternaria* count in the air necessary to elicit allergic symptoms has been estimated to be 100 spores/m3 (Ricci et al., 1995). Compared with the concentration associated with the onset of allergenic symptoms for *Cladosporium* spores (3000 spores/m3) (Gravesen, 1981), that with *Alternaria* are the most widespread mold spores found in aerobiological surveys, the potent allergenicity of *Alternaria* is clear. This is in agreement with a previous study, where the comparison between sensitization to fungi with corresponding fungal spore counts found in the same environment showed that *Alternaria* presented a significantly higher score for sensitization (Beaumont et al., 1985).

Knowledge of the aerodynamic characteristics and distribution of allergens and how and when exposure is increased is essential to try to establish relationships between allergen exposure and specific immune responses. *Alternaria* distribution is reported to be related to the geographic area, season, atmospheric condition and time of day (Rotem, 1994). Given that *Alternaria* species are prevalent phytopathogens, the concentration of allergenic airborne spores can also be linked to the release of spores from infected plants; thus, areas where agricultural activities are prevalent may be conducive to the environmental dissemination of allergenic *A. alternata* spores (Corden et al., 2003).

Fungal exposures differ from prevalent environmental allergens, such as pollens, both in their quantity and in the duration of exposure to the allergenic source. In fact, airborne spore counts are often 1000-fold higher than pollen counts (Salvaggio et al., 1971), and a prolonged intense exposure to *Alternaria* spores normally occurs for months whereas pollen exposure typically occurs for weeks (Dang and Lawrence, 2014).

It has been documented that asthmatic patients sensitized to *Alternaria* tend to suffer a more severe outcome during the late summer and early autumn (Chakrabarti et al., 2012; Canova et al., 2013), when the highest *Alternaria* spore counts are recorded (Rodríguez-Rajo et al., 2005; Segvic and Pepeljnjak, 2006). Pulimood et al. found that epidemic thunderstorm-related asthma is strongly associated with *Alternaria* species sensitivity and fragmentation of *Alternaria* spores results in easily breathable allergenic fragments. A high concentration of fungal spores, their transportation over large distances and other environmental factors that might contribute to bronchial hyper-responsiveness are associated with thunderstorms, such as ozone and a sudden reduction of temperature, and may explain the association. Because *Alternaria* species grow on cereals, it has been proposed that the farming practice contributes to increasing fungal spore levels (Pulimood et al., 2007). Global climatic change and CO2 concentration also appear to stimulate *A. alternata* sporulation and total antigen production (Wolf et al., 2010), consistent with the increase in the prevalence of allergies and asthma severity (Beggs and Bambrick, 2005).

The presence of *A. alternata* allergen indoors has also been detected, namely colonizing in textile home stuffs such as carpets and bedding (Peters et al., 2008). In a US Indoors survey, the prevalence of symptomatic asthma was strongly correlated with *A. alternata* counts in the dust samples of patients' homes (Salo et al., 2006).

Because of the significant negative effects of *A. alternata* on human health and plants, the correct detection of *A. alternata* and its allergens is of great importance not only in the clinical setting but also in environmental, epidemiological and plant pathology studies. Several strategies have evolved to sample, identify and interpret exposure to fungi (Tovey and Green, 2005). The traditional methods of fungi identification and exposure evaluation are based on readily observable morphological features and spore counts (Guarro et al., 1999). The delineation of the *Alternaria* species is complicated by methods that have several associated limitations; these methods are time consuming, laborious, and they are not always reliable because they require the skills of a trained person.

Recently, the epidemiological information obtained by traditional methods has been actively used to protect and enhance the quality of life in the sensitized/allergic subjects, though much work is needed in this field. Several protein- and DNA-based methods have merged and are considered useful tools for monitoring fungal exposure levels in a given indoor or outdoor environment. They can be performed on a large number of samples, often quickly and without the need of technical skills in mycological techniques.

Protein-based methods include immunoassays such as enzyme-linked immunosorbent assays (ELISA) that use polyclonal or monoclonal antibodies to quantify *A. alternata* antigens. Currently, some ELISA kits are commercially available for the specific detection and quantification of the *A. alternata* major allergen, Alt a 1. ELISA techniques are based on the

interaction between antibodies and the specific allergens, and the possibility of cross-reactivity with other nontarget proteins, namely homologues proteins from other sources, can lead to false-positive results. The main advantages of immunochemical assays such as ELISA are that they provide quantitative results and allow direct assessment of the allergen with a low set-up cost, moderated running time, and no special requirements for expert knowledge.

Recently, Meng *et al.* found no significant difference in the detection rate of *Alternaria* between asthmatic and non-asthmatic homes by either spore counting or cultivable airborne detection (Meng et al., 2012). However, in a previous cross-sectional study, a polyclonal anti-*Alternaria* antibody assay was successfully employed to detect *Alternaria* allergens in US homes; exposure to *A alternata* allergens in US homes has been observed to be associated with active asthma symptoms (Salo et al., 2006). The same assay allowed ascertaining that *Alternaria* antigen levels are influenced not only by regional and housing characteristics but also by residents' behavior (Salo et al., 2005). The use of a monoclonal antibody-based ELISA confirmed that risk of respiratory symptoms in patients sensitized to *A. alternata* was significantly correlated with atmospheric Alt a 1 levels (Feo Brito et al., 2012). Capture ELISA was also described as a very sensitive, specific and reproducible assay for Alt a 1 detection in dust samples collected in poultry farms (Prester and Macan, 2010).

Alternatively, development of DNA-based methods has enabled more reliably quantifying fungal species. Several detection and quantification PCR-based methods with acceptable set-up costs and running times have emerged. Given its highly variable content, the large number of copies per cell and deposited sequences in international databases, Internal Transcribed Spacer (ITS) regions of the rRNA gene have been utilized as a target for assessing the fungal diversity in environmental samples (Rittenour et al., 2012). The main advantages of such molecular techniques are their specificity and minimal susceptibility to cross-reactivity phenomena because the chosen target sequence can be adapted from target allergen-encoding sequences to species-specific DNA markers. Despite the advantages of DNA-based methods, PCR is still much contested because when detecting a gene encoding for an allergen, it does not necessarily imply its expression. Consequently, the results obtained by DNA detection do not account for the actual allergenic potential. However, the same happens with some, if not most, ELISA tests that do not necessarily detect the allergenic proteins but rather detect species-specific protein markers. In fact, the detection of a molecular marker gives indirect information regarding the allergenic potential, but provides evidence of the presence of the allergenic ingredient.

A recent molecular study demonstrated that Alt a 1 can be used as a marker to successfully detect allergenic and pathogenic *Alternaria* and related taxa by PCR (Gabriel et al, 2015d). The PCR system using a primer set that was designed based on nucleotide conservation of Alt a 1-encoding sequences was able to detect all of the Alt a 1-producing species that were included in the study (*A. alternata, A. tenuissima, A. infectoria, U. botrytis and S.*

botryosum). In contrast, the use of a second primer set, previously used by other authors for the production of recombinant Alt a 1, allowed the detection of the closely taxonomically related species *A. alternata* and *A. tenuissima*.

6. Allergen based-component diagnosis: a new era of *Alternaria* allergy diagnosis

It is now generally accepted that the diagnosis of an allergy must be based on the clinical history of symptoms and on confirmative assays that may include the determination of allergen specific IgE antibodies by skin prick tests, laboratory-based *in vitro* analyses and/or provocation tests.

According to a large Pan-European GA(2)LEN skin prick test study (Bousquet et al., 2009), an *Alternaria* extract should be included in the minimum standard battery of test inhalant allergens that are used to appropriately assess sensitization across Europe (Table 3).

The combination of skin prick tests and determination of allergen-specific IgE levels in the serum is currently recommended for a reliable assessment of *Alternaria* sensitization. In diagnosis investigations, complex crude extracts are widely used. However, there are several limitations associated with the use of whole complex extracts that include the presence of irrelevant molecules and potentially highly cross-reactive allergens, the limited representation of some allergens and the well-documented variability between preparations of crude extracts (Shreffler, 2011). This could be the origin of the significant low concordance between SPT and serum IgE test results for *Alternaria* (O'Driscoll et al., 2009).

Some studies investigating the biochemical and immunological content of commercially available *Alternaria* skin prick test solutions found high variability with respect to their protein, antigen and allergen contents (Kespohl et al., 2013; Esch, 2004; Vailes et al., 2001). Many factors attributed to the different steps of fungal extract production could be responsible for an inconsistent skin test preparation, which can result in an erroneous diagnosis. The use a specific mold strain and growth conditions preferred by the manufacturers is considered to be the first source of variation. Then, different extraction methods, with or without additives and final allergen quantification systems are also likely to be potential sources of variability (Kespohl et al., 2013,Sáenz-de-Santamaría et al., 2006; Portnoy et al., 1993). Recently, Twaroch *et al.* observed that *Alternaria* strains, media nutritional components and growth periods have an enormous impact on the presence of IgE-binding proteins in the final extract (Twaroch et al., 2015). With regards to the most clinically relevant *Alternaria* allergen, it was demonstrated that Alt a 1 expression is highly dependent on fungal growing time, cultivation media (Ibarrola et al., 2004) and strain (Martínez et al., 2006). In cases of poly-sensitized patients, the application of diagnosis

methods based on extracts obtained from related and non-related allergenic sources where the existence of cross-reactive allergens is well known results in difficulty in identifying the primary sensitizer (Crameri, 2011); therefore, no discrimination is allowed between cross-reactivity and co-sensitization.

To overcome crude extract-based diagnosis limitations, molecular or component-resolved diagnosis (CRD) using individual recombinant or native allergenic molecules tested on a florescence enzyme immunoassay (ImmunoCAP) or a microarray-based assay platform are now widely available in Europe (Shreffler, 2011). The availability of high-quality recombinant allergens by the recent advances in recombinant DNA technology for allergen research has allowed definition of individual sensitization profiles and an understanding of the nature of sensitization as well as noted makers of severity, persistence or cross-reactivity. Thus, more informed choices can be made regarding strategies for allergen-specific immunotherapy (Cromwell et al., 2011).

To date, only two allergens (rAlt a 1, rAlt a 6) are commercially available for the molecular diagnosis of an *Alternaria* allergy. It has been currently accepted that the use of the species-specific *Alternaria* allergen, Alt a 1, in molecular diagnosis, indicates a genuine sensitization to *A. alternata*, even if close homologue allergens might occur across the fungal kingdom (Twaroch et al., 2012). However, in the absence of sensitization to Alt a 1, the application of an individual *A. alternata* allergen with known cross-reactivity can improve diagnostic sensitivity, thereby permitting the identification of cross-sensitization and allowing the unequivocal definition of primary sensitizers in poly-sensitization cases.

In a preliminary study, the use of two recombinant molecules, Alt a 1 and Alt a 6, by skin prick test seemed to correctly diagnose *Alternaria* sensitization in 7 tested subjects (Unger et al., 1999). Asturias et al. observed that replacing *A. alternata* extracts with either natural or recombinant Alt a 1 forms seemed to be sufficient for a reliable diagnosis of *Alternaria* sensitization (Asturias et al., 2005). Recently, Postigo et al. revealed that 2 of the 30 *A. alternata* allergic-patients did not react to Alt a 1 or Alt a 6 but did react to a potentially cross-reactive MnSOD, now officially named Alt a 14; according to the authors, Alt a 14 should be included in the molecular diagnosis allergen array (Postigo et al., 2011).

It is hoped that the identification and characterization of the whole array of *Alternaria* allergens and new techniques based on allergenic protein cloning will allow the preparation of better quality test solutions and improve *Alternaria* allergy diagnoses.

The most important steps to achieve an effective therapy for a fungal allergy are the accurate diagnosis and exposure assessment to *A. alternata* and its individual components. Because *A. alternata* is mainly an outdoor aeroallergen, its avoidance as a preventive therapeutic measure is difficult to achieve; however, some cautions, especially at home, may reduce and/or prevent indoor exposure (Salo et al., 2005; Sanchez and Bush, 2001).

7. Conclusion

Compared to other common environmental allergenic sources, fungi are reported to be neglected and underestimated. As *A. alternata* allergies continue to rise, enabling research on the role of this fungal species and its allergenic components, particularly in asthma, makes sense. Establishing correlations of *A. alternata* exposure and allergic symptoms remains problematic. It is critically important to adopt a multidisciplinary approach using both epidemiologic and molecular tools to accurately evaluate the exposure trends to *A. alternata* and its individual allergens and to determine the potential health effects.

As presented in this review, *A. alternata* allergens may be considered potent markers of phylogeny, sensitization and exposure. Complete characterization of fungal allergens and the analysis of a distinct pattern of allergenic species distribution will aid in understanding and predicting cross-reactivity and improving allergy diagnostic methodologies. Moreover, an understanding of the phylogenetic content of unique and shared allergens will provide insights into fungal biology, allergenicity and fungi immune responses.

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Table 1. Official *A. alternata* allergens

Allergen	Biochemical name	MW (SDS-PAGE) kDa	Clinical relevance	Isoallergen and variants	Nucleotide (NCBI)	Protein (NCBI)	Protein (UniProt)	PDB
Alt a 1		16.4 and 15.3 band	Major allergen	Alt a 1.0101	U82633/U86752	AAB40400/AAB47552	P79085	3V0R
				Alt a 1.0102	AY568627	AAS75297	Q6Q128	4AUD
Alt a 3	Heat shock protein 70		Minor allergen	Alt a 3.0101	U87807/U87808	AAB48042/AAB48043	P78983	-
Alt a 4	Disulfide isomerase	57	Minor allergen	Alta 4.0101	X84217	CAA58999	Q00002	-
Alt a 5	Ribosomal protein P2	11	Minor allergen	Alt a 5.0101	X89222/U87806	CAA55066/AAB48041	P42037	-
Alt a 6	Enolase	45	Minor allergen	Alt a 6.0101	U82437	AAG42022	Q9HDT3	-
Alt a 7	Flavodoxin, YCP4 protein	22	Minor allergen	Alt a 7.0101	X78225	CAA55069	P42058	-
Alt a 8	Mannitol dehydrogenase	29	Minor allergen	Alt a 8.0101	AY191815	AAO91800	P0C0Y4	-
Alt a 10	Aldehyde dehydrogenase	53	Minor allergen	Alt a 10.0101	X78227	CAA55071	P42041	-
Alt a 12	Acid ribosomal protein P1	11	Minor allergen	Alt a 12.0101	X84216	CAA58998	P49148	-
Alt a 13	Glutathione-S-transferase	26	Minor allergen	Alt a 13.0101	AY514673	AAR98813	Q6R4B4	-
Alt a 14	Manganese superoxide dismutase	24	Minor allergen	Alt a 14.0101	KC923297	AGS80276	P86254	-
Alt a 15	Vacuolar Serine protease	58	Minor allergen	Alt a 15.0101	KJ558435	AHZ97469	-	-

The link to World Health Organization and International Union of Immunological Societies(WHO/IUIS) Allergen Nomenclature Sub-committee is http://www.allergen.org/.

Table 2. List of the most common potentially cross-reactive *A. alternata* allergens.

Biochemical function	*A. alternata* allergen	Fungal Homologues IUIS allergens	Species	SDAP E score
Heat shock proteins 70	Alt a 3	Pen c 19	*Penicillium citrinum*	1.9 e-27
		Mala s 10	*Malassezia sympodialis*	1.2 e-3
Ribosomal protein P2	Alt a 5	Fus c 1	*Fusarium culmorum*	3.4 e-27
		Cla h 5	*Cladosporium herbarum*	8.4 e-25
		Asp f 8	*Aspergillus fumigatus*	2.0 e-22
Enolase	Alt a 6	Cla h 6	*Cladosporium herbarum*	1.2 e-157
		Asp f 22	*Aspergillus fumigatus*	1.9 e-154
		Pen c 22	*Penicillium citrinum*	4.7 e-153
		Cur l 2	*Curvularia lunata*	8.4 e-153
		Rho m 1	*Rhodotorula mucilaginosa*	2.5 e-129
Flavodoxin, YCP4 protein	Alt a 7	Cla h 7	*Cladosporium herbarum*	9.4 e-61
Mannitol dehydrogenase	Alt a 8	Cla h 8	*Cladosporium herbarum*	6.6 e-91
Aldehyde dehydrogenase	Alt a 10	Cla h 10	*Cladosporium herbarum*	5.9 e-168
Acid ribosomal protein P1	Alt a 12	Cla h 12	*Cladosporium herbarum*	1.8 e-30
		Pen cr 26	*Penicillium crustosum*	4.2 e-28
		Pen b 26	*Penicillium brevicompactum*	6.8 e-28
Manganese superoxide dismutase	Alt a 14	Asp f 6	*Aspergillus fumigatus*	5.5 e-48
		Mala s 11	*Malassezia sympodialis*	4.9 e-36
Serine protease	Alt a 15	Cur l 4	*Curvularia lunata*	4.3e-152
		Cla h 9	*Cladosporium herbarum*	5.3e-119
		Pen o 18	*Penicillium oxalicum*	2.4e-114
		Asp f 18	*Aspergillus fumigatus*	4.8e-111
		Pen ch 18	*Penicillium chrysogenum*	7.9e-111
		Cla c 9	*Cladosporium cladosporioides*	1.2e-99
		Rho m 2		1.6e-73
		Tri r 2	*Rhodotorula mucilaginosa*	1.6e-37
		Pen ch 13	*Trichophyton rubrum*	1.1e-23
		Asp v 13	*Penicillium chrysogenum*	1.5e-20
		Asp f 13	*Aspergillus versicolor*	2.8e-20

	Asp o 13	*Aspergillus fumigatus*	9.8e-19
	Asp fl 13	*Aspergillus oryzae*	9.8e-19
	Pen c 13	*Aspergillus flavus*	5.3e-15
		Penicillium citrinum	

The link to Structural Database of Allergenic Proteins (SDAP) is http://fermi.utmb.edu/

Table 3. Skin prick test standard allergen battery to assess sensitization across Europe suggested by Global Allergy and Asthma European Network (Bousquet et al., 2009; Bousquet et al., 2012).

Pollen	Grass
	Birch
	Artemisia
	Olive
	Parietaria
	Ambrosia
	Cypress
	Plane
Mites	*Dermatophagoides pteronyssinus*
	Dermatophagoides farinae
Animals	Cat dander
	Dog dander
Molds	Alternaria
	Cladosporium
Insects	Blatella

Chapter 2

Global aims

The main aim of this work was to broaden the knowledge on the repertoire of allergenic proteins produced by *Alternaria alternata,* the main inducer of severe asthma caused by fungi, and use such knowledge to improve prediction, diagnosis and understanding of the human immunological responses to fungal allergens.
The development of specific molecular tools for the unequivocal assessment of the exposure to *A. alternata* is critical in evaluating the epidemiology as well as in predicting the risk and severity of allergic respiratory disorders caused by this fungal specie. Likewise, the identification and characterization of the complete panel of clinically relevant allergens of *A. alternata* and the definition of individual allergen sensitization patterns are crucial for achieving improved understanding, diagnosis and management of the fungal allergic diseases. Particularly, the availability of identified allergens as recombinant proteins will assist the improved component-resolved-diagnosis of fungal allergy and allow for allergen biochemical and immunological characterization. Moreover, the sequence and structural comparison of individual allergens with homologues from other allergenic species as well as the study of IgE cross-reactivity will help to clarify diagnostic problems that are associated with the poly-sensitization phenomena, a common and relevant general clinical feature in the mold allergic population.

With these global aims in mind, the development of the work followed the subsequent tasks:

a) Demonstrate that the phylogenetic information of encoding gene of the *A. alternata* major allergen, Alt a 1, can be used as a molecular marker suitable to be applied in assessment of exposure to *Alternaria* and related species.

b) Cloning and production of a manganese-dependent superoxide dismutase and a serine protease from *A. alternata* as recombinant molecules.

c) Assessment of sequence homologies and IgE cross-reactivity between the newly identified *A. alternata* allergenic proteins and allergen homologues from other fungal species.

d) Practical application of the allergen repertoire of *A. alternata* to explain clinical sensitization data displayed by patients presenting allergic symptoms that seem to be associated to a primary sensitization to *A. alternata*.

e) Recognition by the WHO/IUIS Allergen Nomenclature Sub-committee of the newly characterized IgE-binding proteins as official *A. alternata* allergens.

Chapter 3

Paper II

Development of a PCR-based tool for detecting immunologically relevant Alt a 1 and Alt a 1 homologue coding sequences

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Development of a PCR-based tool for detecting immunologically relevant Alt a 1 and Alt a 1 homologue coding sequences

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Abstract

Alt a 1 has been recognized as the most important allergen produced by the Pleosporaceae family and is a risk factor for asthma development and/or exacerbation. The aim of this study was to develop a detection tool that is highly specific for species that produced airborne Alt a 1. Based on the highly conserved internal nucleotide region of the several Alt a 1 sequences that are available in GenBank, a pair of primers (Alta1CF/Alta1CR) was designed. A set of primers used by other authors for the production of recombinant Alt a 1 (A21F/A21R) was also tested. The molecular analyses were based on the polymerase chain reaction (PCR) amplification and sequencing of the cDNA that was obtained from thirteen common fungal species. The PCR system that utilized Alta1CF/Alta1CR was highly specific, sensitive, and was able to detect an amplicon of approximately 180 bp from Alt a 1 and Alt a 1-like encoding genes from *A. alternata, A. tenuissima, A. infectoria, U. botrytis,* and *S. botryosum.* In contrast, the A21F/A21R primers were specific for the very close taxonomically related species *A. alternata* and *A. tenuissima*. Thus, this rapid, sensitive, and specific detection tool can be used to assess Alt a 1 exposure levels and to inform the implementation of the appropriate public health measures.

Key words: Alt a 1, Alt a 1 homologues, *Alternaria alternata*, allergen monitoring, amplification.

Introduction

Moulds occur in outdoor and indoor environments, where they grow on many different substrates, and are described as significant causes of invasive infections and respiratory allergic diseases in humans [1,2]. Fungal exposure assessments have traditionally relied on species-level identification using indirect methods that are based on spore or fungal colony count and culture techniques [3]. These

methodologies are generally laborious and time-consuming, and it can be difficult to establish or exclude a fungal contamination and its eventual association with a disease. To overcome these limitations and to address the increasing incidence of fungal allergic diseases worldwide, it is necessary to develop faster and more accurate alternative methods for the identification of fungal contamination. Molecular systematic and evolutionary approaches for mould identification have emerged in recent years [4,5].

Alternaria spp. are among the most common fungi, and the genus includes numerous species that are pathogenic to a variety of plants, including cereals, oil-yielding crops, spices, vegetables and fruits [6,7]. Furthermore, *A. alternata* spores are one of the most frequently identified fungal spore types in the atmosphere, and this specie is one of the most important fungal allergen producers [8–10]. In particular, the major allergen, Alt a 1 of *A. alternata* has been demonstrated to be the most important allergen that is produced by the airborne allergenic moulds [11–13]. Sensitization to this allergen is considered an important marker for predicting the risk and severity of respiratory symptoms [14,15]. Several other species of *Alternaria* and related genera have been described to possess Alt a 1 gene homologues [16], and allergenicity and cross-reactivity with some Alt a 1-like proteins from *Stemphylium botryosum* and *Ulocladium botrytis* have been reported [17].

Detection of Alt a 1 and its homologues, by investigating the conserved genes that encode the allergen instead of the species-level identification of *Alternaria*, could be considered an optimal tool for establishing the risk of respiratory symptoms in individuals who are sensitized to Alt a 1 or Alt a 1 homologues. With this in mind, we conducted molecular studies to demonstrate that Alt a 1 can be used as a marker to successfully detect allergenic and pathogenic *Alternaria* and related taxa by PCR.

Materials and method

Fungal strains and culture conditions

The *A. alternata* strains CBS 104.26, CBS 154.31, CBS 106.24, CBS 105.24 (Centraalbureau von Schimmelcultures, Utrech, the Netherlands) and FMR 3292 (Laboratory of Mycology, Rovira Virgili University, Tarragona, Spain), *A. tenuissima* FMR 5813, *A. infectoria* CBS 210.86, *Ulocladium botrytis* FMR 5819, *Stemphylium botryosum FMR 3952, Drechslera tritici-repentis* CBS 265.80, *Pyrenophora teres f. teres* CBS 123932, *Epicoccum nigrum* CBS 684.83, *Curvularia lunata FMR5790, Aspergillus fumigatus* AF54, *Penicillium chrysogenum* PC4 (Municipal Institute for Medical Research, Autonomous University of Barcelona, Spain), *Trichophyton* mentagrophytes CBS 120672 and *Fusarium solani* CECT 20232 (Spanish type Culture collection, University of Valencia, Spain) were used in this study. All of the fungal strains were cultured in 100 ml Erlenmeyer flasks containing 50 ml of Czapek broth medium at 25°C. Mycelia from 5-day-old cultures were harvested by vacuum filtration for RNA extraction. The *A. alternata* CBS 104.26 strain was also grown on Sabouraud dextrose agar plates for 7 days at 25°C and used for *A. alternata* spore suspension preparation. *A. alternata* spores were collected as previously described by Otani *et al.* 1998 [18]. Then, the spore suspension was counted using a hemacytometer, adjusted to a final concentration of 1×10^6 spores/ml and 10-fold diluted with sterile distilled water.

RNA extraction methods

Fungal mycelial mat was ground under liquid nitrogen with mortar and pestle and 100 mg of pulverized sample was resuspended in 1 ml of Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA was extracted from the mycelia according to the instructions provided by Trizol's manufacturer.

On the other hand, a bead beating method was used for extraction of total RNA from *A. alternata* spore suspensions (1×10^1 to 1×10^6 spores/ml). Two hundred microliters of each spore dilution was placed into a 2 ml extraction tube containing 600 mg of 0.1 mm glass beads and 500 μl RLC buffer (provided with the RNeasy® Plant Mini Kit, Qiagen, Germany) that was supplemented with β-mercaptoethanol. The tubes were quickly frozen in liquid nitrogen and placed on ice for 1 min. Then, the spore disruption was achieved by processing samples with the Mikro-Dismembrator U (B. Braun Biotech International, Melsungen, Germany) at 2000 rpm for 1 min, 5 min, and 10 min. The samples were immediately cooled down by placing them in ice in between each bead-beating step for up to 5-min intervals. Then, supernatants were obtained by centrifugation for 2 min at 13000 rpm, and RNA was extracted using RNeasy kit according to the manufacturer's fungi protocol. The final elution step was carried out with 30 μl of sterile distilled water and from this volume, 11 μl was used for reverse transcription. The RNA quality was analyzed with 1.2% agarose gel electrophoresis.

cDNA synthesis, primer design, and PCR amplification

Twenty microliter reverse transcription reactions were carried out with the RevertAid First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). A pair of primers was manually designed based on the highly conserved internal nucleotide region of the several Alt a 1 gene sequences that are available in the GenBank database (Table 1)

Table 1. Genbank accession numbers for Alt a 1 sequences used in multiple alignment analysis.

Species name	GenBank Accesion (Alt a 1 genes)
A. alternata	AF288160.1
A. arborescens	AY563303
A. argyranthemi	AY563280
A. blumeae	AY563291
A. brassicae	AY563309
A. brassicicola	AY563311
A. capsici	AY563298
A. carontiincultae	AY563287
A. cetera	AY563278
A. cheiranthi	AY563290
A. cineraria	AY563308
A.conjuncta	AY563281
A. crassa	AY563293
A. cucumerina	AY563300
A. dauci	AY563292
A. dumosa	AY563305
A. eryngii	AY563313
A. ethzedia	AY563284
A. euphorbiicola	AY563314
A. japonica	AY563312
A. limoniasperae	AY563306
A. longipes	AY563304
A. macrospora	AY563294
A. metachromatica	AY563285
A. mimiculata	AY563310
A.mouchaccae	AY563279
A. oregonensis	AY563283
A. petroselini	AY563288
A. photistica	AY563282
A. porri	AY563296
A. pseudorostrata	AY563295
A. radicina	AY563286
A. solani	AY563299
A. sonchi	AY563307
A. smyrniii	AY563289
A. tagetica	AY563297
A. tenuissima	AY563302
E. allii	AY563322
E. indefessa	AY563323
E. novae-zelandiae	AY563324
E. telluster	AY563325
N. caricis	AY563321
N. scirpicola	AY563320
P. herbarum	AY563277
S. botryosum	AY563274
S. callistephi	AY563276
S. vesicarium	AY563275
U. alternariae	AY563316
U. atrum	AY563318
U. botrytis	AY563317
U. chartarum	AY563319
U. cucurbitae	AY563315

Table 2. Sequence of PCR primers used in this study.

Primer name	Primer sequence (5′→3′)
A21F	GCGGATCCATGTCCTGCCTGTCACCAC
A21R	GCGGATCCTTAAGAGCTCTTGGGGAGAG
Alta1CF	GAGGGYGACTACRTYTGGAAGAT
Alta1CR	CCATGHAGCTGTTCTSGCCRCA

Note: H = A, T or C; R = A or G, S = C or G; Y = T or C

following alignment with the Clustal W analysis program. The primer pair, A21F/A21R, used by Asturias *et al.* [12] to produce recombinant Alt a 1, was also tested. Table 2 summarizes the primer sequences. PCR reactions were performed with Expand High Fidelity System (Roche, Mannheim, Germany) and 10 μl of cDNA as the template. The PCR product was purified with electrophoresis on an agarose gel, subcloned into the pJET1.2/blunt vector, and transformed into *Escherichia coli* DH5 alpha competent cells. The plasmid DNA was purified, and the nucleotide sequence of the cDNA insert was determined. Sequencing reactions were performed using the BigDye Terminator v.3 sequencing kit (Applied Biosystems) according to the manufacturer's specifications and were run in an ABI 3130XL sequencer (Servei de Genòmica, Universitat Autònoma de Barcelona). The amplicon sequence identity was confirmed with NCBI BLAST.

Results

Multiple alignment of the Alt a 1 gene sequences allowed us to identify a core conserved region for the Alta1CF/Alta1CR primer set design (Fig. 1). Clustal W results revealed that the sequence identities of the Alt a 1 gene and its homologues ranged from 69.4% to 100%. Additionally, of the approximately 510 nucleotides that constitute the Alt a 1 genes, 158 were 100% conserved between all *Alternaria* and related species. Eighty-three of these invariant sites were located in the gene region that was covered by the Alta1CF/Alta1CR primer set (Fig. 1). The primers were designed so that the amplified region would include the coding information for the two most important Alt a 1 immunoglobulin E (IgE)-binding linear epitopes (K41-P50 and Y54-K63) that were previously described by Kurup *et al.* [19]. The Alta1CF/Alta1CR primer specificity was assessed by analyzing the cDNA that was obtained from 13 fungal species, including 9 species that belong to the Pleosporaceae family (*A. alternata, A. tenuissima, A. infectoria, U. botrytis, S. botryosum, D. tritici-repentis, P teres f. teres, E. nigrum,* and *C. lunata*) and 4 that were non-Pleosporaceae allergenic relevant species (*A. fumigatus, T. metagrophytes, F. solani,* and *P. citrinum*). Additionally,

to eliminate the influence of the inter-strain variability, 5 different *A. alternata* strains were also included. The PCR amplification reactions with the Alta1CF/Alta1CR primer pair produced a single PCR product of approximately 180 bp for the *A. alternata, A. tenuissima, A. infectoria, U. botrytis* and *S. botryosum* species. This amplicon was also obtained for all five *A. alternata* strains (Figs 2A and 3). Sequence analysis of the 180 pb amplicons that were obtained by this assay revealed sequence identities that ranged from 87.3% to 100%.

Furthermore, the PCR amplification experiments with the A21F/A21R primer set produced the expected 390 bp amplicon in the *A. tenuissima* strain and the five *A. alternata* strains, but no PCR product was detected for the other species that were included in this study (Figs 2B and 3).

Additionally, to determine the PCR sensitivity (the lowest *A. alternata* counts detected) the *A. alternata* spore suspensions that ranged from 1×10^1 to 1×10^6 spores/ml were used. This system included a bead-beating process for breaking up the spores in the sample and the Alta1CF/Alta1CR primer set to amplify the fungal RNA that was retrieved from spore breakage. The lowest spore counts detected were 2×10^3 *A. alternata* spores, which existed in the 200 μl of 1×10^4 *A. alternata* spores/mL suspension that was used for RNA extraction (Fig. 4). This corresponded to an RNA detection limit of approximately

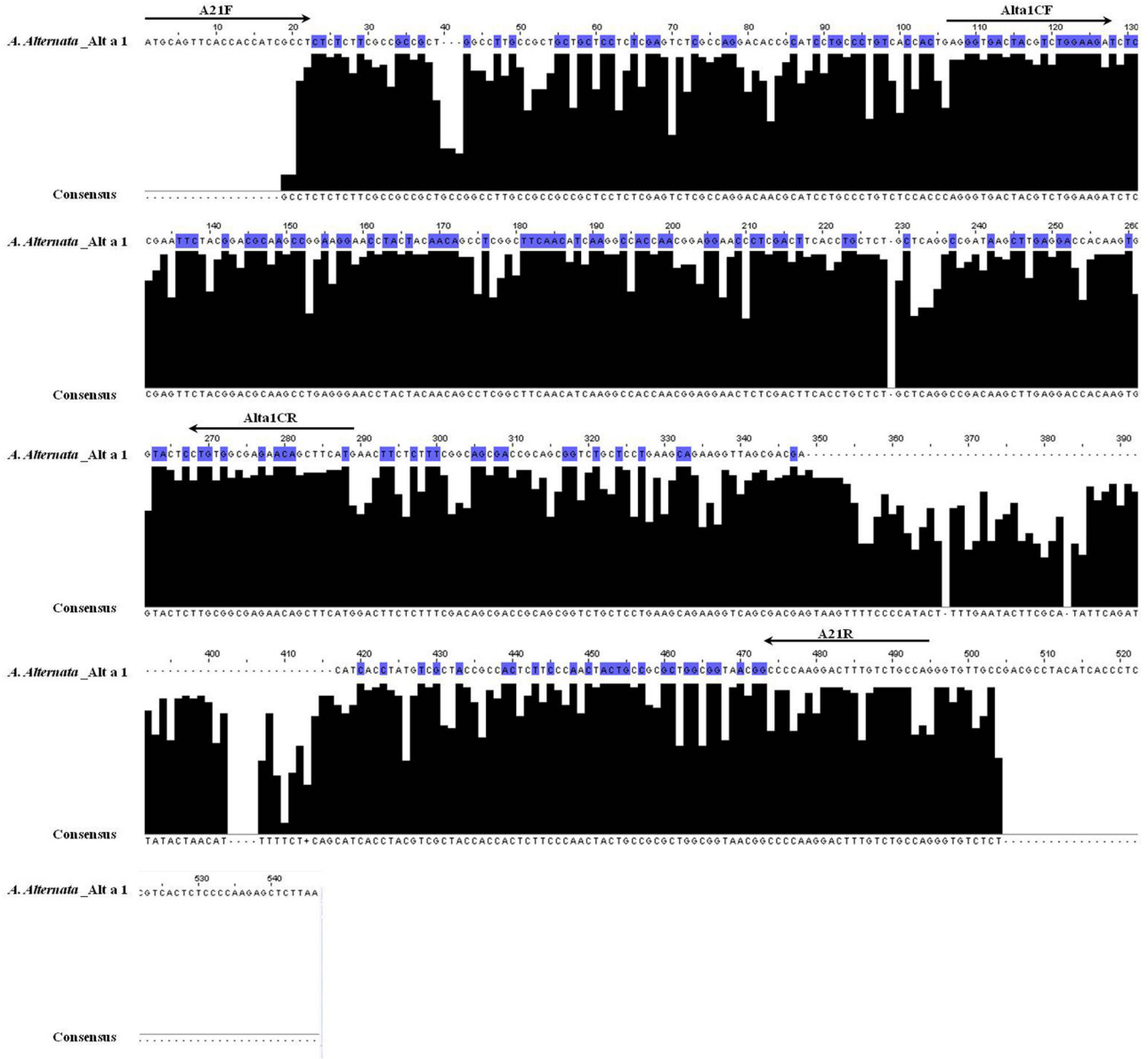

Figure 1. Nucleotide identity found by multiple alignment of Alt a 1 nucleotide sequences of *Alternaria* and related species. The higher bar indicates higher homologies. Nucleotides that 100% identical (invariant) among all Alt a 1 sequence are boxed. The primer positions are shown by arrows.

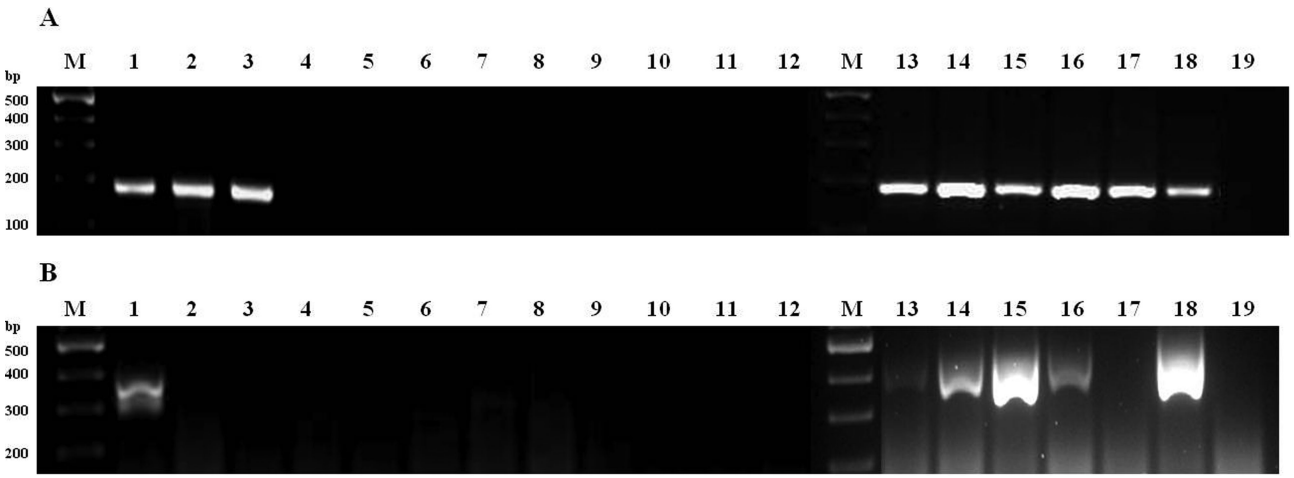

Figure 2. Electrophoretic analysis of Alt a 1 PCR products obtained using (A) Alta1CF/Alta1CR and (B) A21F/A21R primers from different allergologically important fungal species. Samples in lanes correspond to: *Alternaria alternata* strain CBS 104.26 (1), *Ulocladium botrytis* FMR 5819 (2), *Stemphylium botryosum* FMR 3952 (3), *Drechslera tritici-repentis* CBS 265.80 (4), *Pyrenophora teres f. teres* CBS 123932 (5), *Epicoccum nigrum* CBS 684.83 (6), *Curvularia lunata* FMR 5790 (7), *Aspergillus fumigatus* AF54 (8), *Trichophyton* mentagrophytes CBS 120672 (9) and *Fusarium solani* CECT 20232 (10), *Penicillium chrysogenum* PC4 (11), negative control (12 and 19), *Alternaria alternata* strains CBS 154.31 (13), CBS 106.24 (14), CBS 105.24 (15) FMR 3292 (16), *A. infectoria* CBS 210.86 (17), *A. tenuissima* FMR 5813 (18), GeneRuler™ DNA Ladder Mix (M).

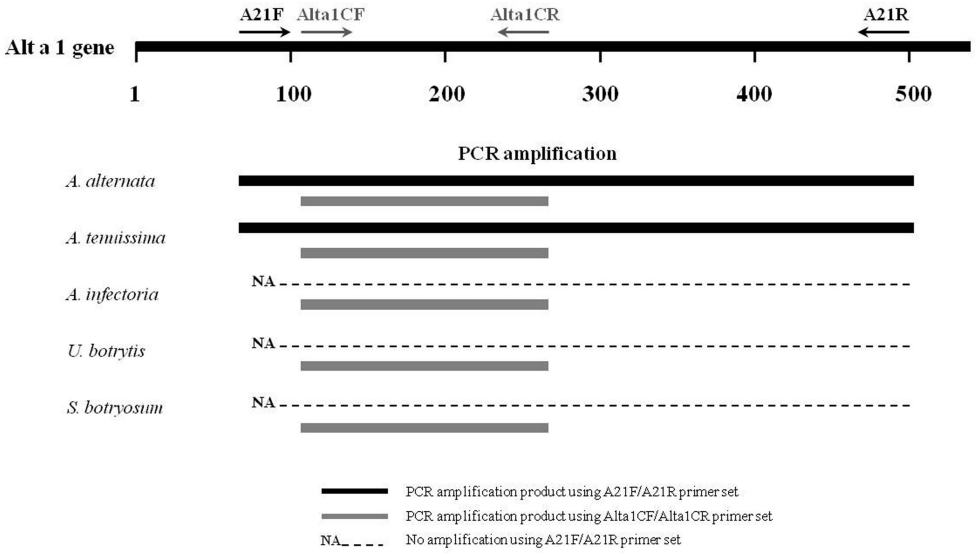

Figure 3. Position of the primers A21F, A21R, Alta1CF and Alta1CR in the Alt a 1 gene (534 pb) and scheme of amplicons obtained by PCR amplification using cDNA from *A. alternata*, *A. tenuissima*, *A. infectoria*, *U. botrytis* and *S. botryosum*.

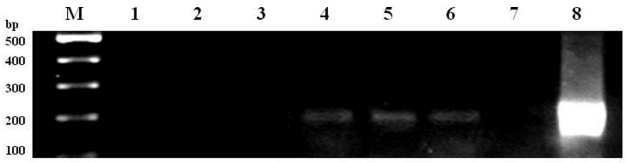

Figure 4. Electrophoretic analysis of Alt a 1 PCR products obtained using Alta1CF/Alta1CR primer set from 10-fold dilutions of an *A. alternata* spore suspension. Samples in lanes correspond to *Alternaria alternata* strain CBS 104.26 spore suspensions adjusted to a concentration of: 1 × 10^1 (1), 1 × 10^2 (2), 1 × 10^3 (3), 1 × 10^4 (4), 1 × 10^5 (5), and 1 × 10^6 (6) spores/ml; Negative control (7); positive control (8) and GeneRuler™ DNA Ladder Mix (M).

366 spore per PCR reaction. All of the amplified products were purified and sequenced. The sequencing results and BLAST analysis indicated that the fragments indeed matched the Alt a 1 DNA sequences.

Discussion

The foreign nature degree of the proteins that we encounter in the surrounding environment is highly dependent on their evolutionary distance from human proteins. Therefore, the human immune response can mirror the phylogenetic

relationships of allergenic sources. Particularly, among the allergologically relevant fungal species, the evolutionary process seems to be responsible for the observation that a limited number of species produce a specific allergen that is highly recognized by the human immune system. Additionally, a close relationship between molecular fungal systematic and fungal IgE sensitization was recently reported [20]. With regards to Alt a 1, the cross-reactivity phenomenon that is triggered by the presence of multiple closely related Pleosporaceae allergen homologues [17] is one explanation for the clinical observation that the majority of patients displaying *A. alternata* allergies are polysensitized to several fungal species. With this in mind, to correctly assess the respiratory allergenic disorders epidemiology that is caused by *Alternaria* and its related species, it will be important to detect all Alt a 1 homologues that are allergenic and potentially cross-reactive. Here, we took advantage of the phylogenetic information that was obtained from investigating the nucleotide conservation of the genes encoding the Alt a 1 homologues and developed a method for detecting the presence of Alt a 1 with a PCR-based system.

A multiple alignment analysis of the different fungal Alt a 1 nucleotide sequences allowed for the identification of a highly conserved sequence region. Primers with the potential to amplify all Alt a 1-encoding sequences were designed for this conserved region. These oligonucleotides were applied in the PCR experiments using cDNA as the amplification target. The use of cDNA instead of gDNA was considered more suitable because it is a better indicator of cell viability, which allows for the specific detection of viable fungal spore producers of Alt a 1-like proteins. Of the 13 fungal species included in the study, cDNA that was prepared from five species (*A. alternata, A. tenuissima, A. infectoria, U. botrytis,* and *S. botryosum*) generated a 180 bp amplicon in the PCR assay. These results suggest that genes encoding Alt a 1 (and its homologues) are restricted to specific groups of fungi including *Alternaria* and its related species. These observations are in accordance with previous studies [16]. These findings also confirmed that the studied Alt a 1 conserved "core" region is shared by all of the species in which an Alt a 1-like gene was described. The detection system sensitivity of the pure *A. alternata* spore samples was 366 spore RNA equivalents per PCR reaction, which corresponded to 1×10^4 spores/ml of sample.

A study using Alt a 1 synthetic peptides identified two linear epitopes (K41-P50 and Y54-K63) that were shown to strongly bind IgE from the sera of patients who were allergic to *A. alternata* [19]. Recently, Chruszcz *et al.* demonstrated that these epitopes were surface exposed in the Alt a 1 three-dimensional structure and, therefore, they were easily accessible for interactions with the antibodies [21]. Based on these reports and because the conserved region of the Alt a 1 gene that was amplified by the Alta1CF/Alta1CR primers includes the nucleotide sequences that encode these two epitopes, we suggest that the fragment detected by this system is not only a phylogenetic marker but also an Alt a 1 allergenicity marker. In the DNA amplification assays with the A21F/A21R primers, only a 390 bp PCR product was obtained from *A. alternata* and *A. tenuissima* cDNA. This observation may be explained by the high homology that is shared between the *A. alternata* and *A. tenuissima* Alt a 1 genes due to their close phylogenetic relationship. The A21F/A21R primers may be a suitable *alternata* species-group-specific primer pair for screening fungal species in environment monitoring. Further studies using air and/or dust samples are required to validate the applicability of the method proposed by this work. Nevertheless, the most common Alt a 1-producing species were included in this study, suggesting that this PCR system may be a valuable tool for Alt a 1 allergen detection. Moreover, because *Alternaria* species are important pathogens of a wide range of fruits and vegetables [6,7], and because Alt a 1 is a competitive inhibitor of plant defense proteins belonging to the PR5-TLP family, and it is most likely a fungal infection pathogenicity marker [22], this technique may also be a valuable tool for the identiffication of Alt a 1-producing fungal species and as a quality and biosecurity marker for food stuffs. Our findings are in line with some reports that have demonstrated the applicability of PCR-based techniques and sequencing assays that targeted the internal transcribed spacer (ITS) region of ribosomal RNA genes and some species-specific conserved genes for fungal species detection and identification [23–25].

In summary, because Alt a 1 sensitivity is a compelling epidemic asthma predictor, and although the environment usually contains much higher *A. alternata* spore levels than those of the other Pleosporaceae species that express an Alt a 1-like protein, the detection of the total Alt a 1 levels, independent of its fungal source, as proposed in this work, may help to clarify the relationship between the inhaled allergen and the patient's allergic response.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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Paper III

Characterisation of *Alternaria alternata* manganese-dependent superoxide dismutase, a cross-reactive allergen homologue to Asp f 6

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Characterisation of *Alternaria alternata* manganese-dependent superoxide dismutase, a cross-reactive allergen homologue to Asp f 6

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ABSTRACT

It is well known that *Alternaria alternata* presents a significant level of allergenic cross-reactivity with several other phylogenetically related and non-related allergenic moulds. To improve the molecular diagnosis, the identification and characterisation of all clinically relevant allergens, including both species-specific and cross-reacting proteins, is required. In this study we report the molecular and immunological characterisation of the *A. alternata* manganese-dependent superoxide dismutase (Alt a MnSOD) and its cross-reactivity with Asp f 6, a diagnostic marker allergen in allergic bronchopulmonary aspergillosis (ABPA). The cDNA coding for Alt a MnSOD sequence was isolated by RACE and PCR. Alt a MnSOD is a protein of 191 amino acids that presented significant homology and potential cross-reactive epitopes with Asp f 6. The recombinant protein was produced in *Escherichia coli* and the immunoreactivity was evaluated in patient sera. Immunoblotting analyses showed that seven of sixty-one *A. alternata*-sensitised patient sera and two ABPA patient sera reacted with the recombinant Alt a MnSOD. The native counterpart contained in both *A. alternata* and *Aspergillus fumigatus* extracts inhibited IgE binding to the recombinant molecule. The allergen was named Alt a 14 by the official Allergen nomenclature subcommittee. Thus, Alt a 14 is a relevant allergen in *A. alternata* sensitisation that may be used to improve diagnostic procedures. Evidence of cross-reactivity between Asp f 6 and Alt a 14-recognition by ABPA patient sera suggest the existence of an Alt a 14-mediated mechanism that, similar to Asp f 6, may be related to the pathogenesis of ABPA.

Introduction

Fungi are recognised as important sensitisers and triggers of respiratory allergies (Simon-Nobbe et al., 2008). Among the common airborne fungal species, *Alternaria alternata* is one of the principal species associated with allergic disease (Simon-Nobbe et al., 2008; Bush and Portnoy, 2001; Bousquet et al., 2009; Mari et al., 2003). Exposure and sensitisation to this mould have been increasingly recognised as risk factors for the development and persistence of asthma, asthma severity, and potentially fatal asthma exacerbations (Salo et al., 2006; Black et al., 2000; Kobayashi et al., 2009; Brito et al., 2012).

Among several allergens described in *Alternaria*, Alt a 1 has been demonstrated to be the most important, being recognised by approximately 80% of *A. alternata*-sensitised patients (Unger et al., 1999; Morin et al., 2012; Asturias et al., 2005). Other fungi from the Pleosporaceae family (*Stemphylium*, *Ulocladium*, *Nimbya* and *Embellisia*) have been described to possess Alt a 1 gene homologues (Hong et al., 2005). However, to date, evidence of allergenicity and cross-reactivity with Alt a 1 from *A. alternata* has only been reported for *Stemphylium botryosum* and *Ulocladium botrytis* Alt a 1 homologues (Gutierrez-Rodriguez et al., 2011). Although in addition to Alt a 1, 12 other proteins have been accepted by the International Union of Immunological Societies (IUIS) as official *A. Alternata* allergens (http://www.allergen.org), the clinical observations do not appear to be completely explained by the previously identified allergens. In fact, the *A. alternata* sensitisation data are

Abbreviations: ABPA, allergic bronchopulmonary aspergillosis; Alt a MnSOD, *A. alternata* manganese-dependent superoxide dismutase; sIgE, specific immunoglobulin E.

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0171-2985/

quite complex, showing a significant level of cross-reactivity and poly-sensitisation with several other phylogenetically related and non-related moulds (Crameri, 2011; Katotomichelakis et al., 2012; Postigo et al., 2011).

Recently, Postigo et al. (2011) identified a new 24 kDa IgE-reactive protein from *A. alternata*, included in the manganese-dependent superoxide dismutase (MnSOD) protein family. Other fungal MnSODs have also been identified as important allergens in *Aspergillus fumigatus* (Asp f 6) and *Malassezia sympodialis* (Mala s 11) (Lambou et al., 2010; Andersson et al., 2004). In particular, Asp f 6 has been widely described as an allergen that is strongly associated with allergic bronchopulmonary aspergillosis (ABPA) and frequently used as a tool to distinguish ABPA from fungal sensitisation (Fricker-Hidalgo et al., 2010; Schwienbacher et al., 2005).

In the present study, we report the cloning, expression, immunologic characterisation of the *A. alternata* MnSOD and its cross-reactivity with Asp f 6.

Materials and methods

Strains and fungal extracts

A. alternata strain CBS 104.26 (Centraalbureau von Schimmelcultures, Utrech, the Netherlands) and *A. fumigatus* strain AF54 (Municipal Institute for Medical Research, Barcelona, Spain) were used. Fungal extracts were prepared as previously described by Martinez et al. (2006).

Human sera

Sera from sixty-one individuals sensitised to *A. alternata* were selected according to a positive skin prick test (ALK-Abelló *A. alternata* extract, >3 mm wheal diameter) and positive *A. alternata*-specific IgE (sIgE) levels (>0.35 kU/L).sIgE to rAlt a 1 and rAsp f 6 was quantitatively determined using the ImmunoCAP™ System (Phadia AB, Uppsala, Sweden). Additionally, one *Dermatophagoides pteronyssinus*-sensitised patient serum sample that was negative for *A. alternata*- and *A. fumigatus*-specific IgE was used as a negative control. Two sera from patients with ABPA were also used. The diagnostic features for both ABPA subjects were intradermal test reactivity to *A. fumigatus* extract, an *A. fumigatus* sIgE level higher than 10 kU/L, positive precipitins to *A. fumigatus* extract, central bronchiectasis, positive sputum culture to *A. fumigatus* and positive Asp f 6 sIgE (>3 kU/L). All sera were acquired from our sera collection (C.0002774, registered in the Institute of Health Carlos III, Ministry of Economy and Competitiveness, Government of Spain). Written informed consent to participate in the study was obtained from all individuals. The study was approved by the local ethics committee and was conducted in accordance with the Declaration of Helsinki.

cDNA cloning and sequencing

Total RNA was extracted from the fungal mat of 5-day-old *A. alternata* using TRIzol (Invitrogen, Carlsbad, CA), and reverse transcription was performed using a RevertAid First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturers' instructions. Degenerated primers iSODF (5′-GTCKCCGTGCAGCARGCSATCAAG-3′) and iSODR (5′-CTCCCACATRTCSAYRCCAAAGAC-3′) were designed based on the highly conserved nucleotides of the several available fungal MnSOD sequences in GenBank and used to amplify an internal fragment of the Alt a MnSOD. The PCR product was purified, subcloned and sequenced. Then, full-length cDNA was obtained using Rapid Amplification of cDNA ends (RACE) as previously described by Tripathi et al. (2011). Products of the RACE reactions were purified, subcloned and sequenced. New primers, flSODF (5′-ATCATGGAGCTGCATCACAGCAAG-3′) and flSODR (5′-TTGAGAGTGAATGCGTGTCTTTG-3′), were designed for the full-length sequence, and cDNA was amplified, cloned and sequenced.

Computational analysis and homology search

The Alt a MnSOD gene and deduced protein sequences were analysed and compared with those in the GenBank database and the Structural Database of Allergenic Proteins (SDAP). Sequence alignments were performed using the ClustalW Analysis Programme (http://searchlauncher.bcm.tmc.edu).

Theoretical protein parameters were calculated with the ProtParam programme (http://web.expasy.org/protparam/). The sequences were also analysed by Gene Runner software (http://www.generunner.net/). The three-dimensional structure of the MnSOD from *A. fumigatus* (Asp f 6, Protein Database Bank (PDB) code 1KKC) was used as a template to build the homology model of the Alt a MnSOD. Monomeric and tetrameric molecular models were generated using the programme ESyPred3D (http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred/) (Lambert et al., 2002). Visualisation of the models was performed with the programme Swiss-Pdb Viewer (http://spdbv.vital-it.ch).

Expression of the recombinant protein

The open reading frame encoding Alt a MnSOD was amplified using gene-specific primers, i.e., a forward primer with an Nco I (underlined) restriction site (5′-CCATGGATGAGCTGCATCACAGCA-3′) and a 3′ reverse primer introducing a Hind III (underlined) restriction site (5′-AAGCTTGATGGAAGCCTTGAGCTC-3′). Then, it was cloned into the plasmid expression vector pBAD-TOPO (Invitrogen) and transformed into One Shot® TOP10 Chemically Competent *Escherichia coli* cells (Invitrogen). The cells were grown at 37 °C in Luria–Bertani broth medium containing 100 μg/ml ampicillin to an $OD_{600\,nm}$ of 0.5 and induced by treatment with 0.02% (w/v) L-arabinose for 4 h. Then, induced cells were pelleted at 10,000 × *g* for 10 min and lysed with B-PER bacterial protein extraction reagent (Thermo Scientific, Rockford, IL, USA). After inclusion bodies were solubilised using an inclusion body solubilisation reagent, recombinant protein was purified by affinity chromatography using His Pur™ Ni-NTA (Ni^{2+}-nitrilotriacetate) resin (Thermo Scientific). Eluted protein was refolded by dialysis against decreasing concentrations of urea and, finally, against Tris buffer (25 mM Tris–HCl, 150 mM NaCl, pH 7.5).

SDS-PAGE and immunoblotting

Proteins were separated by 12% SDS-PAGE gels. For immunoblotting, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA), as described by Towbin et al. (1979). Briefly, the membrane was cut into strips and incubated overnight with patient sera at 4 °C. Then, bound IgE was detected by incubation with mouse anti-human IgE horseradish peroxidase (HRP) conjugate (Southern Biotech, Birmingham, AL, USA). The blots were developed by the addition of chemiluminescent reagents (ECL+, Amersham Biosciences, Bucks, UK). To detect the $[His]_6$-fusion protein, western blotting using an anti-His-HRP antibody (Invitrogen) was also performed.

For the immunoblotting inhibition assays, a serum sample from a patient sensitised to *A. alternata* with positive immunoblotting to rAlt a MnSOD was preincubated at 4 °C overnight with *A. alternata* and *A. fumigatus* extracts (5 mg protein/mL) or PBS. Then, the inhibited sample was tested for binding to rAlt a MnSOD blotted onto a PVDF membrane.

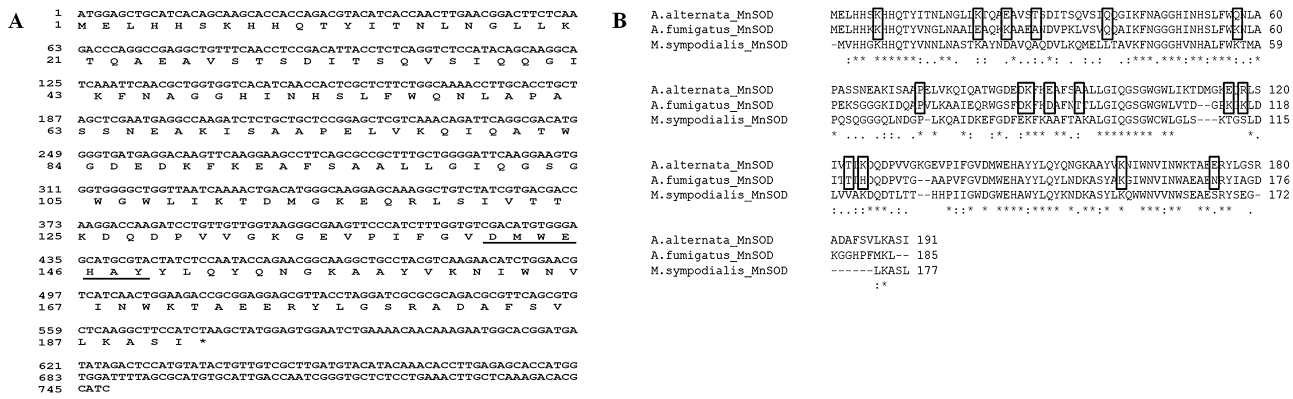

Fig. 1. (A) The nucleotide and deduced aminoacid sequences of Alt a MnSOD (GenBank accession No. KC923297). The stop codon TAA is shown (*). The manganese and iron superoxide dismutase signature is underlined. (B) Sequence alignment of Alt a MnSOD protein with fungal MnSOD allergens, Asp f 6 from *Aspergillus fumigatus* and Mala s 11 from *Malassezia sympodialis*. Identical aminoacids exposed to the solvent (FSASA > 0.3) in both *A. fumigatus* and Alt a MnSOD structures are boxed.

Mass spectrometry

The 26 kDa band, revealed by western blotting, was identified by MALDI-TOF/TOF. The MALDI result was obtained in the form of a MASCOT search chart.

Specific IgE ELISA inhibition

The serum of an *A. alternata*-positive patient (25 μL) was incubated with different concentrations (0.2–2000 ng) of purified recombinant protein and *A. alternata* extract separately, overnight at 4 °C. Serum from a *D. pteronyssinus*-sensitised patient was used as a control. IgE quantification was performed as described by Tawde et al. (2006). Briefly, microtiter plates were pre-coated with 50 ng of the purified rAlt a MnSOD diluted in 50 mM pH 9.6 carbonate-bicarbonate buffer. The binding of IgE was detected by mouse anti-human IgE-HRP, and the reaction was developed with Sigma-Fast OPD (o-phenylenediamine dihydrochloride). The OD was measured in a LabSystems Multiskan Bichromatic plate reader (LabSystems, Helsinki, Finland) at 495 nm.

Results

Cloning of cDNA coding for A. alternata *MnSOD*

Full-length Alt a MnSOD cDNA clones were identified using RACE-PCR, standard cloning techniques and nucleotide sequencing. The complete cDNA sequence of 748 bp was submitted to the GenBank database under accession number KC923297. Alt a MnSOD-encoding cDNA consists of 576 bp, which corresponds to a deduced amino acid sequence of 191 residues (Fig. 1A). A conserved motif, DMWEHAY (142–148 residues), was detected in the sequence of the deduced protein. A motif search using Gene Runner software identified this sequence as a manganese and iron superoxide dismutase signature. Its theoretical molecular weight was 21.3 kDa, with an isoelectric point of 6.6.

Bioinformatic analysis of the deduced protein sequence

Comparisons of the Alt a MnSOD cDNA sequence with the NCBI database showed homology with manganese-dependent superoxide dismutases from various sources at both the DNA and protein levels. At the protein level, it was similar to the MnSODs from *Pyrenophora tritici-repentis* (94%) XP_001937231.1, *Leptosphaeria maculans* (90%) XP_003842884.1, and *Curvularia lunata* (89%) AAQ87929.1. Further, it also showed significant homology with two fungal allergens in the SDAP database, namely Asp f 6 from *A. fumigatus* (59%, e-score: 7e-80) AAB60779 and Mala s 11 from *M. sympodialis* (48%, e-score: 2e-57) CAD68071, and two non-fungal allergens, Hev b 10 from *Hevea brasiliensis* (45%, e-score: 8e-34) CAB53458 and Pis v 4 from *Pistacia vera* (45%, e-score: 4e-33) EF470980.

Molecular modelling of Alt a MnSOD and identification of putative cross-reactive IgE-binding epitopes

Sequence alignment revealed that Alt a MnSOD shares significant sequence homology with MnSODs from *A. fumigatus* and *M. sympodialis*, two important fungal allergens (Fig. 1B). In all known MnSOD structures, the metal ion is bound by four invariant protein ligands: one aspartate and three histidines. In fact, the conserved amino acids include the three histidines at positions 4, 52 and 146 and the aspartate at position 142 in the Alt a MnSOD sequence, which are involved in the binding of Mn^{2+} metal.

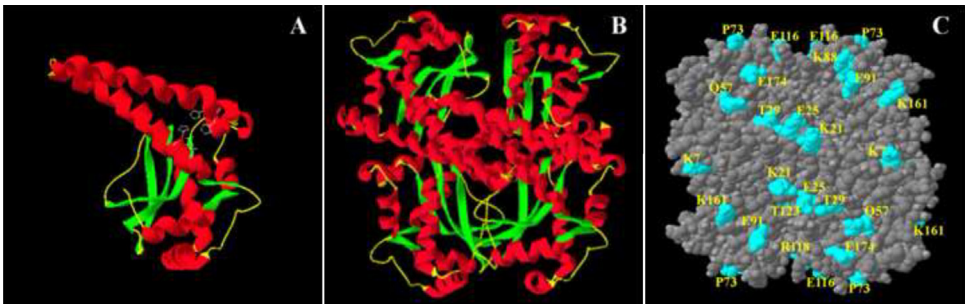

Fig. 2. Structural models. (A) Ribbon representation of the Alt a MnSOD monomer. α-helices are shown in red, β sheets in green and turns in yellow. (B) Ribbon diagram of the Alt a MnSOD tetramer. (C) Alt a MnSOD molecular surface. Identical aminoacids that are solvent-exposed (FSASA > 0.3) in both Asp f 6 and Alt a MnSOD models are shown in blue.

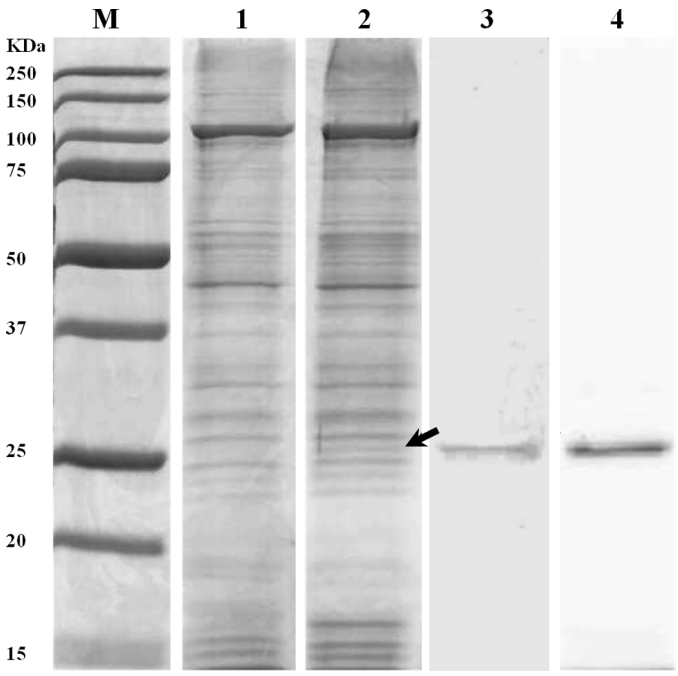

Fig. 3. Expression of Alt a MnSOD. Lane M – molecular mass marker, Lane 1 – non-induced *E. coli* inclusion bodies fraction, Lane 2 – L-arabinose-induced *E. coli* inclusion bodies fraction. Arrow indicates position of recombinant Alt a MnSOD band identified by MALDI, Lane 3 – rAlt a MnSOD purified by Ni-NTA resin, Lane 4 – anti-His Western Blotting.

A three-dimensional structural model of Alt a MnSOD was generated using the previously solved crystal structure of Asp f 6 (Fluckiger et al., 2002). Model data are available in the PMDB database under the accession number PM0079554. The monomer exhibits an α/β fold (Fig. 2A) in accordance with the 3D structure of Asp f 6. Based on the oligomeric state of the template, the tetrameric state of the target molecule was created (Fig. 2B).

To determine which of the residues shared by Asp f 6 and Alt a MnSOD could be critical for cross-reactivity, conserved residues that are exposed to the solvent on the surface of the molecular models were identified. It was observed that although Asp f 6 and Alt a MnSOD share 59% of the primary amino acid sequence, only 17 of the pairwise conserved residues, shown in boxes in Fig. 1B, were at least 30% exposed to the solvent in both models. As shown in Fig. 2C, these critical residues are scattered over the entire surface of the Alt a MnSOD molecule.

Expression of recombinant A. alternata *MnSOD in* E. coli

The coding region of the isolated cDNA was fused with a short DNA sequence encoding an extra peptide containing a V5 epitope and a $[His]_6$-tag at the C-terminus of the protein. Alt a MnSOD recombinant protein was expressed in *E. coli* TOP 10, and it was detected, recovered and purified from the inclusion body fraction. It presented an apparent molecular mass of approximately 26 kDa, as estimated by SDS-PAGE and western blotting with an anti-histidine antibody (Fig. 3). The MALDI results showed that MnSODs from *Pyrenophora teres f. teres* and *P. tritici-repentis* are the closest homologues of the recombinant protein produced in this study.

Human IgE recognition of the recombinant protein

The levels of sIgE to Alt a 1 and Asp f 6 in the 64 study serum samples measured by ImmunoCAP are shown in Table 1. Among the 61 serum samples from *A. alternata*-sensitised patients (patients no. 1–61), 54 had Alt a 1 sIgE, and only 6 presented detectable rAsp f 6 sIgE.

Investigation of the IgE reactivity of rAlt a MnSOD by immunoblotting revealed that 7 out of 61 *A. alternata*-sensitised patient sera reacted with the recombinant molecule (Fig. 4). Thus,

Table 1
Results of allergen-specific IgE detection in serum samples from *A. alternata*-sensitised patients (1–61), a subject mono-sensitised to *D. pteronyssinus* (62) and patients with diagnosed ABPA (63–64).

Serum I.d.	ImmunoCap sIgE (kU/L)		Immunoblotting sIgE
	Asp f 6	Alt a 1	Alt a 14
1	0	>100	–
2	0	>100	–
3	0.55	78.2	+
4	0	0	–
5	0	6.39	–
6	0	15.2	–
7	0	11.1	–
8	0	44.2	–
9	0	64.3	–
10	0	4.29	–
11	0	66.4	–
12	0	0	–
13	0	19	–
14	0	4.32	–
15	0	7.24	–
16	0	3.42	–
17	0	3.85	–
18	0	0	–
19	0	10.1	–
20	0	13.6	–
21	0.44	33.7	+
22	0	9.21	–
23	0.46	36	+
24	0	3.84	–
25	0	1.97	+
26	0.37	1.58	+
27	0	0	–
28	0	18.5	–
29	0	1.58	–
30	0	20.1	–
31	0	8.75	–
32	0	>100	–
33	0	34.5	–
34	0	3.03	–
35	0	9.68	–
36	0	15.7	–
37	0	19	–
38	0	36.5	–
39	0	0	–
40	0	24.2	–
41	0	17	–
42	0	1.55	–
43	0	96	–
44	0	13.1	–
45	0	0	–
46	0	0	–
47	0	10.4	–
48	0	7.37	–
49	0	73.4	–
50	0	30	–
51	0	>100	–
52	0	13.7	–
53	0	0	–
54	0	11.3	–
55	0	3.62	–
56	0	4.66	–
57	0	97.1	–
58	0	35	–
59	12.8	0.76	+
60	1.04	0.59	+
61	0	7.89	–
62	0	0	–
63	5.11	0	+
64	3.20	0	+

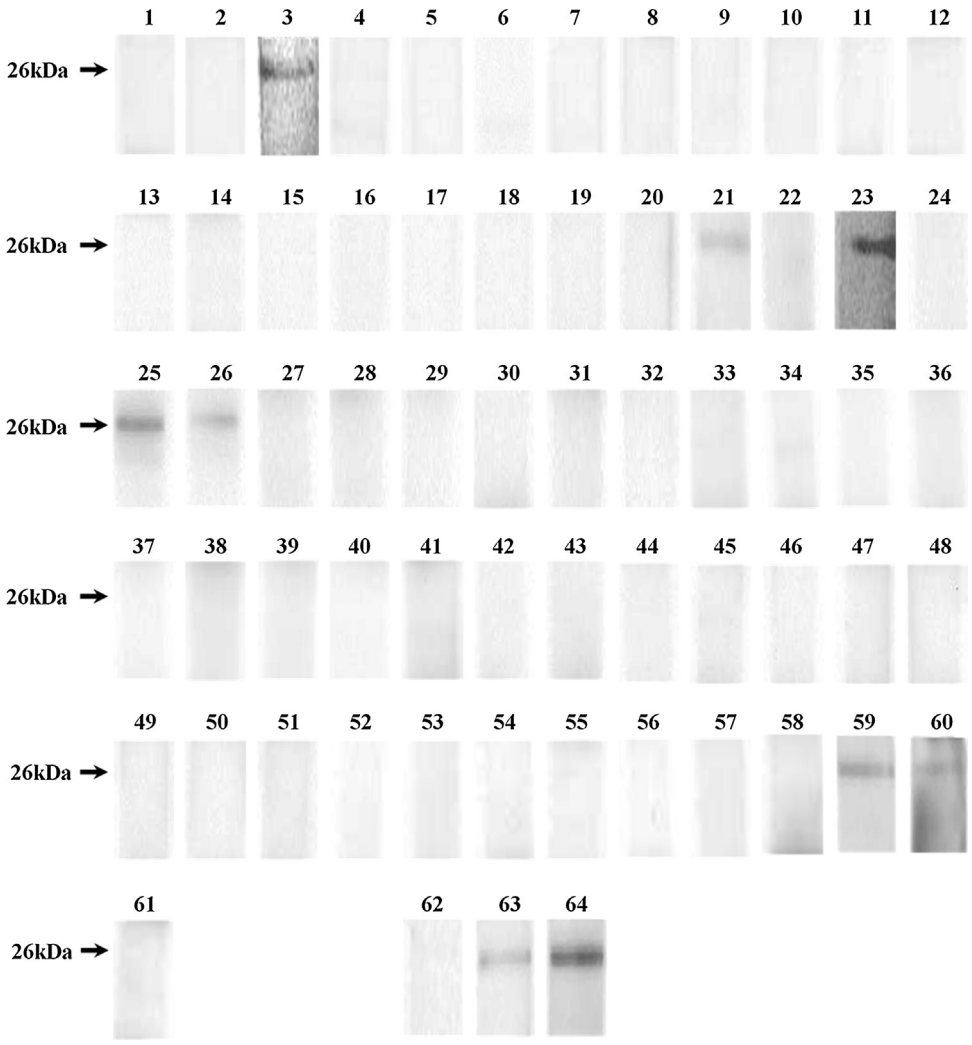

Fig. 4. IgE-immunoblot of rAlt a MnSOD using 64 selected serum samples. Blotted rAlt a MnSOD probed with: Lanes 1–61 – individual *A. alternata*-sensitised patientsí sera, Lane 62 – serum from a subject sensitised to *D. pteronyssinus*, Lanes 63–64 – probed with sera from patients with ABPA.

all sera from *A. alternata*-sensitised patients containing sIgE to rAsp f 6 (Table 1) labelled the rAlt a MnSOD protein (Fig. 4, patients/lanes 3, 21, 23, 26, 59, and 60). Additionally, rAlt a MnSOD was able to bind IgE from ABPA individuals (patients 63 and 64) who presented rAspf 6 sIgE.

Immunoblotting inhibition was assayed using serum from patient no. 59. The inhibition of sera with *A. alternata* and *A. fumigatus* extracts resulted in an evident inhibition of the protein band at 26 kDa (Fig. 5A). Furthermore, the ELISA results (Fig. 5B) showed that IgE binding of patient number 59s serum to Alt a MnSOD could be inhibited in a dose-dependent manner by preincubation with increasing amounts of the purified rAlt a MnSOD or the *A. alternata* mycelial extract.

Alt a MnSOD was submitted to the International Union of Immunological Societies Allergen Nomenclature Sub-Committee (http://www.allergen.org/Allergen.aspx) and was officially named Alt a 14.

Discussion

Apparent sensitisation to multiple fungi, caused by co-sensitisation and/or cross-reactivity, represents a frequent clinical observation in patients with mould allergy that makes an accurate diagnosis of mould allergy difficult (Crameri et al., 2009). In recent years, component-resolved diagnosis has been presented as a valuable tool to discriminate between cases of co-sensitisation and/or cross-reactivity (Mothes et al., 2006; Vieira et al., 2012). To improve the molecular diagnosis, the identification and characterisation of all clinically relevant allergens, including both species-specific and cross-reacting proteins, is required. With this in mind, in the present work, we aimed to characterise the recently identified *A. alternata* Asp f 6 homologue (Alt a 14) and to demonstrate that it is an *A. alternata* clinically relevant allergen showing cross-reactivity with Asp f 6.

The strategy used in this work takes advantage of the similarity of the different fungal MnSOD nucleotide sequences submitted to the GenBank database, which allowed the design of degenerate oligonucleotides and the cloning of Alt a 14-encoding cDNA by RACE experiments. A sequence similarity search revealed that Alt a 14 presents high homology with MnSODs from other members of Pleosporales (*P. tritici-repentis* (94%), *L. maculans* (90%), and *C. lunata* (89%)). The percent identity decreased when Alt a 14 and homologous molecules from fungi belonging to other orders were compared. Significant homology with the allergens contained in the official list, Asp f 6 (59%), Mala s 11 (48%), Hev b 10 (45%) and Pis v 4 (45%), was also detected. Additionally, comparison between the multiple sequence alignments and the surface accessibility of conserved amino acids in the 3D models of Alt a 14 and Asp f 6 indicated that although the majority of the residues are identical, only seventeen are at least 30% solvent exposed in both

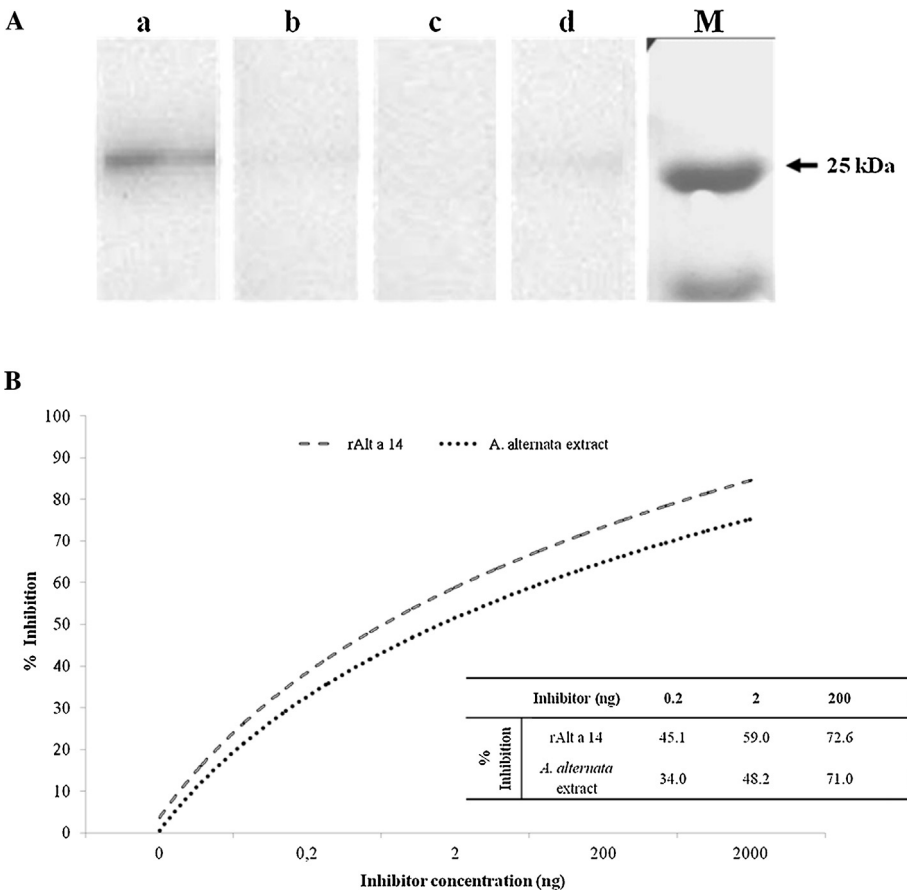

Fig. 5. (A) Inhibition of the IgE-immunoblot reactivity of a serum sample that has shown positive reactivity to rAlt a 14. Strips represent results of immunoblot obtained by incubating the PVDF blots with the same serum sample that has been mixed and pre-incubated with: (a) PBS, (b) *A. alternata* metabolic extract, (c) *A. alternata* cellular extract and (d) *A. fumigatus* cellular extract. (B) Inhibition of IgE-binding to recombinant Alt a 14 coated on the solid phase. Serum from an Alt a 14-sensitised patient was preincubated with increasing amounts of recombinant Alt a 14 and *A. alternata* extract.

structures and therefore likely to be accessible for interactions with cross-reactive antibodies. These residues appear scattered over the entire surface of the Alt a 14 molecule, suggesting that the entire surface is potentially cross-reactive with Asp f 6. The exposed amino acids, either alone or together with surrounding residues, are likely to define cross-reactive conformational B cell epitopes shared between Alt a 14 and Asp f 6 and possibly among MnSODs from other species. In this regard, based on the solvent-exposed amino acid distribution profile in the 3D structure, two regions including the residues K21, E25, T29 and Q57 and the residues P73, K88, E91 and K161 could be defined as potential cross-reactive areas. Both areas are present in at least two different sections of the tetramer surface, satisfying the requirement needed to induce the cross-linking of allergen-specific IgE bound to the surface of effector cells.

The recombinant production of Alt a 14 was performed in *E. coli* and revealed an apparent molecular mass of 26 kDa. Assessment of the allergenic significance of Alt a 14 in *A. alternata*-sensitised individuals by immunoblotting showed that 11.5% had sIgE to the 26 kDa band, suggesting that Alt a 14 is an *A. alternata* minor allergen. The results of immunoblotting and ELISA inhibition showed that native Alt a 14 contained in the *A. alternata* extracts strongly inhibited the reaction between rAlt a 14 and the sIgE of an Alt a 14-sensitised patient's serum, suggesting that the allergenic ability of the native Alt a 14 is also present in the recombinant polypeptide.

Data obtained from the investigation of sIgE to single fungal allergens, including Alt a 1, Alt a 14 and Asp f 6, revealed that among the *A. alternata*-sensitised individuals ($n = 61$), 86.9% were sensitised to Alt a 1, 11.5% to Alt a 14 and 9.8% to Asp f 6. Thus, only one individual who was sensitised to Alt a 14 was not sensitised to Asp f 6 (Table 1). To explore the potential cross-reactivity between Alt a 14 and the recognised clinically relevant *A. fumigatus* allergen Asp f 6, two sera from patients with diagnosed ABPA and detectable Asp f 6-sIgE were tested by immunoblotting. This assay demonstrated that both sera recognised rAlt a 14. This finding, together with the results obtained from immunoblotting-inhibition assays using *A. fumigatus* extract suggest that both allergens possess allergen-cross-reactive IgE epitopes.

The evidence of cross-reactivity between Alt a 14 and Asp f 6, demonstrated for the first time in this study, raises some questions because of the recognised importance of Asp f 6 in allergic diseases to moulds. In fact, Asp f 6 is regarded, together with Asp f 4, as a specific marker for ABPA (Fricker-Hidalgo et al., 2010; Hemmann et al., 1999). Currently, ABPA is associated with an array of immune responses to *A. fumigatus* and has been reported to occur primarily in asthmatic individuals and in patients suffering from cystic fibrosis (Knutsen and Slavin, 2011; Baxter et al., 2013). In this regard, and based on the recognised fact that most *A. alternata*-sensitised patients are asthmatic and the recent revelation that *A. alternata* contains an allergen that is homologous and cross-reactive to Asp f 6, it will be important to determine whether Alt a 14-sensitised individuals are at risk of ABPA. Interestingly, this is in line with the findings of Jubin et al. (2010), who demonstrated that ABPA was significantly associated with a prior or concomitant sensitisation to *Alternaria* species and defined *Alternaria* sensitisation as a

risk factor for ABPA. The existence of cross-reactive proteins from *Alternaria* and *Aspergillus* species, such as the MnSOD described in this study, could explain a similar mechanism of sensitisation that may contribute to the pathogenesis of ABPA. Considering the results reported by Agarwal et al. (2012, 2013) the possibility that the individuals who presented positive levels of anti-Asp f 6 and/or Alt a 14 IgE, are in an asymptomatic phase of ABPA with controlled asthma, stage 0 of ABPA, must not be excluded. For this reason, they should be evaluated for ABPA to aid in the early diagnosis of the disorder before the onset of bronchiectasis. Furthermore, in the clinical practice, it is not unusual to find patients who present negative skin-pick test results to an *A. fumigatus* extract but a positive Asp f 6-specific IgE serum levels. The sensitisation to the Asp f 6-like from *A. alternata*, Alt a 14, may explain this observation.

In conclusion, this study characterised Alt a 14 as a minor allergen of *A. alternata* that can trigger a cross-reactive IgE response to Asp f 6. The data disclosed by this study suggest that the Asp f 6-like allergen found in *A. alternata* may be related to ABPA and should greatly contribute to improving the accuracy of the diagnosis and, subsequently, the understanding and management of IgE-mediated fungal diseases.

Databases

GenBank: nucleotide sequence data are available in the GenBank database under the accession number KC923297.

PMDB: model data are available in the PMDB database under the accession number PM0079554.

Allergen database: this new allergen has been submitted, accepted and assigned with an official name by the International World Health Organisation and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee (http://www.allergen.org).

Conflict of interest

The authors declared no conflict of interest.

Acknowledgements

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Paper IV

Alt a 15 is a new cross-reactive minor allergen of *Alternaria alternata*

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ABSTRACT

Alternaria alternata is one of the most common saprophytes worldwide that is clinically and epidemiologically associated with severe asthma. Therefore, the identification and characterization of all *A. alternata* allergens are of major clinical importance. This study describes a new cross-reactive *A. alternata* allergen that was officially named Alt a 15 by the official Allergen Nomenclature Subcommittee. The complete coding region for Alt a 15 was amplified using 5′ and 3′ rapid amplification of cDNA ends and PCR. The recombinant protein was produced in *Escherichia coli* as a 65-kDa fusion protein, and the protein sequence exhibits high homology with several important fungal allergens. Immunoblotting analyses revealed that IgE antibodies from *A. alternata*-sensitized patients ($n = 59$) bound to rAlt a 15 with a prevalence of 10.2%. All patients who presented sIgE to rAlt a 15 were apparently poly-sensitized to *A. alternata* and *C. lunata*. The extensive cross-reactivity between *A. alternata* and *C. lunata* serine proteases was confirmed using immunoblotting inhibition assays. Overall, Alt a 15 is an important new cross-reactive allergen of *A. alternata* that explains some allergies to *A. alternata* without Alt a 1 sensitization and initial diagnostic errors for allergies to *Alternaria*. This molecule may improve the accuracy of the diagnosis, the understanding, and the management of IgE-mediated fungal diseases.

1. Introduction

Exposure to ubiquitously present fungal spores, primarily from *Alternaria*, *Cladosporium* and *Aspergillus* species, may significantly impact the human immune system, which makes fungal allergy a well-known worldwide problem (Simon-Nobbe et al., 2008; Pant et al., 2009). Several reports found links between sensitization to common airborne fungi and the risk for the development of severe IgE-mediated respiratory symptoms as well as between spore prevalence and asthma (Denning et al., 2006; Delfino et al., 1997). Although a predominant role for fungi has been demonstrated in airway disease, fungal allergens are largely neglected in molecular allergology.

Compelling evidence shows that apparent sensitization to multiple fungi is a frequent clinical observation in patients with mold allergy, which may be a consequence of the cross-reactivity between fungal proteins (Gupta et al., 2002; Crameri et al., 2009). Serine proteases (SPs), particularly subtilisin-like SPs, belong to one of the most important families of allergenic proteins (Radauer et al., 2008). Many fungal SPs exhibit significant cross-reactivity, likely because of conserved protein sequences and shared IgE-binding epitopes, which prompted the designation of SPs as a cross-reactive allergen group of prevalent airborne fungal species (Shen et al., 2007; Yike, 2011), including major allergens in the *Aspergillus*, *Cladosporium*, *Curvularia*, *Penicillium*, *Rhodotorula* and *Trichophyton* species (Pöll et al., 2009). In addition to the phenomenon of cross-reactivity, several lines of evidence suggest that proteases appear to play an important role in the mechanisms underlying allergenicity and to be essential in the eliciting of Th2 responses, which exert a greater impact on the pathogenesis of respiratory allergies (Yike et al., 2011; Wills-Karp et al., 2010 Wills-Karp et al., 2010).

Alternaria alternata is one of the most common environmental fungal species that has been clinically and epidemiologically

Abbreviations: CCD, cross-reactive carbohydrate determinants; RACE, rapid amplification of cDNA ends; SP, serine protease; sIgE, specific IgE; SDAP, structural database of allergenic proteins; PVDF, polyvinylidene difluoride.

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associated with severe asthma and life-threatening acute exacerbations of asthma (Neukirch et al., 1999; Zureik et al., 2002; Black et al., 2000). Alt a 1 is the most important specific allergen of the *A. alternata* allergens and is used to identify genuine IgE-mediated allergy to *Alternaria* (Unger et al., 1999; Asturias et al., 2005). In addition to Alt a 1, however, ten other *A. alternata* allergens have been identified and characterized thus far. The biological role of *Alternaria* allergens in the development of allergy and asthma is poorly understood. Therefore, a more comprehensive definition of the *A. alternata* allergen repertoire appears crucial for the accurate understanding, diagnosis and management of allergic disease.

Several studies recently demonstrated that intrinsic SP-specific activity of *A. alternata* extracts plays an important role in the physiopathogenesis of asthma via the elicitation of an increase in the permeability of bronchial epithelial cells (Leino et al., 2013) and the promotion of a rapid and robust release of early innate mediators and prolonged Th2 inflammation (Kouzaki et al., 2009; Boitano et al., 2011; Snelgrove et al., 2014). The above-mentioned data support the need to further investigate the allergenic properties of *A. alternata* SPs.

The present study describes a new cross-reactive minor allergen of *A. alternata*, which is included in the vacuolar serine protease protein family. The IUIS allergen nomenclature subcommittee approved this allergen, which is officially named Alt a 15.

2. Methods

2.1. Strains and fungal extracts

The *A. alternata* strain CBS 104.26 (Centraalbureau von Schimmelcultures, Utrecht, the Netherlands) and *Curvularia lunata* strain FMR5790 (Laboratory of Mycology, Rovira Virgili University, Tarragona, Spain) were used. Cellular extracts were prepared as previously described by Sáenz-de-Santamaría et al. (2006).

2.2. Human Sera

Sera from fifty-nine respiratory allergic adults sensitized to *A. alternata* were selected from our serum collection (C.0002774, registered in the Institute of Health Carlos III, Ministry of Economy and Competitiveness, Government of Spain) based on a positive skin prick test (ALK-Abelló *A. alternata* extract, >3 mm wheal diameter), positive *A. alternata*-specific IgE (sIgE) levels (>0.35 kU/L) and data availability of skin prick test reactivity to a panel of other fungal extracts. The results of skin prick tests with mold extracts from the database of samples from the serum collection included in this study revealed that 67.8% of the patients were sensitized to *Curvularia*, 54.2% were sensitized to *Aspergillus*, and 66.1% were sensitized to *Cladosporium*. Selected sera origins were the Medical Mycology Laboratory from Municipal Institute for Research, Barcelona, Spain (54 sera) and the Allergy department of University General Hospital of Alicante, Alicante, Spain (6 sera).

sIgEs to rAlt a 1, *C. lunata* extract, rAlt a 6 and cross-reactive carbohydrate determinants (CCDs) were quantitatively determined using the ImmunoCAP™ System (Phadia AB, Uppsala, Sweden). One *Dermatophagoides pteronyssinus*-sensitized patient serum sample that was negative for fungal extracts was also included as a negative control.

2.3. cDNA cloning and sequencing

Total RNA was extracted from the fungal mat of 5-day-old *A. alternata* using Trizol (Invitrogen, Carlsbad, CA, USA), and reverse transcription was performed using a RevertAid First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturers' instructions. The following degenerate primers were designed based on the highly conserved nucleotides of the several available fungal SP sequences in GenBank and used to amplify an internal fragment of the Alt a 15 cDNA using PCR: iSPF (5′-GCGAATTCATGGTYGTYAAGGGTGTCGAGT-3′) and iSPR (5′-GCCTCGAGCAGAGTCCTTRGCRGGCTGG-3′). The PCR products were purified, subcloned into pJET1.2 (Fermentas) and transformed into *E. coli* DH5 alpha competent cells (Invitrogen). Plasmid DNA was purified, and the nucleotide sequence of the insert was determined. Full-length cDNA was obtained using 5′- and 3′-end Rapid Amplification of cDNA ends (RACE), as previously described by Tripathi et al. (2011). Products of the RACE reactions were purified, subcloned and sequenced. New primers, flSPF (5′-CCTCAGCGCCAGATGGATTCG-3′) and flSPR (5′-TTAGGCGGGAGGAAAGGTTAA-3′), were designed for the full-length sequence, and cDNA was amplified, cloned and sequenced.

2.4. Computational analyses and homology search

The Alt a 15 cDNA and deduced protein sequences were analyzed and compared with sequences in the GenBank database and the Structural Database of Allergenic Proteins (SDAP). Sequence alignments were performed using the ClustalW Analysis Program (http://searchlauncher.bcm.tmc.edu).

Theoretical protein parameters were calculated using the ProtParam program (http://web.expasy.org/protparam/). The presence of putative signal peptides and propeptide cleavage sites was predicted using the SignalP program (http://www.cbs.dtu.dk/services/SignalP/) and ProP 1.0 Server (http://www.cbs.dtu.dk/services/ProP/). The sequences were also analyzed by Gene Runner software (http://www.generunner.net/).

2.5. Expression of the recombinant protein

The open reading frame encoding Alt a 15 was amplified using gene-specific primers: a forward (5′-ATGGATACGGCCAAGGAGGTGCC-3′) and a 3′ reverse primer (5′-AAGCTTCTGGCTAGCGACGCG-3′). The open reading frame was cloned into the plasmid expression vector pBAD-TOPO (Invitrogen) and transformed into One Shot® TOP10Chemically Competent *E. coli* cells (Invitrogen). The cells were grown at 37 °C in Luria–Bertani broth medium containing 100 μg/ml ampicillin to an OD_{600nm} of 0.5 and induced by treatment with 0.02% (w/v) L-arabinose for 4 h. Induced cells were pelleted at 10 000 × *g* for 10 min and lysed using B-PER bacterial protein extraction reagent (Thermo Scientific, Rockford, IL, USA). Inclusion bodies were solubilized using an inclusion body solubilization reagent, and recombinant protein was purified by affinity chromatography using HisPur™ Ni-NTA (Ni^{2+}-nitrilotriacetate) resin (Thermo Scientific). Eluted proteins were refolded using dialysis against decreasing concentrations of urea and Tris buffer (25 mM Tris–HCl, 150 mM NaCl, pH 7.5).

2.6. SDS-PAGE and immunoblotting

Proteins were separated using 12% SDS-PAGE according to Laemmli (1970). Proteins for immunoblotting were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA), as described by Towbin et al. (1979). Briefly, the membrane was cut into strips and incubated overnight with patient sera at 4 °C. Bound IgE was detected via incubation with a mouse anti-human IgE horseradish peroxidase (HRP) conjugate (Southern Biotech, Birmingham, AL, USA). The blots were developed by the addition of chemiluminescent reagents (ECL+, Amersham Biosciences, Bucks, UK). Western blotting using an anti-His-HRP

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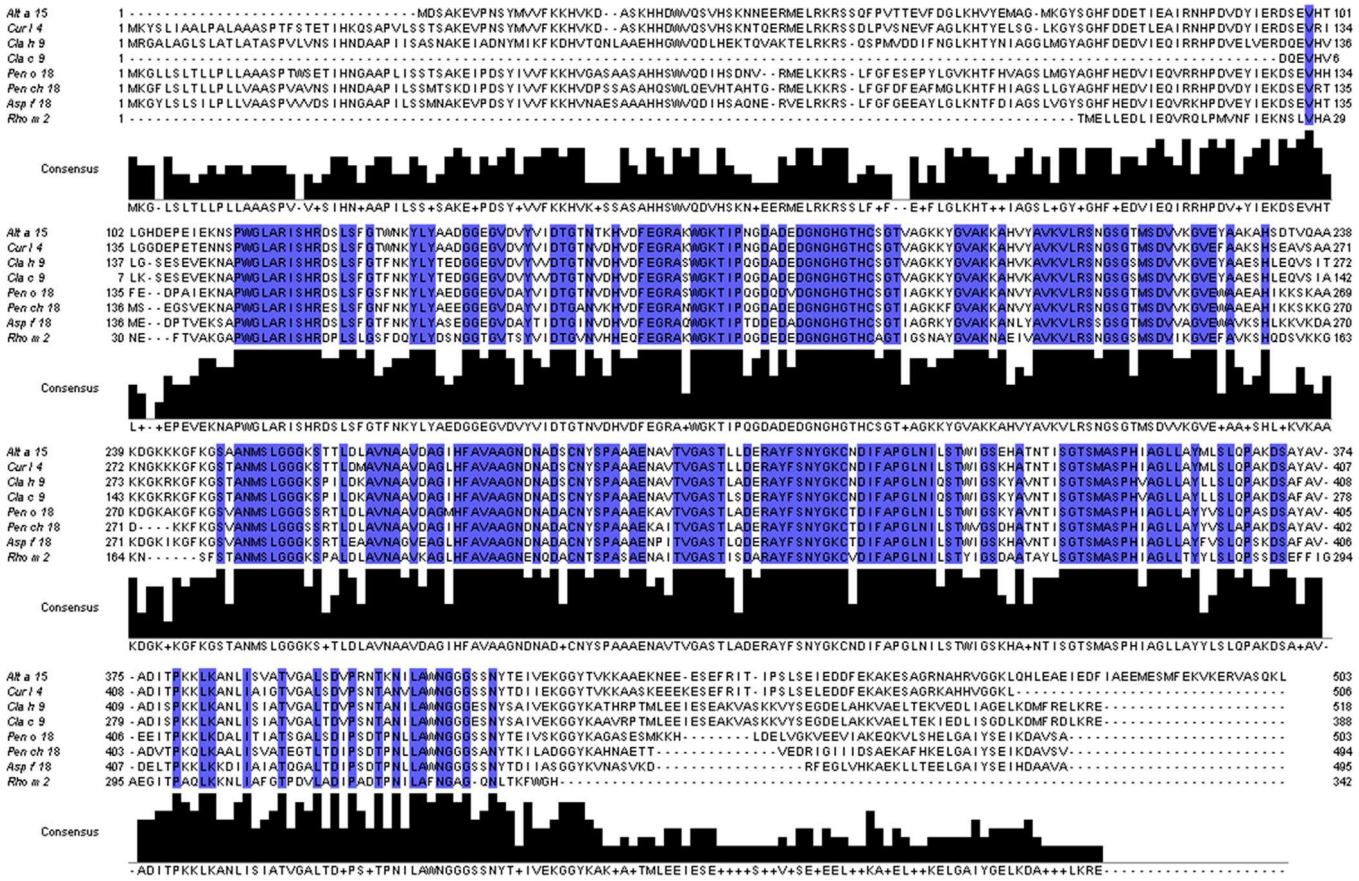

Fig. 1. Nucleotide identity found by multiple alignment of SDAP fungal vacuolar serine proteases protein sequences. The higher bar indicates higher homologies. Nucleotides that 100% identical (invariant) among all sequences are boxed.

antibody (Invitrogen) was also performed to detect the $[His]_6$-fusion protein.

2.7. Mass spectrometry

The purified 65-kDa band revealed by the anti-His-HRP antibody was sent to the Proteomic Unit of the Spanish National Centre for Biotechnology (Madrid, Spain) for identification using MALDI-TOF/TOF. The MALDI result was obtained in the form of a MASCOT search chart.

2.8. IgE immunoblotting and ELISA inhibition assays

The serum of an Alt a 15-sensitized patient (25 μL) was separately incubated overnight at 4 °C with different total protein concentrations (0.2–2000 ng) of purified recombinant protein and *A. alternata* extract. IgE quantification was performed as described by Tawde (Tawde et al., 2006). Briefly, microtiter plates were pre-coated with 50 ng of purified rAlt a 15 diluted in 50 mM carbonate-bicarbonate buffer, pH 9.6. The binding of IgE was detected using a mouse anti-human IgE-HRP, and the reaction was developed with Sigma-Fast OPD (o-phenylenediamine dihydrochloride). The OD was measured in a LabSystems Multiskan Bichromatic plate reader (LabSystems, Helsinki, Finland) at 495 nm. The percent inhibition was calculated as (1 - OD_{495nm} with inhibitor/ OD_{495nm} without inhibitor) × 100.

A serum sample from a patient sensitized to *A. alternata* with positive immunoblotting to rAlt a 15 was preincubated at 4 °C overnight with *A. alternata* and *C. lunata* crude extracts (5 mg protein/mL) or PBS for the immunoblotting inhibition assays. The inhibited sample was tested for binding to rAlt a 15 blotted onto a PVDF membrane, and bound IgE was detected using the procedure described above.

3. Results

3.1. Cloning and sequencing of the alternata vacuolar serine protease

The complete cDNA sequence of Alt a 15 obtained using RACE, PCR and sequencing assays was submitted to the National Center for Biotechnology Information (NCBI) GenBank database under accession number KJ558435. The open reading frame encodes a deduced 503-amino acid polypeptide presenting a theoretical molecular weight and an isoelectric point of 54.4 kDa and 5.8, respectively.

3.2. Bioinformatic analyses of deduced protein sequence

A motif search of the coding sequence of Alt a 15 using Gene Runner software detected the presence of two conserved serine protease motifs of the subtilase family, HGTHCSGTVAG (182–192 residues) and GTSMASPHIAG (346–356 residues). The active-site residues for serine proteases (Asp150, His182 and Ser348) andthree potential N-glycosylation sites that are conserved in several vacuolar serine proteases (Shen et al., 2003) were also noted.

Comparisons of the Alt a 15 sequence with the NCBI database revealed a high similarity to a family of subtilisin-like serine proteases from other filamentous fungi. The protein sequence showed significant homology with fourteen recognized fungal allergens, namely, Cur l 4, Cla h 9, Pen o 18, Asp f 18, Pen ch 18, Cla c 9,

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Table 1
Homology of Alt a 15 protein sequence with allergens in SDAP database.

	Allergen	Source	Sequence accession number Swissprot/NCBI/PIR	Sequence length	(%) Identity	E score
Vacuolar Serine proteases	Cur l 4	*Curvularia lunata*	ACF19589	506	90	4.3e–152
	Cla h 9	*Cladosporium herbarum*	AAX14379	518	70	5.3e–119
	Pen o 18	*Penicillium oxalicum*	AAG44478	503	68	2.4e–114
	Asp f 18	*Aspergillus fumigatus*	P87184	495	71	4.8e–111
	Pen ch 18	*Penicillium chrysogenum*	AAF71379	494	71	7.9e–111
	Cla c 9	*Cladosporium cladosporioides*	ABQ59329	388	76	1.2e–99
	Rho m 2	*Rhodotorula mucilaginosa*	Q32ZM1	342	64	1.6e–73
Alkaline Serine proteases	Tri r 2	*Trichophyton rubrum*	AAD52013	412	42	1.6e–37
	Pen ch 13	*Penicillium chrysogenum*	AAF23726	397	40	1.1e–23
	Asp v 13	*Aspergillus versicolor*	ADE74975	403	42	1.5e–20
	Asp f 13	*Aspergillus fumigatus*	P28296	403	40	2.8e–20
	Asp o 13	*Aspergillus oryzae*	CAA35594	403	41	9.8e–19
	Asp fl 13	*Aspergillus flavus*	AAD47202	403	40	9.8e–19
	Pen c 13	*Penicillium citrinum*	AAD25926	397	39	5.3e–15

Rho m 2, Tri r 2, Pen ch 13, Asp v 13, Asp f 13, Asp o 13, Asp fl 13 and Pen c 13, in the SDAP database (Table 1).

Analysis of the deduced protein sequence of Alt a 15 using the ProP 1.0 program predicted the presence of a propeptide cleavage site that is typically located at the N-terminal end of fungal vacuolar serine proteases between Arg46 and Ser47.

Lai et al. (2004) demonstrated that two critical amino acids played a role in IgE recognition in patient sera, using site-directed mutagenesis mapping of these on immunodominant epitopes in Pen ch 13, and these amino acids are highly conserved in all fungal serine protease allergens. Alignment of the allergenic vacuolar SP sequences (Fig. 1) identified that the conservation of these two amino acids was also observed in Alt a 15 (His157 and Phe160). Moreover, the critical core amino acid residues recognized by the monoclonal antibody that was produced against different epitopes of the serine proteinase major allergens in prevalent *Penicillium* and *Aspergillus* species, which has been generally accepted as a monoclonal antibody against serine protease pan-fungal allergens (FUM20) (Lin et al., 2000; Lee et al., 2007), were also conserved in the Alt a 15 protein sequence (Lys111, Pro114 and Trp115).

3.3. *Immunological characterization of the* alternata *vacuolar serine protease*

The recombinant *A. alternata* vacuolar SP was successfully expressed in *E. coli* and detected in the inclusion bodies fraction. His-tagged Alt a 15 was purified using Ni-NTA affinity chromatography and subsequently visualized at approximately 65 kDa using SDS-PAGE and western blotting with an anti-histidine antibody (Fig. 2). The 65-kDa protein was subjected to MALDI-TOF analysis to confirm its function. The peptide sequences were searched in the matrix science database, showing that the subtilisin-like serine protease from *C. lunata* is the closest homologue of the produced 65-kDa recombinant protein. The expressed protein was purified by one-step affinity chromatography, which yielded approximately 0.2 mg of rAlt a 15 from 1 L of bacterial culture. Investigation of the IgE reactivity of purified rAlt a 15 using immunoblotting revealed that six (10.2%) of the tested sera from atopic patients with *A. alternata* sensitization demonstrated IgE reactivity to this recombinant molecule (patient nos. 10, 32, 35, 37, 44 and 47, Fig. 3 A and Table 2).

The levels of sIgE in study serum samples measured using ImmunoCAP (Table 2) revealed that 51 (86.4%) of the 59 *A. alternata*-sensitized patients (patients no. 1-59) presented sIgE to Alt a 1, 26 (44.1%) patients presented sIgE to *C. lunata* extract, and 15 (25.4%) patients presented sIgE to Alt a 6. Positive levels of sIgE to *C. lunata* in all rAlt a 15-immunoreactive sera were also revealed using ImmunoCAP. Only one serum from a patient who was not sensitized to Alt a 1 (patient no. 35) presented IgE reactivity to rAlt a 15 using

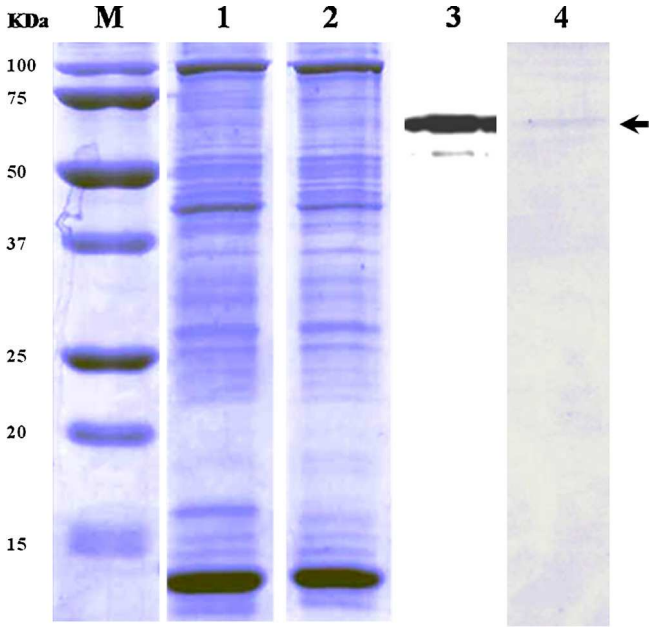

Fig. 2. Expression of Alt a 15. Lane M- Molecular mass marker, Lane 1-Non-induced *E. coli* inclusion bodies fraction, Lane 2- L-arabinose-induced *E. coli* inclusion bodies fraction, Lane 3- anti-His Western Blotting. Lane 4- rAlt a 15 purified by Ni-NTA resin Arrow indicates position of recombinant Alt a 15 band identified by MALDI.

immunoblotting. This patient exhibited negative IgE responses to Alt a 1 and Alt a 6 but high IgE responses to *C. lunata*. Fifteen of the 26 patients who were sensitized to *C. lunata* exhibited sIgE to Alt a 6. Only one of the six *C. lunata* and rAlt a 15-reactive subjects exhibited IgE reactivity to fungal enolase (patient no. 44).

Inhibition ELISA assays were performed using serum from patient no. 37 to assess the relative reactivity of recombinant and native Alt a 15. The preincubation of this patient's serum with increasing amounts of *A. alternata* mycelial extract yielded an evident dose-dependent inhibition of sIgE-binding to purified rAlt a 15 immobilized in the solid phase (Fig. 4). One nanogram of purified recombinant protein was required for 50 percent inhibition (IC50) of the solid phase-bound rAlt a 15, but 51 ng of total protein content in the *A. alternata* extract was required for the same outcome.

The IgE cross-reactivity between *A. alternata* SP and *C. lunata* SP was demonstrated using the immunoblotting inhibition assay and serum from patient no. 35. *C. lunata* and *A. alternata* extracts completely inhibited the binding of serum IgE to rAlt a 15 (Fig. 3 B).

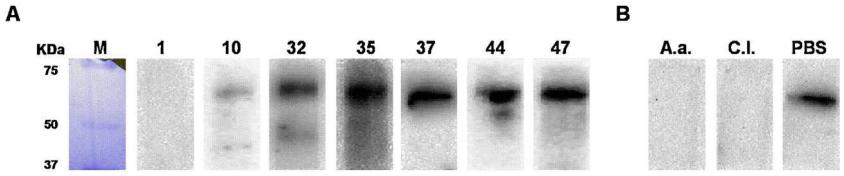

Fig. 3. A. IgE-immunoblotting with representative sera from 59 *A. alternata*-sensitized patients showing IgE reactivity to rAlt a 15 (patient/lane 10, 32, 35, 37, 44 and 47) and one example of an Alt a 15-sIgE negative sera (patient/lane 1). B. Inhibition of the IgE-immunoblotting reactivity of a serum sample no 35 that has been mixed and pre-incubated with: (A.a.) *A. alternata* extract, (C.l.) *C. lunata* extract and (PBS) phosphate-buffered saline.

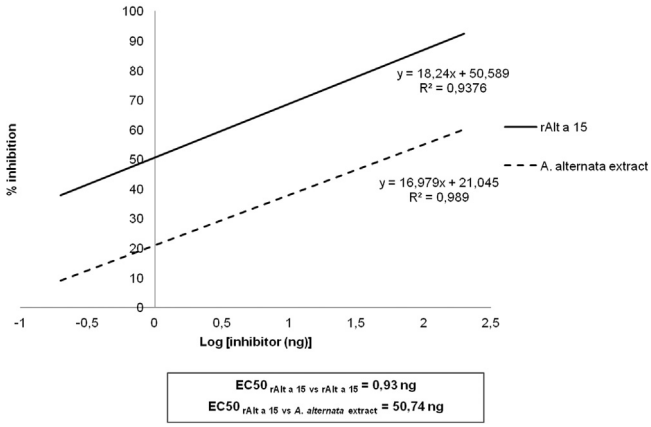

Fig. 4. ELISA-inhibition of IgE-binding to recombinant Alt a 15 coated on the solid phase. Serum from an Alt a 15-sensitized patient was preincubated with increasing amounts of recombinant Alt a 15 and *A. alternata* extract.

4. Discussion

Several fungal allergens have been produced as recombinant proteins and applied in molecular-based allergy diagnostic approaches in the past few decades, which allows for the definition of individual reactivity profiles for each patient (Canonica et al., 2013; Sastre, 2010). An understanding of the complete panel of allergens contained within an allergenic source and the identification of markers of sensitization are key issues for achieving a correct diagnosis and subsequent prescription of allergen-specific immunotherapy (Mothes et al., 2006; Valenta and Niederberger, 2007).

This study cloned, expressed and assessed the allergenic relevance of a vacuolar serine protease from the most important fungal specie that produces airborne allergens, *A. alternata*. The similarity of different fungal SP nucleotide sequences in the GenBank database was analyzed and used in the design of degenerate oligonucleotides, which allowed the isolation and cloning of the Alt a 15-encoding cDNA using RACE experiments. Sequence comparisons of the deduced Alt a 15 protein sequence and allergenic members of the SP protein family from a variety of molds, including Cur l 4, Cla h 9, Pen o18, Asp f 18, Pen ch 18, Cla c 9 and Rho m 2, detected significant identities higher than 50%. This sequence conservation may reflect the high extent of IgE cross-reactivity that has been described within members of this protein family.

The expression of Alt a 15 as a recombinant protein in *E. coli* yielded low quantities of soluble protein. SDS–PAGE revealed that the purified protein had an apparent molecular mass that was higher than expected, and this difference may be associated with the irregular migration patterns of histidine-tagged proteins (Pico de Coaña et al., 2010). The immunological relevance of the recombinant *A. alternata* vacuolar SP was analyzed in an *A. alternata*-sensitized population using immunoblotting. Alt a 15 was found to be a minor allergen of *A. alternata* with the IgE-reactivity to rAlt a 15 demonstrated in 10.2% of study population. The ELISA inhibition assays using purified rAlt a 15 in the solid phase and an Alt a 15-sensitized patient serum preincubated with *A. alternata* crude extract and rAlt a 15 demonstrated similar slope values for the log-transformed dose-dependent inhibition curves. This outcome indicated that the produced recombinant polypeptide and the natural Alt a 15 contained in the *A. alternata* crude extract exhibited similar immunochemical characteristics for Alt a 15-specific IgE antibodies. This similarity was also reflected in immunoblotting inhibition results, in which a complete inhibition of IgE binding to rAlt a 15 was obtained using the same *A. alternata* extract.

An increasing number of authors suggest that it would be prudent to search for sensitization to other allergenic sources when *A. alternata* sensitization is diagnosed because a significant level of cross-reactivity and poly-sensitization with several other phylogenetically related and non-related molds is reported for *A. alternata* (Katotomichelakis et al., 2012; Crameri, 2011; Postigo et al., 2011; Gutierrez-Rodriguez et al., 2011). Accordingly, our recorded skin prick test results revealed that 67.8% of selected *A. alternata*-sensitized individuals were apparently multiple fungi sensitized to at least *Curvularia*. Curiously, measurement of sIgE to *C. lunata* extract revealed that this rate was much lower (44.1%). In general, patients with multiple fungi sensitization present a trend of IgEs that react to several protein components in *Alternaria* extracts, which suggests that a complete panel of cross-reacting molecules is needed to completely define poly-sensitization patterns (Mari et al., 2003). Previously, Postigo et al. (2011) examined 30 subjects who were allergic to *A. alternata* and demonstrated via molecular diagnosis that most of the apparently poly-sensitized patients were sensitized to enolase Alt a 6 or other *Alternaria* allergenic proteins, which present cross-reactive homologues in other fungal species. Our sIgE investigations verified that the sensitization to Alt a 6 justified the majority (57.6%) of poly-sensitization cases ($n = 26$). However, sIgE levels to *C. lunata* extract and immunoreactivity to rAlt a 15 clearly highlighted that all Alt a 15-sensitized patients exhibited in vitro IgE reaction to *C. lunata* extract, which suggests that 23.1% of poly-sensitization cases in this group of patients are likely mediated by a cross-reactivity to vacuolar SP. Moreover, since only one poly-sensitized patient presented sensitization to both Alt a 6 and Alt a 15 allergens, the sensitization of the 23.1% of poly-sensitized patients to other *A. alternata* cross-reacting molecules must be studied. Our research group recently characterized a manganese-dependent superoxide dismutase, Alt a 14, as a minor *A. alternata* allergen that likely participates in cross-reactivity, particularly with *A. fumigatus* (Gabriel et al., 2015). This allergen and the other *A. alternata* cross-reacting molecules identified to date, such as the enolase Alt a 6, helped clarify the diagnostic problems that are associated with cross-reactivity and multiple sensitization phenomena.

Data from sIgE investigations also revealed that 86.4% of the patients who were clinically labeled as sensitized to *A. alternata* exhibited IgE responses to the *A. alternata* major allergen, Alt a 1. Among the eight *A. alternata* sensitized non-reactive to Alt a 1 and Alt a 6 allergens, only one subject presented positive anti-rAlt a 15 IgE immunoblotting. Interestingly, this patient exhibited high IgE responses to *C. lunata* extract (patient no. 35). The complete

Table 2
Results of allergen-specific IgE detection in serum samples from *A. alternata*-sensitized patients (1–59), a subject mono-sensitized to *D. pteronyssinus* (60).

ID	Skin Prick test	Specific IgE (KU/L)				Immunoblotting sIgE
	A.a.	Alt a 1	C. l.	Alt a 6	CCD	Alt a 15
1	+	>100	2.3	16.00	<0.35	–
2	+	>100	2.48	7.60	<0.35	–
3	+	<0.35	<0.35	<0.35	<0.35	–
4	+	6.39	<0.35	<0.35	<0.35	–
5	+	15.20	<0.35	<0.35	<0.35	–
6	+	11.10	<0.35	<0.35	<0.35	–
7	+	44.20	2.74	<0.35	<0.35	–
8	+	64.30	<0.35	<0.35	<0.35	–
9	+	4.29	1.80	<0.35	<0.35	–
10	+	1.90	5.10	<0.35	<0.35	+
11	+	<0.35	<0.35	<0.35	<0.35	–
12	+	19.00	<0.35	<0.35	<0.35	–
13	+	4.32	<0.35	<0.35	<0.35	–
14	+	4.40	<0.35	<0.35	<0.35	–
15	+	3.42	<0.35	<0.35	<0.35	–
16	+	<0.35	<0.35	<0.35	<0.35	–
17	+	10.10	<0.35	<0.35	<0.35	–
18	+	13.60	3.71	5.05	<0.35	–
19	+	9.21	<0.35	<0.35	<0.35	–
20	+	3.84	<0.35	<0.35	<0.35	–
21	+	1.97	<0.35	<0.35	<0.35	–
22	+	1.58	<0.35	<0.35	<0.35	–
23	+	< 0.35	<0.35	<0.35	<0.35	–
24	+	18.50	0.94	<0.35	<0.35	–
25	+	1.58	<0.35	<0.35	<0.35	–
26	+	20.10	3.69	<0.35	<0.35	–
27	+	8.75	4.20	4.30	<0.35	–
28	+	>100	4.21	11.82	<0.35	–
29	+	34.50	25.31	6.35	<0.35	–
30	+	3.03	<0.35	<0.35	<0.35	–
31	+	9.68	<0.35	<0.35	<0.35	–
32	+	15.70	10.84	<0.35	<0.35	+
33	+	19.00	<0.35	<0.35	<0.35	–
34	+	36.50	3.57	<0.35	<0.35	–
35	+	<0.35	23.21	<0.35	<0.35	+
36	+	24.20	3.23	5.91	<0.35	–
37	+	17.00	8.62	<0.35	<0.35	+
38	+	1.55	<0.35	<0.35	<0.35	–
39	+	96.00	5.70	<0.35	<0.35	–
40	+	13.10	6.00	8.53	<0.35	–
41	+	<0.35	<0.35	<0.35	<0.35	–
42	+	<0.35	<0.35	<0.35	<0.35	–
43	+	10.40	4.25	3.35	<0.35	–
44	+	4.10	2.43	4.28	<0.35	+
45	+	73.40	19.71	7.35	<0.35	–
46	+	30.00	<0.35	<0.35	<0.35	–
47	+	8.70	9.55	<0.35	<0.35	+
48	+	13.70	1.80	2.25	<0.35	–
49	+	<0.35	<0.35	<0.35	<0.35	–
50	+	11.30	<0.35	<0.35	<0.35	–
51	+	3.62	<0.35	<0.35	<0.35	–
52	+	4.66	<0.35	<0.35	<0.35	–
53	+	97.10	4.28	10.10	<0.35	–
54	+	66.40	3.51	4.35	<0.35	–
55	+	7.24	<0.35	<0.35	<0.35	–
56	+	7.37	<0.35	<0.35	<0.35	–
57	+	>100	5.80	2.80	<0.35	–
58	+	1.70	<0.35	<0.35	<0.35	–
59	+	1.90	<0.35	<0.35	<0.35	–
60[a]	–	<0.35	<0.35	<0.35	<0.35	–

ID, patient identification number; A.a., *Alternaria alternata*; C.l., *Curvularia lunata*; CCD, cross-reactive carbohydrate, +, positive result; -, negative result. The cut offs for positive results were 3 mm for SPT and 0.35 KU/L for detection of specific IgE by ImmunoCAP.

[a] Negative control.

inhibition of IgE binding to rAlt a 15 by the *C. lunata* extract using the serum from this patient strongly suggests that his IgE reactivity to *A. alternata* was the result of cross-reactivity between highly homologous SPs, and it is possible that his antibodies were primarily elicited by SPs from other allergenic sources, likely *C. lunata*. The extensive cross-reactivity between Alt a 15 and Cur l 4 is not surprising, given the high level of overall sequence identity (90%). In fact, some patients who are primarily sensitized by a cross-reactive allergen from one fungal species may develop allergic symptoms by exposure to other environmental fungi because of cross-reacting IgE antibodies that often cause diagnostic errors.

The stimulating/allergenic features of proteases from *A. alternata* and correlations between the severity of the allergic disease and proteolytic activity of *Alternaria* extracts were clearly demon-

strated in various models (Leino et al., 2013; Kouzaki et al., 2009; Boitano et al., 2011; Snelgrove et al., 2014). The inherent allergenic properties of proteases appear to be not only structural but also biochemical in nature, though this hypothesis has not been unequivocally proven and remains a topic of discussion. Therefore, the percentage of the clinical relevance of Alt a 15 that was found using immunoblotting may not represent the real influence of this protein in the development of allergic disease. Based on sequence homology to other serine proteases and on the knowledge that serine proteases are expressed as large precursor molecules, which are post-translationally cleaved to form the mature enzymes, it will be a challenge to produce enzymatically active rAlt a 15 by removal of the prosequence.

In conclusion, the *A. alternata* vacuolar serine protease Alt a 15 behaves as a cross-reactive minor allergen in an *A. alternata*-sensitized population. Alt a 15 (or the highly homologous protein Cur l 4) may also be considered a marker for *C. lunata* sensitization in the absence of Alt a 1 sensitization, which may underlie the initial diagnostic errors that occur for allergies to *Alternaria*. Thus, this molecule can be added to the panel of cross-reacting components needed to completely define polysensitization patterns and solve diagnostic problems associated with cross-reactivity and/or co-sensitization phenomena.

Therefore, the recombinant protein and immunological information provided in this study are useful to improve immunodiagnoses and understanding of IgE-mediated fungal diseases for the development of therapeutic strategies.

Conflict of interest

The authors declared no conflict of interest.

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Databases

Genbank nucleotide sequence data are available in the GenBank database under the accession number KJ558435.

Allergen database: this new allergen has been submitted, accepted and assigned with an official name by the International World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee (http://www.allergen.org).

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Paper V

From respiratory sensitization to food allergy: Anaphylactic reaction after ingestion of mushrooms (*Agaricus bisporus*)

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From respiratory sensitization to food allergy: Anaphylactic reaction after ingestion of mushrooms (*Agaricus bisporus*)

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ABSTRACT

We report a case of a 38-year-old mold-allergic patient who developed episodes of generalized urticaria and systemic anaphylactic shock immediately after ingesting button mushrooms. A manganese-dependent superoxide dismutase (MnSOD) and a NADP-dependent mannitol dehydrogenase (MtDH) from *Agaricus bisporus* mushroom were identified as patient-specific IgE-binding proteins. Cross-reactivity between *A. bisporus* MnSOD and mold aeroallergens was confirmed. We conclude that prior sensitization to mold aeroallergens might explain severe food reactions to cross-reacting homologs mushroom proteins.

1. Introduction

Fungi are recognized as one of the principal causes of type I allergies, which lead to severe respiratory and cutaneous allergic diseases [1, 2]. Exposure to fungal allergens can occur by inhalation of mold spores, skin contact with saprophytic species or ingestion of edible mushrooms [3]. The most important known allergenic fungi are molds belonging to the genera *Alternaria, Aspergillus* and *Cladosporium* [4]. An association between sensitization to airborne molds and systemic reactions caused by ingestion of mushrooms have been suggested but not extensively investigated [5, 6]. Among edible mushroom species, *Agaricus bisporus* is the most extensively cultivated and consumed throughout the world [7].

Here, we describe a case of a woman with bronchial asthma who experienced episodes of urticaria and anaphylaxis after ingesting a button mushroom. The allergens implicated were identified using the patient's serum.

2. Case

A 38-year-old woman was referred because she developed generalized urticaria immediately after ingesting chicken with mushrooms. One month later and a few minutes after the intake of fried champignon mushrooms, she suffered an episode of anaphylaxis, which needed treatment in the Emergency Department.

She had a previous history of perennial childhood asthma treated with immunotherapy to *Alternaria alternata*. Seven years before food allergy reaction to mushroom, this patient revealed positive skin prick tests (SPT) to *Alternaria*, *Penicillium*, *Aspergillus*, dog, mites and grass pollen. Specific IgE tests were positive for *Aspergillus* (RAST class 3), *Penicillium* (RAST class 3) and mites (RAST class 2). During recent years, she has been stable without asthma exacerbations, and only minimal medication with anti-asthmatic drugs has been necessary.

Currently, she related oral itching after the ingestion of several fruits belonging to the family Rosaceae, and she was tolerant of banana and tropical fruits (pineapple or mango).

Serum from the patient was obtained with a written consent agreeing to participate in the study.

After the anaphylactic episode, SPT to commercially available common inhalants (ALK-Abello SA, Madrid, Spain) were positive for airborne molds (*A. alternata, Cladosporium herbarum, Penicillium notatum and Aspergillus fumigatus*) but negative for other common inhalants, including mite, dander and pollens. SPT using extracts of *Pleurotus ostreatus*, *Lentinus edodes*, *Boletus edulis* and *A. bisporus* (Bial-Aristegui SA, Spain) were also positive. Total serum IgE was 271 kU/L. Specific IgE by Fluoro Enzyme ImmunoAssay (ImmunoCAP, Thermo-scientific, Sweden) to the different species and allergens gave the following results (kU/L): *A. alternata*, 3.7;

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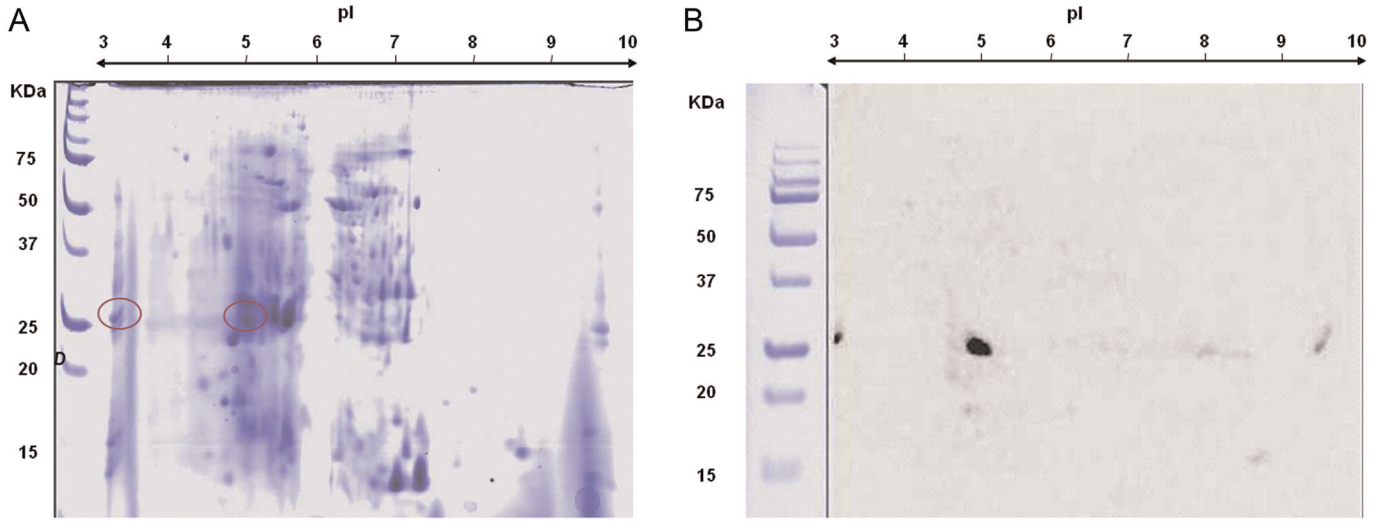

C

Accession number*	Protein name	MW (KDa)		pI		MASCOT score†	Matched peptides	Peptide
		Observed	Theoretical	Observed	Theoretical			
Q9P4T6	Superoxide dismutase (Mn), *Agaricus bisporus*	24	22.2	5.2	6.5	161.55	3	YVEATQGSKL RYVEATQGSK DAIDQTFGGLDNLKK
O93868	NADP-dependent mannitol dehydrogenase, *Agaricus bisporus*	27	28.0	3.2	8.2	156.14	5	VGKEFGVK DAVEVTEKVGK SAKDAVEVTEK GLAAEWASAGIR DHQASNIPLNR

* UniProt database accession number

† Probability-based score used by MASCOT program

Fig. 1. (A) Two-dimensional electrophoresis and (B) Two-dimensional IgE-immunoblotting of *Agaricus bisporus* crude extract. Immunoblotting was performed using the patient's serum. (C) Proteins identified by liquid chromatographic electron spray ionization tandem mass spectrometric analysis. MW, molecular weight, pI, isoelectric point.

A. bisporus, 2.1; *P. ostreatus*, 1.2; *L. edodes*, 0.5; *B. edulis*, <0.35; rAsp f 6, 2.3 and Pru p 3, 20.4. Component-resolved diagnosis using the ISAC method revealed the following specific IgE results (ISU): rAlt a 1, 1.3; rAsp f 3, 2.0; rAsp f 6, 2.9 and nPru p 3, 14.

Two dimensional electrophoresis IgE immunoblotting revealed two spots with apparent molecular weights of approximately 24 and 27 kDa and approximated pIs of 5.2 and 3.2, respectively (Fig. 1). Both protein spots were excised and identified by liquid chromatography coupled to mass spectrometry as a manganese-dependent superoxide dismutase (MnSOD) and NADP-dependent mannitol dehydrogenase (MtDH) with significantly high scores (Fig. 1C).

BLAST alignment between MnSOD and MtDH protein sequences from *A. bisporus* (GenBank accession nos. Q9P4T6 and AAC79985, respectively) and fungal aeroallergen homologs revealed significant homologies (Table 1).

ImmunoCap-inhibition results demonstrated that the *A. bisporus* crude extract was able to strongly inhibit IgE-binding to rAsp f 6 (68%) but not to rPru p 3 (Table 2).

3. Discussion

Strong allergic reactions to mushrooms have been increasingly recognized [8–10], and there are few studies reporting an association between aeroallergens and mushroom ingestion-related symptoms. In this regard, Herrera et al. in some of their works found a relationship between allergenicity to airborne molds

Table 1
Homologies of *Agaricus bisporus* manganese-dependent superoxide dismutase and NADP-dependent mannitol dehydrogenase protein sequences with officially recognized WHO/IUIS mold allergens.

Allergen	Source	Accession number Swissprot/NCBI/PIR	% Identity	*E* score
Manganese-dependent superoxide dismutases				
Asp f 6	*Aspergillus fumigatus*	Q92450	49	8e−61
Alt a 14	*Alternaria alternata*	AGS80276	43	1e−40
NADP-dependent mannitol dehydrogenase				
Alt a 8	*Alternaria alternata*	P0C0Y4	36	9e−47
Cla h 8	*Cladosporium herbarum*	AAO91801	35	4e−45

Table 2
ImmunoCAP inhibition assay results. Recombinant Prup 3 and Asp f 6 were used in the solid phase and different concentrations of *Agaricus bisporus* as liquid phase inhibitor.

Inhibitor concentrations (*A. bisporus* crude extract) (mg/ml)	% of inhibition (rPru p 3 in solid-phase)	% of inhibition (rAsp f 6 in solid-phase)
0	0	0
0.0001	0	10
0.001	0	30
0.01	0	45
0.1	0	56
1.0	0	68

(*A. alternata, C. herbarum and A. fumigatus*) and food allergies to *A. bisporus* mushrooms and spinach, demonstrating the existence of some non-identified cross-reacting proteins [6–11]. Another study attributed one form of oral allergy syndrome in adults to heat labile mushroom proteins of 43–67 kDa in size that also seem cross-react to molds [5]. In fact, knowledge of allergenic proteins that cause recognized clinically relevant cross-reactions between molds and food is still limited, and research in this field is needed to identify the causative allergens and to understand the immunological events that take place.

In this work, we describe a case of a patient with a previous clinical history of respiratory symptoms associated with mold aeroallergens and treated by immunotherapy with *A. alternata* extract. Years later, this patient suffered anaphylactic shock after intake of mushrooms. IgE-mediated sensitization to *A. alternata*, *C. herbarum*, *P. notatum*, *A. fumigatus*, *A. bisporus*, *P. ostreatus*, and *L. edodes* was confirmed, and circulating IgE levels reactive to Alt a 1, Asp f 3, Asp f 6 and Pru p 3 were detected. Moreover, two IgE-binding components from *A. bisporus* were identified as a MnSOD and a MtDH. To date, six MnSODs have been identified and officially recognized as allergens in *A. alternata*, *A. fumigatus*, *Hevea brasiliensis*, *Olea europaea*, *Malassezia sympodialis* and *Pistacia vera*, whereas only two MtDH from *A. alternata* and *C. herbarum* are listed in the WHO/IUIS allergen database (www.allergen.org). Although both families of proteins are described as important cross-reactive allergens [1–12], cross-reactivity between MnSODs from different sources has been more intensively studied and reported [13–15].

Looking at the specific IgE levels, skin prick test results, and the course of development of allergic symptoms in this patient, it is assumed that her mushroom-related symptoms could be due to cross-reactive IgE initially raised against airborne mold homolog allergens. However, because this patient also reported symptoms to several fruits belonging to Rosaceae, probably because of cross-reactive non-specific lipid transfer protein (LTP)-specific IgE antibodies, a relationship between LTP sensitization and allergy to mushrooms was also not excluded. With this in mind, to determine whether immunologic phenomenon that originated in systemic reactions upon ingestion of *A. bisporus* were due to the presence of cross-reacting antibodies to MnSODs or LTPs, IgE-inhibition assays using Asp f 6 and Pru p 3 in the solid-phase were performed. Blast alignment and inhibition assay results suggest that the *A. bisporus* MnSOD homolog has some common epitopes causing cross-reactivity between mushrooms and common airborne molds.

In conclusion, our findings suggest that at least the cross-reactive *A. bisporus* MnSOD may lead to severe food allergic reaction in mold-sensitized patients, justifying the well-recognized relationship between sensitization to airborne molds and allergy to mushrooms ingestion. However, more in-depth knowledge of the role of MnSOD and MtDH as causal agents of severe food reactions is necessary to improve diagnosis and to allow effective avoidance and prevention of further systemic reactions.

Conflict of interest

There are none.

Ethical form

Written informed consent was obtained from the patient for publication of this Case report.

Acknowledgements

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Chapter 4

Concluding remarks

Compared to other common environmental allergenic sources, fungi are reported as neglected and underestimated. Among fungi associated with allergenic diseases, *A. alternata* produces a broad panel of allergenic proteins that may impact the immune system of atopic patients, frequently resulting in severe respiratory manifestations. Research on the array of allergenic components from *A. alternata*, prediction of cross-reactivities and the role of these proteins in the development of respiratory allergies seem to be very relevant keys in establishing correlations between *A. alternata* exposure and allergic symptoms as well as understanding how and why *A. alternata* is associated with a wide range of serious respiratory symptoms. Taking this into account, the studies included in this doctoral work were focused on broadening knowledge on *A. alternata* allergen repertoire, analyzing how these allergens are phylogenetically distributed across other allergological important fungal sources and determining how they may be employed to justify the complex IgE binding patterns and clinical manifestations exhibited by patients sensitized to *A. alternata*.

In accordance with previous reports, from a phylogenetic perspective, it was demonstrated that the gene encoding for the *A. alternata* major allergen, Alt a 1, is limited to a restrict number of *Alternaria* related species, exclusively belonging to the Pleosporaceae family. Species that are able to produce homologues of this important fungal allergen are also potentially capable of stimulating the immune system of atopic patients. Two PCR-based systems targeting the detection of the variant and invariant regions of the gene that encodes Alt a 1 and Alt a 1 homologues were successfully developed. Results of such assays supported that the Alt a 1 gene is a specie-specific molecular marker that may be used as a valuable tool in assessing Alt a 1 exposure levels and contamination/colonization by *A. alternata* and other Alt a 1-producing species. This approach constitutes a faster, simpler and optimal alternative tool to the traditional species-level identification. Moreover, in this way it is possible to establish a relationship between exposure to Alt a 1, independent of its fungal origin, and the risk of respiratory symptoms as well as to provide information on the implementation of the appropriate public health measures.

To complete the identification of the allergen panel triggering allergic reactions to *A. alternata* and the understanding of poly-sensitization clinical data were the major goals of our research. Taking into account the recent identification of a manganese-dependent superoxide dismutase as an IgE reactive component of *A. alternata*, and the fact that serine proteases are well recognized fungal pan-allergens that had never been characterized in *A. alternata*, we intended to proceed to the analysis of the potential of these two *A. alternata* proteins in diagnosis of mold allergy. Since both MnSOD and SP are evolutionarily conserved proteins, a combinatorial strategy involving sequence similarity analysis, RACE-PCR and sequencing was successfully applied to isolate the respective full-length cDNA sequences. The

obtained sequences were deposited in the GenBank database and are now publicly available. The recombinant *A. alternata* MnSOD and SP were produced and they were shown to bind to specific IgE from 11.5% (n=61) and 10.2% (n=59), respectively, of the patients diagnosed as being sensitized to *A. alternata* included in the study. These two new minor *A. alternata* allergens were approved by the WHO/IUIS Allergen Nomenclature Sub-Commitee. Consequently, they were added to the panel of *A. alternata* allergens and were officially named Alt a 14 (*A. alternata* MnSOD) and Alt a 15 (*A. alternata* SP). The observation that both recombinant proteins exhibited similar immunochemical properties for sIgE antibodies to the respective counterpart found in the crude extract suggests that the produced polypeptides could be used as efficient diagnostic reagents. This work also provided evidence that Alt a 14 and Alt a 15 show IgE cross-reactivity with similar homologue allergens from other allergological important fungal species, such as *A. fumigatus* and *C. lunata*, respectively. In addition to allergenicity and cross-reactivity evidences, both new allergens seem to play important roles in the development of particular allergic responses. In fact, for Alt a 14 we detected that it was able to bind sIgE from ABPA diagnosed patients suggesting that sensitivity to this allergen may be related to ABPM. On the other hand, sensitivity to Alt a 15 was shown to be highly specific to fungi poly-sensitized patients and appeared to explain *A. alternata* allergy in some cases of patients who did not presented reactivity to the genuine sensitization marker, Alt a 1.

Lastly, in this doctoral work, a strong allergic reaction to mushrooms was found to be attributed to two IgE-binding proteins from the mushroom *A. bisporus,* a MtDH and a MnSOD which present their homologues in some airborne molds, including *A. alternata*. Patient clinical history of respiratory allergic symptoms associated to mold aeroallergens, together with evidences of cross-reactivity mediated at least by MnSODs, supported the theory that environmental mold allergens could act as primary sensitizers' agents for homologue food allergens and lead to severe food allergic reaction. Moreover, this study reveals that although they are characterized as minor allergens, the newly identified cross-reactive allergenic proteins, namely Alt a 14, may have serious clinical implications and may be used in clinical practice as a prognostic marker of potential systemic reaction to mushrooms.

Overall, the findings achieved in this doctoral research work allowed for the consideration of new perspectives for improving the accuracy of the assessment of allergen exposure, diagnosis, management and understanding of IgE-mediated fungal diseases.

Future Trends

Due to the fact that fungal allergy continues to be a poorly studied area both in basic research as well as in clinical practice, there is substantial room for improvement. In this regard, the results presented in this doctoral thesis have not only contributed to a significant increase in the understanding of this area of study, but have also paved the way for future investigations:

- In order to prove its value in monitoring the exposure to Alt a 1 allergen, the simple, sensitive and specific PCR system that was developed and presented in this work, should be applied in epidemiological studies using environmental air/dust and food samples. This will allow establishing potential correlations of allergic manifestations with Alt a 1 exposure levels existing in the environment surrounding atopic patients and provide information on the implementation of appropriated personal/public protective health measures.

- The molecular studies performed in this work have also supported the theory that evolutionary events have conditioned the profile of mold components that are recognized by the human's immune system resulting in a complex range of allergic manifestations. Thus, also based in recent reports which strengthen the same hypothesis, additional phylogenic studies should be performed in order to correlate the evolutionary process of the genes coding for allergen, its potential role in fungal biology and allergic response.

- The produced recombinant proteins may be applied in improving molecular diagnosis allowing for the definition of individual IgE reactivity patterns, mainly in fungal poly-sensitized population and information of prognosis of clinical manifestations and potential cross-reactivities. This may also lead to a more effective specific immunotherapy using a single or a few allergenic molecules.

- The availability of the recombinant *A. alternata* allergens may also be applied in studies with animal models in order to elucidate the biological role of *A. alternata* in the physiopathogenesis of allergy and asthma.

- Given the apparent association between sensitization to Alt a 14 and ABPA verified in this work, asthmatic patients presenting IgE reactivity to Alt a 14 should be extensively evaluated to ascertain if they are at risk of developing ABPM or if they are already in the asymptomatic phase of the disease (stage 0 of ABPA).

- Since proteolytic active serine proteases are described as potent adjuvant of inflammatory response in allergy and asthma, Alt a 15 should be produced in its enzymatic active form and further studied.

Appendices

Appendix A - Sequence data of the gene encoding for Alt a 14 available from the GenBank.

Appendix B - Sequence data of the gene encoding for Alt a 15 available from the GenBank.

Appendix C - Alt a 14 allergen information from the WHO/IUIS Allergen Nomenclature Database.

Appendix D - Alt a 15 allergen information from the WHO/IUIS Allergen Nomenclature Database

Appendix A

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  TITLE     Molecular cloning, expression and characterization of the manganese
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  TITLE     Direct Submission
  JOURNAL   Submitted (17-APR-2013) Department of Immunology, Microbiology and
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  TITLE     Direct Submission
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Appendix C

ALLERGEN NOMENCLATURE
IUIS Allergen Nomenclature Sub-Committee

- Home
- Search
- Tree View
- Publications
- Standardization
- Executive Committee
- Submission Form
- Log In

Member Login

Username or Email: Password:

Login

Fungi Ascomycota Pleosporales *Alternaria alternata* Alt a 14

Allergen Details:

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Allergenicity ref.:	21255138
Food allergen:	No
Date Created:	2014-02-19 07:45:08
Last Updated:	2014-02-19 07:57:57
Submitter Info:	
Name:	Marta F. Gabriel
Institution:	University of the Basque Country, Department of Immunology, Microbiology and Parasitology
City:	Vitoria, Spain
Email:	mgabriel20023@gmail.com
Date:	2013-07-22

Comments

Table of IsoAllergens:

+/-	Isoallergen and variants	GenBank Nucleotide	GenBank Protein	UniProt	PDB
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Appendix D

ALLERGEN NOMENCLATURE
IUIS Allergen Nomenclature Sub-Committee

- Home
- Search
- Tree View
- Publications
- Standardization
- Executive Committee
- Submission Form
- Log In

Member Login

Username or Email: Password:

Login

Fungi Ascomycota Pleosporales *Alternaria alternata* Alt a 15

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Food allergen:	No
Date Created:	2014-11-11 14:36:16
Last Updated:	2014-11-11 14:36:16
Submitter Info:	
Name:	Marta F. Gabriel
Institution:	University of Basque Country, Dept. of Immunology, Microbiology and Parasitology
City:	Vitoria, Spain
Email:	mgabriel20023@gmail.com
Date:	2014-09-30

Comments

Table of IsoAllergens:

+/-	Isoallergen and variants	GenBank Nucleotide	GenBank Protein	UniProt	PDB
▶	Alt a 15.0101	KJ558435 (To be released upon publication)			