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Research paper

# High-throughput screening of T7 phage display and protein microarrays as a methodological approach for the identification of IgE-reactive components

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ABSTRACT

Olive pollen and yellow mustard seeds are major allergenic sources with high clinical relevance. To aid with the identification of IgE-reactive components, the development of sensitive methodological approaches is required. Here, we have combined T7 phage display and protein microarrays for the identification of allergenic peptides and mimotopes from olive pollen and mustard seeds. The identification of these allergenic sequences involved the construction and biopanning of T7 phage display libraries of mustard seeds and olive pollen using sera from allergic patients to both biological sources together with the construction of phage microarrays printed with 1536 monoclonal phages from the third/four rounds of biopanning. The screening of the phage microarrays with individual sera from allergic patients enabled the identification of 10 and 9 IgE-reactive unique amino acid sequences from olive pollen and mustard seeds, respectively. Five immunoreactive amino acid sequences displayed on phages were selected for their expression as His6-GST tag fusion proteins and validation. After immunological characterization, we assessed the IgE-reactivity of the constructs. Our results show that protein microarrays printed with T7 phages displaying peptides from allergenic sources might be used to identify allergenic components -peptides, proteins or mimotopes- through their screening with specific IgE antibodies from allergic patients.

## 1. Introduction

Type I allergy, or immediate hypersensitivity, is a generalized health problem in industrialized countries with an increasing incidence in the last years, which affects about 30% of the population (Asher et al., 2006; Eder et al., 2006; Bousquet et al., 2009). The starting point for triggering the allergic symptoms -rhinitis, conjunctivitis or asthma- consists of IgE overproduction against allergenic substances, innocuous for the rest of the population (Dahl et al., 2004; Stone et al., 2010).

In Mediterranean countries, large areas of California and Australia, olive (*Olea europaea*) pollen is a major allergenic source with a high clinical relevance (Rodriguez et al., 2001; Vereda et al., 2011; Villalba et al., 2014). In Spain, olive pollen is the second cause of pollinosis after grass pollen, and the first one in some localized areas of Extremadura and Andalusia, where olive trees are extensively cultivated. Thirteen allergens (Ole e 1–13) from olive pollen have been described so far, but its complex allergogram remains to be completed (Rodriguez et al., 2001; Villalba et al., 2014). Indeed, by western blot (WB) analyses there are several IgE-recognized protein bands not yet identified as allergens, and about 20% of patients are negative to the major olive pollen allergens Ole e 1, Ole e 7 and Ole e 9 (Alcántara et al., 2017). On the other hand, yellow mustard (*Sinapis alba*) seeds are responsible for severe allergic symptoms (angioedema and anaphylaxis reactions) in allergic patients in multiple countries (Rance, 2003; Vereda et al., 2011). The use of yellow mustard as a condiment in many foods or a hidden component in many pre-packaged meals produces unexpected reactions and makes mustard an important food allergen in developed countries. Four allergens from mustard seeds have been described (Sin a 1–4), remaining other IgE-reactive components to be identified (Rance, 2003; Sirvent et al., 2009; Vereda et al., 2011).

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Saline protein extracts are commonly used for isolation of allergens and to produce allergenic extracts for skin testing diagnosis and immunotherapy. However, the use of saline protein extracts to identify IgE-reactive components limits this process to those soluble molecules after the extraction procedure. Thus, to identify new IgE-reactive molecules and to contribute to the completion of allergograms of allergenic sources with high clinical relevance, new methodological approaches allowing for the identification of undetectable molecules by conventional methods are needed. The combination of the high-throughput screening of phage display and protein microarrays might offer an interesting approach to identify new allergenic components.

Phage display technology allows for the *E. coli* expression of exogenous peptides on the surface of viral particles using coat proteins as fusion proteins. The construction of phage libraries displaying foreign peptides and their subsequent screening against a desired target are the basis of this method, which has been mainly used for monoclonal antibody production, and epitope mapping, among others (Rhyner et al., 2004; Pande et al., 2010). On the other hand, protein microarrays are a powerful tool for large scale testing of biological samples, which allow for the high-throughput simultaneous screening and rapid analysis of thousands of proteins. These tools permit surveying antigens and antibodies in blood samples or other biological fluids using very low volumes of samples, and have been commonly used for the identification of biomarkers (Kodadek, 2001; Ramachandran et al., 2008). The combination of phage display and protein microarrays increases the throughput of both assays, and has already been used to identify molecules with diagnostic purposes in malignancies different from allergy (Wang et al., 2005; Bradford et al., 2006; Chatterjee et al., 2006a; Freckleton et al., 2009; Babel et al., 2011).

Here, we combined the screening of T7 phage libraries displaying peptides and proteins encoded by cDNA from olive pollen and yellow mustard seeds with protein microarrays as a new methodological approach for the identification of IgE-reactive molecules of these highly relevant clinical allergenic sources. First, two T7 phage libraries displaying amino acid sequences encoded by the cDNA from yellow mustard seeds and olive pollen were constructed. After biopanning of the libraries with sera from allergic patients, 768 monoclonal phages of each library were printed on nitrocellulose microarrays, and probed with sera from both types of allergic patients (Fig. 1). The subsequent screening and bioinformatic analyses of the phage microarrays enabled the identification of allergenic peptides or mimotopes -defined as peptides capable of binding to the paratope of an antibody but unrelated in sequence to the natural protein that the antibody actually recognizes- (Geysen et al., 1986). In total, 10 and 9 unique allergenic peptides or mimotopes from olive pollen and mustard seeds were found, respectively. After expression as His6-GST N-terminal tagged fusion proteins of 5 peptides or mimotopes, we confirmed their allergenicity and observed that the purified allergenic molecules of mustard seeds in combination were able to abrogate about 80% of the specific IgE binding to mustard seeds extract.

## 2. Materials and methods

### 2.1. Non-atopic control serum, mustard and olive pollen allergic patients' sera

The Institutional Ethical Review Boards of the Complutense University of Madrid approved this study on the identification of allergenic proteins. Written informed consent was obtained from all patients. Serum samples were obtained from a collection of sera previously characterized from allergic patients to mustard seeds from the Fundación Jiménez Díaz Hospital or olive pollen from the Complejo Hospitalario de Jaén (Sirvent et al., 2009; Vereda et al., 2011; Villalba et al., 2014). In addition, non-atopic controls were obtained from the Fundación Jiménez Díaz Hospital.

For selection of T7 phage libraries, three non-atopic controls sera for negative selection and allergic patients' sera with > 500 kU/l of total IgE (four sera from mustard seeds and four from olive pollen) for the positive selection of IgE immunoreactive phages were used. The allergic patient's sera were selected for their high specific IgE levels to multiple allergenic protein bands visualized by WB analysis of olive pollen and mustard seeds extracts. For microarray analysis, serum samples from 5 allergic patients to mustard seeds and 5 olive pollen allergic patients' sera were analysed. For validation, an independent cohort of 32 mustard seed allergic patients (Sirvent et al., 2009; Vereda et al., 2011), 92 serum samples from olive pollen allergic patients (Villalba et al., 2014), and three non-atopic control sera were used. As the concentration of IgE in non-atopic controls is minimal, three patients were considered optimal to be used as negative controls in the assays. All samples were handled anonymously according to the ethical and legal guidelines of the Complutense University of Madrid. Skin prick tests (SPT) were performed according to standard procedures. A wheal diameter < 3 mm was considered negative. All patients included in this study reported a clear immediate IgE-mediated allergic reaction with mustard or olive pollen, and had positive SPT to mustard or olive pollen extracts. Olive pollen allergic patients did not report any episode of food allergy and their SPT were negative for commercial food extracts. On the other hand, mustard allergic patients were selected because of a positive SPT to mustard extract, and no positive SPT to pollen extracts. In addition, mustard seeds allergic patients were also positive by SPT to tree nuts and/or peanut. Quantification of specific IgE levels in serum was performed by ELISA using mustard seeds or olive pollen extracts. Each OD value was the mean of 2 determinations after blank subtraction. OD values < 0.1 were considered negative.

### 2.2. T7 phage display cDNA library synthesis, biopanning, printing and quality control of phage microarrays

We used 4 μg of mRNA isolated from olive pollen and mustard seeds, respectively, for cDNA synthesis. Then, T7 phage cDNA display libraries were constructed using the OrientExpress cDNA synthesis and cloning systems (Novagen) according to manufacturer's instructions. Both cDNAs were synthesized using oligo(dT) primer by RT-PCR, size separated to get rid of small cDNA fragments, and cloned indistinctly into T7Select 415-1 (able to display 415 copies of peptides about 50 amino acids in length) and T7Select 10-3b vectors (able to display an average of 5–15 copies of peptides and proteins up to 1200 amino acids in length) according to the manufacturer instructions. They were grown on *E. coli* BL21 or BLT 5403 strains, respectively. The four T7 libraries on T7Select 415-1 and T7Select 10-3b vectors were titrated using serial dilutions and plaque assays. Library sizes were higher than $10^6$ pfu/mL.

Biopanning was performed in solution using tosil-activated magnetic beads (Invitrogen) covalently attached with an anti-IgE monoclonal antibody (ALK-Abelló) according to the manufacturer instructions. First, a negative selection was performed. Beads were incubated with 120 μL pooled control sera diluted 1:10 at 4 °C overnight. After washing the beads, phage biopanning was performed separately using the 2 libraries constructed with mustard seeds cDNA and the 2 libraries constructed with olive pollen cDNA. In addition, to avoid for the selection of phages displaying sequences from previously known allergens from both allergic sources, the pool of sera of olive pollen allergic patients was preincubated with 10 μg of Ole e 1, profilin, rOle e 3, Ole e 9, rOle e 10, rOle e 11 and rOle e 12, and the pool of sera of mustard allergic patients with 10 μg of profilin, rSin a 1, rSin a 2 and rSin a 3. The flow-through phages were then separately incubated with tosil-activated anti-IgE mAb magnetic beads coated with IgE antibodies captured from the pools of sera of allergic patients in the same conditions. Two different pools of serum samples from mustard seeds or olive pollen allergic patients were used to enrich for IgE reactive phages using respectively phage display cDNA libraries from mustard seeds cDNA or olive pollen cDNA. Finally, after washing with phosphate-buffered saline (PBS) containing Tween 20 0.1%, bound phages were