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Invited critical review

Proteomic approaches for identifying new allergens and diagnosing allergic diseases

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Abstract

Background: Allergic diseases are (IgE)-mediated hypersensitivity reactions affecting more than 25% of the world's population. Proteomic technologies have been increasingly used in the field of allergy and include the use of protein microarrays and two-dimensional gel electrophoresis coupled with immunoblotting.
Methods: The literature relevant to proteomic approaches to allergic diseases was searched using MEDLINE database. We reviewed proteomics approaches and applications, focusing specifically on two-dimensional immunoblotting techniques and allergen microarrays.
Results: The results obtained show that proteomic approaches using two-dimensional immunoblotting appear to be a powerful strategy for the identification of allergenic proteins. Likewise, the use of allergen microarrays allows a large number of IgE antibodies to be simultaneously identified.
Conclusions: Proteomic approaches are only beginning to be applied to the study of allergy. In the field of *in vitro* diagnosis, allergen microarrays provide a promising tool not routinely used in the allergy laboratory. In the near future this powerful technique will be used as a standard technique for *in vitro* diagnosis of allergy.

Keywords: Allergen; Allergic diseases; Immunoblotting; Microarrays; Proteomics

Contents

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1. Introduction

Allergic diseases are a heterogeneous group of type I Immunoglobulin E (IgE)-mediated hypersensitivity reactions affecting more than 25% of the world's population of developed countries. Type I allergy is initiated by the generation of IgE antibodies after the introduction of an allergen into the immune system. In a second allergen exposure, the binding of the allergen to the preformed specific IgE antibodies attached to the surface of mast cells and basophils causes the release of inflammatory mediators (*e.g.* histamine, leukotrienes, cytokines, proteases), which produce the clinical manifestations (*e.g.*, rhinitis, urticaria, anaphylaxis).

The term "proteome" was first used by Marc Wilkins' group in 1995 to describe the whole protein content of an organism [1]. Later, the term "proteomics" was introduced to refer to the study of the proteome [2]. In a more recent definition, the proteome is the set of all the proteins of a cell, organism or biological medium at a given moment. This includes all the proteins modified by alternative splicing of primary transcripts, posttranslational processing or a combination of both.

Studies in proteomics employ two main technological approaches: on one hand, a combination of a protein separation method and a technique to identify the proteins separated and, on the other, protein microarrays. In the field of allergy, the former approach is mainly used to discover new allergens while the second one aims to identify the allergens to which individuals are sensitive (Fig. 1). The aim of this review is to explore the current status of the application of proteomic strategies to the study and analysis of allergic diseases. The literature relevant to proteomic approaches to allergic diseases was searched using MEDLINE database. The main reference terms used for the search were "proteomics AND allergy", and "microarrays AND allergy".

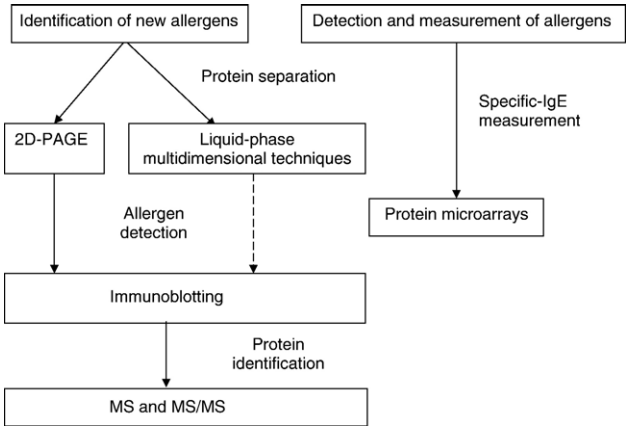

Fig. 1. Proteomic technological approaches used in allergy.

2. Proteomic methods

2.1. Protein separation

To date, the standard method for protein separation in proteomic studies is two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) [3]. The proteins are first subjected to isoelectric focusing (IEF), which separates them in the first dimension according to their isoelectric point (pI), and subsequently, in the second dimension, to sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis, which separates them on the basis of their relative masses. Once separated, the proteins are stained and individual spots of interest are recovered from the gel for protein identification by mass spectrometry. In the search for allergens, a fundamental technique is immunoblotting. Proteins resolved by 2D-PAGE are electrotransferred onto a nitrocellulose membrane. Identification of allergens on the immunoblots is performed using either allergic patients' serum or monoclonal antibodies. Immunoreactivity is revealed by incubation with labelled anti-human IgE, followed by detection of the labelling.

In recent years, liquid phase-based multidimensional separation techniques have been used most frequently in the field of proteomics [4]. These approaches combine several chromatographic methods or chromatography and capillary electrophoresis. These separation techniques can be applied directly to separate proteins or can be applied to the separation of trypsin-generated peptides of sample proteins. As with 2D-PAGE, protein identification is achieved using mass spectrometry. To date, multidimensional separation techniques have not yet been applied to the search for new allergens, but these fast growing methodologies should be of great use in the field in the near future.

2.2. Protein identification

In proteomic analyses the identification of proteins is accomplished by means of mass spectrometry (MS). To identify a protein by MS using trypsin digestion of the proteins, two main approaches can be used. The first is peptide mass fingerprinting [5] and the second is peptide sequencing [6]. Peptide mass fingerprinting is usually performed with MALDI-TOF MS, whereas peptide sequencing uses tandem MS (MS/MS).

Mass fingerprinting involves measuring the molecular masses of all the tryptic products. The molecular masses are matched with the theoretical sizes of the trypsin fragments from known protein sequences. Peptide sequencing by MS uses two mass spectrometers connected in series. After ionization, the mixture of charged peptides enters the first mass spectrometer, where the peptides are separated according to their mass/charge ratios. The instrument is adjusted so that only a specific species is directed into a collision cell, where the peptide is broken down into a series of fragments, resulting from the sequential removal of individual amino acids from the end of the peptide ion. The fragments are separated in the second mass spectrometer. The differences in molecular weight between successive fragments, which differ by the mass of one amino acid, identify the peptide sequence.

2.3. Protein microarrays

The other main technological approach in proteomics is protein microarrays [7,8]. In allergy, microarray technology is directed to *in vitro* diagnosis. With an immunoassay format, this technology allows the simultaneous analysis of a large number of IgE antibodies with only a small amount of serum sample. Allergen microarrays are built by immobilizing multiple allergens onto a modified glass slide in an arrayed fashion. The incubation of microarrays with the samples gives rise to the binding of allergen-specific IgE from the samples with its corresponding allergen in the array. After a washing step to remove non-reacting material, a labelled anti-human IgE antibody is added. After a second washing step, the label is detected. The secondary anti-human IgE antibody can be labelled fluorescently or enzymatically. The assessment of reactivity is achieved with fluorescence, chemiluminescence or visible or UV absorbance.

3. Identification of new allergens

Conventional identification and characterization of allergens requires extensive effort and a large amount of starting material, although the search for clinically relevant allergens has progressed dramatically within the last several years. Recently, proteomic technologies using 2D-PAGE and immunoblotting have been applied in the identification of new allergens.

Allergy to latex has been investigated with proteomic approaches. Immunoblots of two-dimensional electrophoretic separations of non-ammoniated natural rubber latex proteins were used to compare the reactivity of several different polypeptides against IgE, IgG and subclasses of IgG [9]. Several antigens were identified as significant owing to their reactivity with antibodies in latex-allergic patients. Four proteins reacted with the IgE, IgG1, IgG3 and IgG4 of only spina bifida patients with latex allergy, while one other protein reacted only with healthcare workers. These proteins were not identified. It is well known that children with spina bifida have a high incidence of latex-allergic reactions due to a high degree of exposure to latex products as a consequence of repeated surgical procedures, the implantation of latex-containing materials and catheterization. At present, it is known that latex-sensitive patients with spina bifida react preferentially to the Hev b 1 and Hev b 3 latex allergens, whereas latex-sensitive healthcare workers produce IgE antibodies preferentially directed to Hev b 5 and Hev b 6. In another work, proteins extracted from fresh *Hevea brasiliensis* latex were separated by 2D-PAGE and IgE-reactive proteins were analyzed by immunoblotting with sera from healthcare workers with latex allergy [10]. Protein microsequencing and monoclonal antibodies were used to identify the latex allergens. Seven of the soluble IgE-reactive protein spots showed homology with enolase, superoxide dismutase, triosephosphate isomerase, the proteasome subunit, and chitinase, and they represent previously undescribed latex allergens. In contrast, nine protein spots corresponded to known latex allergens, namely prohevein, hevein, the prohevein C-domain, and hevamine. Chardin et al. pointed out that 2-D analysis of the sensitization to latex allergens allows the identification of allergen isoforms and the characterization of individual response diversity [11]. More recently, Yagami et al. also used a proteomic strategy to identify allergens to latex proteins [12]. Five previously reported allergens (Hev b 9, Hev b 7, Hev b 11, Hev b 6 and hevamine) and five new allergen candidates (UDP-glucose pyrophosphorylase, isoflavone reductase, rotaminase, thioredoxin and citrate-binding protein) were identified.

Dermatophagoides farinae mite allergens, extracted from whole cultures in the presence of a mild detergent, were separated by 2D-PAGE with subsequent immunoblotting [13]. IgE-binding proteins were detected with individual mite-sensitive patient sera and the anti-*Dermatophagoides pteronyssinus* human sera pool. In addition to identification of the Der f 1, Der f 2, and Der f 3 allergens, new allergens were characterized as Der f 4, Der f 5, and 2 high-molecular mass allergens. Microsequencing of peptides from the latter allergens revealed significant homologies with the Mag 3 allergen from *D. farinae* and with chitinase from the prawn *Penaeus japonicus*.

Food allergies have also been studied using proteomic technologies to identify allergens. Sander et al. identified four new allergens by means of 2D immunoblotting [14], and demonstrated the interindividual variation of wheat flour sensitization in baker's asthma. Beyer et al. identified four sesame seed allergens using 2D-PAGE and Edman sequencing [15]. Two of the allergens belonged to the group of seed storage proteins that are well known as allergens in peanuts and various tree nuts. The authors further described a sequence homology between an IgE-binding site on the peanut allergen Ara h 1 and one of these sesame seed allergens, Ses i 3, which belongs to the same group of proteins. Recognition of this IgE-binding site in certain patients could result in clinical reactivity to both peanuts and sesame seeds.

Costantin et al. compared IgA- and IgE-reactive antigens in wheat using sera from patients with celiac disease and food allergy to wheat by two-dimensional immunoblotting [16]. The results obtained indicated that the IgA antibodies from celiac patients and the IgE antibodies from allergic patients recognized distinct profiles of wheat antigens. The study demonstrates that wheat contains antigens/epitopes that are preferentially recognized by celiac patients, whereas others elicit IgE-mediated food allergy. This finding suggests that the nature of a food antigen may influence the quality of the pathological immune response in the gut and has implications for the diagnosis and therapy of hypersensitivity to wheat. Two-dimensional electrophoresis with an immobilized pH gradient followed by acetic acid/urea-polyacrylamide gel electrophoresis has been developed for the detection of low-molecular weight wheat allergens [17]. Following separation, proteins were transferred to a polyvinylidene difluoride membrane. The electroblotted membrane was immunolabelled with serum from an individual allergic to wheat in order to identify allergenic proteins. Mass spectrometry analysis revealed that these proteins were alpha-amylase/trypsin inhibitors and lipid transfer proteins [17].

A novel shrimp allergen, designated Pen m 2, was identified using two-dimensional immunoblotting and sera from subjects with shrimp allergy [18]. The novel allergen was purified by anion exchange chromatography and was shown to have arginine kinase activity, to react with the serum IgE from shrimp-allergic patients, and to induce immediate-type skin reactions in sensitized patients. This novel allergen could be useful in allergy diagnosis and in the treatment of crustacean-derived allergic disorders [18].

Cow's milk allergens have been identified by proteomic analysis [19]. In the group of 20 patients studied, the prevalence of cow's milk allergens was as follows: 55% α_{s1}-casein, 90% α_{s2}-casein, 15% β-casein, 50% κ-casein, 45% β-lactoglobulin, 45% bovine serum albumin, 95% IgG-heavy chain, 50% lactoferrin, and undetectable α-lactalbumin [19].

Pollen allergens have been analyzed with proteomic approaches. Japanese cedar (*Cryptomeria japonica*) pollen proteins were separated with 2D-PAGE, and sera from subjects allergic to *C. japonica* pollen were used to detect the allergens [20]. Mass fingerprinting was used to elucidate the diversity of the major allergens. Twelve isoforms of Cry j 1 and 3 isoforms of Cry j 2, the best-characterized allergens of this pollen, were detected. In total, 31 spots were found to be more reactive than the highest IgE-reactive isoform of Cry j 2. The authors concluded that proteomic

approaches reveal considerable interindividual variation in IgE-binding patterns to *C. japonica* proteins and contribute to the repertoire of numerous *C. japonica* allergens other than Cry j 1 and Cry j 2.

More recently, Corti et al. used a proteomic approach to detect grass allergens from natural protein extracts [21]. A five-grass commercialized pollen extract used for diagnosis and immunotherapy was resolved by 2D-PAGE and assayed with sera from pollen-allergic patients whose sensitization profile was dissected using IgE reactivity to recombinant allergens. They identified 6 out of 8 expected allergens, together with different molecular isoforms of single allergens.

Petersen et al. performed a study of the allergen composition and the allergenic potency of maize (*Zea mays*) pollen in comparison with pollen from timothy grass (*Phleum pratense*) using proteomic technologies [22]. They found that 65% of the sera reactive to timothy pollen also bound to maize pollen proteins. They failed to find any novel, maize-specific pollen allergens, but they did find cross-reacting allergens belonging to groups 1 and 13 (Zea m 1 and 13), both having high IgE prevalence, as well as the presence of the less important group 3 and 12 allergens. Zea m 1 and Zea m 13 showed sequence identities of 72 and 70%, respectively, to the corresponding Phl p 1 and Phl p 13 allergens of timothy grass pollen. The authors concluded that maize pollen plays a less important role as a sensitizer than the pollen of common grass species. They reported that this may be explained in terms of morphological differences, as well as the apparently lower allergen content and the lower number of allergen groups found in maize pollen.

The results obtained to date show that proteomic approaches using two-dimensional immunoblotting appears to be a powerful strategy for the identification of allergenic proteins. The principal advantage of these methods is the low amount of sample needed. However, further studies are necessary.

4. Diagnosis of allergic diseases

4.1. Conventional methods

A detailed history regarding the temporal pattern of symptoms may indicate the allergens to which the patient is sensitive. IgE-mediated sensitivity can be established through skin testing. Confirmation of *in vivo* tests is obtained by IgE antibody testing with *in vitro* laboratory assays (Fig. 2). *In vitro* test methods include several immunoassay formats with solid-phase supports. For a review of *in vitro* assays for the diagnosis of IgE-mediated disorders, see Hamilton and Adkinson [23].

4.2. Allergen production for in vitro diagnosis

In vitro diagnostic techniques in allergic diseases depend on the ability to demonstrate the existence of specific IgE antibodies directed against an allergen. This requires the use of allergens in their native form. The quality of antigens used in *in vitro* assays strongly influences the specificity of IgE antibody measurements. The first tests used crude or purified extracts of the allergen sources. In recent years, however, researchers and manufacturers

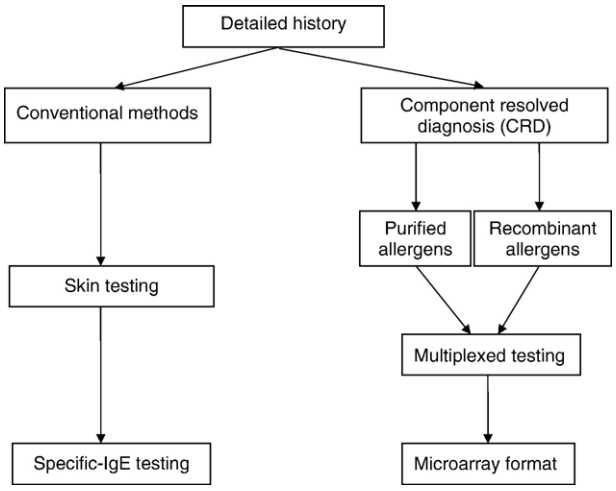

Fig. 2. General schema for the diagnosis of allergic diseases.

have improved the quality of the allergenic material used in diagnostic tests. The European Union has promoted the CREATE Project (Full title of the project: Development of Certified Reference Materials for Allergenic Products and Validation of Methods for their Quantification) with the aim of improving allergen standardization [24].

With allergen extracts it is only possible to determine whether a patient is sensitized to undefined allergens from a given allergen source, but the disease-eliciting components cannot be identified with these tests. Most patients raise IgE antibodies to only some allergenic components. The increasing availability of allergen panels derived from several different sources enables a detailed analysis of the sensitization profile in individual patients to be made. This concept has been defined as "component resolved diagnostics" (CRD) [25]. The purpose of CRD is to establish significant associations between specific subpopulations of specific IgE, measured by the use of individual allergen components or parts thereof, and clinically relevant aspects of the allergic disease [26]. Also, knowledge of the specific allergen is of critical importance for developing the correct composition of vaccines for specific immunotherapy.

The purification and characterization of a specific allergen is a complex procedure involving a combination of analytical and preparative methods, together with immunochemical and biological procedures. Aqueous extracts are subjected to several preparative chromatographic steps in order to obtain a sufficient amount of material for finer analytical separations.

4.3. Recombinant allergens

The identification of specific allergens, together with recombinant DNA techniques, has allowed investigators to obtain large quantities of specific allergens for use in *in vitro* tests. For the recombinant production of allergens, suitable expression systems, culture conditions, and purification steps must be established for each individual allergen. Knowledge of the glycosylation state, the occurrence of internal disulphide bonds, and the overall stability of the protein are useful information for the establishment of the expression system. *Escherichia coli* is a suitable host for

proteins that are not glycosylated or where glycosylation is not necessary. In certain cases IgE reactivity is directed to the carbohydrate moiety of the allergen glycoprotein. Glycosylated proteins can be produced in several eukaryotic expression systems, such as the yeast *Pichia pastoris*, baculovirus in host insect cells, and various plants [27,28]. However, in many instances the degree of glycosylation is not comparable with that of the natural protein, which can reduce reactivity.

Purified recombinant allergens must be carefully investigated as regards their biochemical, biophysical and immunological properties. For diagnostic purposes it is essential that the recombinant molecules exhibit IgE reactivity equal to the natural wild-type molecules.

4.4. Microarrays for the diagnosis of allergic diseases

While specific IgE assays have mainly been designed as single allergen tests, protein array technologies allow the simultaneous measurement of IgE antibodies of multiple specificities with the same serum sample; *i.e.*, multiplex analysis (Fig. 2). The first experimental microarray system for allergy diagnosis was reported by Wiltshire et al. [29]. Those authors described the production of a microarray of multiple allergen extracts and demonstrated the usefulness of this microarray in combination with an immunoassay with rolling-circle amplification to simultaneously detect allergen-specific IgEs for multiple allergens in patient samples. Two years later, Kim et al. also used crude allergen extracts in another microarray [30]. They attached unpurified *D. pteronyssinus*, egg white, milk, soybean, and wheat allergen extracts to silica chips. The report provided few conclusive data about the accuracy and precision of chip IgE assay results with crude allergen extracts other than from *D. pteronyssinus*.

The use of purified native allergens, recombinant allergens or both should provide better results. This alternative has been applied to microarrays. Hiller et al. reported an international collaborative study using a microarray with 78 recombinant and 16 natural allergen molecules representative of major allergen groups (*e.g.* mites, fungi, insect venoms, animal epidermis, and trees) [31]. A single fluorescent monoclonal anti-human IgE antibody allowed the detection of IgE reactivities to immobilized allergens. The performance of the allergen microarray was assessed by examining reproducibility and correlation with skin-prick testing or the recognition of allergens spotted onto nitrocellulose under conditions of an allergen excess.

Fall et al. described a microarray with allergen extracts and recombinant/purified allergens (24 preparations) for the screening of allergen-specific IgE [32]. They used an HRP-labelled antibody to detect the reactions. The slide was placed in the flow cell of a PASA (parallel affinity sensor array) device and a chemiluminescent substrate was pumped into the flow cell. Chemiluminescence intensities were detected with a CCD camera. The authors reported adequate reproducibility.

Several studies have been reported that used microarrays produced by VBC-GENOMICS (Vienna, Austria). Jahn-Schmid et al. [33] compared the analytical performance of this chip IgE assay with that of the Pharmacia CAP system and an in-house ELISA. They used purified recombinant grass allergens (Phl p 1, 2, 5, and 6) and birch tree pollen (Bet v 1 and 2). The results showed coefficients of correlation greater than 0.900 between the microarray technology and currently used methods. Later, Deinhofer et al. [34] applied this microarray technology to create a multi-allergen test system, based on microarrayed recombinant allergens. The authors pointed out that although chip-based allergy diagnosis may equal well-established allergy test systems in terms of sensitivity and specificity, several problems must be properly addressed before allergen chips can be used for routine testing. Microarray assays are generally prone to producing artificial signals, because defects in the glass substrate and partial or complete dehumidification may give rise to artificially increased signals [34].

Most of the microarray assays presented for allergic diagnosis use fluorescence or chemiluminescence detection. Lebrun et al. developed a colorimetric microarray assay for allergen-responsive human IgE [35]. Three common allergens (mould, dustmite, grass) were arrayed. The results obtained indicated that this system reliably detects allergen-specific IgE below 0.35 IU, the current WHO standard cutoff.

Recently, Wöhrl et al. have reported a study in which they comparatively analyzed a new component-based allergen microarray (ISAC version CRD-50, VBC-GENOMICS, Vienna, Austria) and ImmunoCAP for their clinical relevance in patients with allergic rhinoconjunctivitis to five aeroallergens (house dust mite, cat dander, birch, grass and mugwort pollen) [36]. They concluded that component-based testing and the whole-allergen CAP are equally efficient in the diagnosis of grass-, birch- and cat-allergic patients. Although slightly less sensitive, the microarray performs sufficiently well for the diagnosis of house dust mite-allergic patients, but needs alternative and/or additional components to adequately detect mugwort allergy. Ott et al. [37] used a customized, commercially available allergen microarray (ISAC Atopy; VBC-GENOMICS, Vienna, Austria) containing recombinant birch (rBet v 1, rBet v 2), alder (rAln g 1), hazel (Cor a 1) and timothy grass pollen (rPhl p 1, rPhl p 5, rPhl p 6 and rPhl p 7) components to detect IgE antibodies in capillary blood samples from patients with atopy. They obtained high or very high correlation coefficients between the microarray results after capillary and venous serum sampling.

As indicated by Joos and Berger, despite many years of development protein microarrays are not widely used in diagnostics [38]. They indicated that two factors are mainly responsible for this situation. First, most efforts in the past have been directed towards nucleic acid systems. Second, the manufacturers of a diagnostic test need to ascertain that the correct component (in our case the allergen) is functionally immobilized on the microarray. These compounds need to undergo all kinds of quality control procedures. The required regulatory procedures command a substantial share of the development budget of new tests.

4.5. Mapping allergenic epitopes

Microarrays have also been used to map allergenic epitopes. The IgE epitope mapping of allergens might reveal relevant information about antigen structure, and the patient's immune response, and is fundamental for designing hypoallergenic

immunotherapeutic agents. Shreffler at al. developed a peptide microarray-based immunoassay to map peanut epitopes using microliter quantities of serum [39]. A set of 213 overlapping 20-residue peptides was synthesized corresponding to the primary sequences of Ara h 1, Ara h 2, and Ara h 3. These were arrayed in triplicate along with the corresponding recombinant proteins onto glass slides and used for immunolabelling with serum from 75 peanut-allergic or -sensitized individuals, 10 atopic controls, and 5 non-atopic individuals. The results indicated a remarkable heterogeneity in the number and patterns of epitope recognition. High epitope diversity was found in patients with a history of more severe allergic reactions.

Later, the same group used a microarray immunoassay for IgE and IgG4 epitope mapping of the previously characterized peanut allergen Ara h 2 [40]. By using peptides of 10, 15, or 20 amino acid residues, they were able to define 11 antigenic regions. The IgE and IgG4 epitopes recognized by patients were largely the same, and there was a positive association between the IgE and IgG4 signals, suggesting coordinate regulation. Cluster analysis of peptide-binding patterns confirmed the specificity of antibody-peptide interactions and was used to define core epitopes ranging from 6 to 16 residues in length.

5. Conclusions

At present proteomic approaches are only beginning to be applied to the study of allergy. On one hand, these technologies are being used to discover new allergens. On the other, proteomic approaches using allergen microarrays allow a large number of anti IgE antibodies to be measured simultaneously with this multiplexed format. However, allergen microarrays are not yet routinely used in allergy laboratories. We anticipate that in the near future this promising technique will be used routinely in the *in vitro* diagnosis of allergy.

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