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Research Article

A novel multiplex method for the simultaneous detection and relative quantitation of pollen allergens

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Standardization of pollen protein extracts is essential in order to ensure efficiency and safety in allergy diagnosis and immunotherapy. In this paper, we have optimized a multiplex Western blotting method for the simultaneous detection of four olive pollen allergens (Ole e 1, Ole e 2, Ole e 5, and Ole e 9) on a single blot using a monoclonal antibody from mouse and three polyclonal antibodies raised in rabbit. We utilized unconjugated Fab antibody fragments for blocking rabbit primary antibodies, and fluorescence-based detection. These changes allowed an accurate and reliable comparative quantitation of these allergens among pollen-protein samples from six olive cultivars. In addition, we also tested the IgE-binding capacity of these pollen extracts by reprobing the same blot with a pool of sera from eight patients allergic to olive and detection with enzyme conjugated antibodies. A noticeable variability regarding allergen content and IgE-reactivity was found among the olive cultivars analyzed. Moreover, we could easily confirm the identity of some of the IgE-binding proteins by simply overlapping both fluorescence and chemiluminescence images. This method is versatile since it can be applied to other allergogenic plant species and extended to other allergens.

Keywords:

Allergen / Fab fragment / Multiplex Western blotting / Pollen / Standardization
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1 Introduction

Olive pollen produces seasonal respiratory allergy in the Mediterranean area and in other temperate climates of the world where this species is intensively cultivated. To date, up to 11 allergenic proteins have been identified and characterized in olive pollen extracts, called Ole e 1 to Ole e 11 [1–4]. Ole e 1 is the most prevalent sensitizing agent, affecting more than 70% of patients suffering from olive pollinosis [2]. Others proteins like Ole e 4, Ole e 9, Ole e 10, and Ole e 11 have been also reported as major allergens [1–4]. In addition, Ole e 6 and Ole e 7 allergens reach high values of clinical incidence [1, 2] and Ole e 2, Ole e 3, and Ole e 7 are involved in cross-reactivities, being classified as panallergens. Some of these proteins exhibit a significant polymorphism in 1-D and 2-D polyacrylamide gels, shown as a pattern of multiple bands or spots, respectively [4, 5]. This variability is due to the existence of several protein forms of the allergen as a consequence of amino acid substitutions, PTMs (e.g. glycosylation), and/or

the presence of multimeric forms [4, 5]. Moreover, some allergens show conspicuous quantitative differences, depending on the genetic origin (i.e. cultivar) of pollen [6, 7]. Currently, this molecular variability is underrepresented in the commercial protein extracts of olive pollen used for diagnosis and treatment of olive pollen allergy. The relative abundance of individual protein forms of an allergen with distinct IgE-reactivity might affect the total allergenic potency of the extract. The standardization of commercial olive pollen extracts is of great importance in order to warrant efficiency and safety in the diagnosis and immunotherapy procedures. Therefore, it is important to ensure that the allergenic variability in standardized protein extracts resembles as much as possible that observed in the natural sources.

Current procedures for preparation of protein extracts frequently determine the IgE response of a population, represented by a pool of sera from allergic patients. Analysis of patient's reactivity is performed in vivo by SPT (skin prick test), as well as in vitro by competitive assays like RAST (radioallergosorbent test) and ELISA inhibition tests among others. These procedures do not routinely discriminate individual allergens in the case of olive, and hence do not explain their individual contribution to reactivity. Therefore, further

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Abbreviations: Ab, antibody; SOD, superoxide dismutase

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analysis of the variability in both the number and nature of those allergens present in the mixture would greatly benefit standardization. Methods based on electrophoretic separation of proteins and immunoblotting could be used in parallel to in vitro competitive assays for this purpose [8, 9]. Allergen-specific antibodies can be used to detect and quantitate allergens in nonstandardized extracts by immunoblotting, yet it is a time-consuming procedure. The standard method for visualizing the profile of two or more specific antigens at the same time involves either the production of blot replicas, or additional stripping and reprobing steps for each additional protein of interest [10]. This fact often makes difficult the normalization and comparison between protein bands because alignment of different blots is often compromised. In addition, the stripping procedure also tends to elute proteins from blots, resulting in decreasing signal intensities after each round of blot stripping and reprobing [11, 12]. Simultaneous Western blot analyses of two allergens can be easily carried out by using primary antibodies raised in different animal species (e.g. mouse and rabbit), in such a way that detection can be performed by using secondary antibodies conjugated to different reporting molecules, such as fluorochromes or enzymes. In this paper, we have developed a novel multiplex approach, which allows the concurrent fluorescent detection and quantitation of two major (Ole e 1 and Ole e 9) and two minor (Ole e 2 and Ole e 5) allergens on the same blot. The putative applications and further developments of this multiplex are discussed.

2 Material and methods

2.1 Plant material

Mature pollen grains were harvested from selected olive trees (cvs. "Picual", "Arbequina", "Cornicabra", "Verdial de Vélez-Málaga" and "Lechín de Sevilla") grown in the province of Granada, Spain (37°9'56"N 3°35'28"W). For this purpose, flowering shoots at anthesis were vigorously shaken inside large paper bags. The pollen was purified by sieving through a nylon mesh of 50 µm in pore diameter, frozen in liquid nitrogen, and stored at –80°C until use. An olive pollen batch of uncertain varietal origin was also purchased from Iberpolen S.L. (Jaén, Spain).

2.2 Olive allergic patients' sera

Serum samples from eight olive allergic individuals were obtained at the Allergy Service, Hospital Universitario San Cecilio, in Granada (Spain). All selected patients had a clinical history of rhinitis and/or asthma and were immunoCAP- (Phadia) and SPT-positive to olive pollen protein extracts. Serum samples were individually frozen in liquid nitrogen and stored at –80°C until use.

2.3 SDS-PAGE

Crude pollen protein extracts were prepared by stirring 0.1 g of pollen in 1 mL of extraction buffer (40 mM Tris–HCl, 2% (v/v) Triton X-100, 1 mg/mL ascorbic acid, and 10 µL/mL of a protease inhibitor cocktail) for 4 h at 4°C. After centrifugation at 14 000 × g for 30 min at 4°C, the resulting supernatants were filtered through a 0.20 µm diameter syringe filter, and stored in aliquots at –20°C until use. Protein concentration in the samples was measured using the Bio-Rad reagent (Bio-Rad Corp., USA) based in [13] and BSA as standard, following the manufacturer's instructions.

For each sample, 30 µg of total protein were diluted in Laemmli sample buffer, heated at 100°C for 15 min, and allowed to cool to room temperature. SDS-PAGE was performed in 12% (w/v) polyacrylamide gels according to Laemmli [14]. Proteins were separated using a Mini-Protein III system (Bio-Rad) at 200 V for 1 h. ECL Plex Fluorescent Rainbow markers (Amersham Biosciences, Sweden) were used as weight standards. Gels were electrophoresed in duplicates, the first gel being stained with silver nitrate [15] and the second gel electroblotted for immunodetection purposes as described below.

2.4 Antibody production

The anti-Ole e 2 polyclonal antibody (Ab) was produced by immunization of rabbits with a keyhole limpet hemocyanin (KLH)-linked synthetic peptide (AQSATFPQFKPEEM) designed from the predicted amino acid sequence of an olive profilin (Ole e 2). Specificity of the antibody was already reported by Western blotting experiments and immunolocalization of the allergen [16]. The anti-Ole e 9 polyclonal Ab was produced as described above using a synthetic peptide (YPYFAYKNQPTPDT) from the Ole e 9 amino acid sequence [17, 18]. We also purchased a commercially available polyclonal Ab that recognizes a chloroplastidic isoform of Cu/Zn-superoxide dismutase (SOD) from *Arabidopsis thaliana* (Agrisera, Sweden, Product No. AS06 170), with probed cross-reactivity to Ole e 5 [19]. Finally, the anti-Ole e 1 mAb (10H1), widely used for multiple applications [5, 6, 20–22], was kindly provided by Dr. Carlos Lahoz.

2.5 Western blotting experiments

Total proteins were transferred from polyacrylamide gels onto PVDF membranes in a Trans-Blot Semi-Dry transfer cell (Bio-Rad) according to standard procedures. Prior to antibody incubations, PVDF membranes were incubated with a blocking solution containing 0.3% (v/v) Tween 20, and 10% (w/v) skimmed dried milk in TBS for 2 h. Primary Abs were diluted in blocking solution and incubated for 2 h at room temperature, whereas secondary Abs were diluted in TBS buffer containing 0.3% (v/v) Tween 20 (TBST) and incubated for 1 h at room temperature in the dark. After Ab

Table 1. Antibodies and dilutions used for Multiplex Western blotting and IgE-binding experiments.

Target	Primary antibody (source) ^{a)}	Dilution	Secondary antibody (source)	Dilution
Ole e 1	Mouse anti-olive Ole e 1 mAb (Lauzurica et al. 1988) [20]	1:20 000	Goat anti-mouse IgG Ab, Alexa fluor 488-conjugated (Molecular Probes, product reference A11017)	1:10 000
Ole e 2	Rabbit anti-olive Ole e 2 PoAb (Morales et al. 2008) [16]	1:20 000	Donkey anti-rabbit IgG (Fab fragment) Ab, Cy3-conjugated (Jackson ImmunoResearch, product reference 711-167-003) Donkey anti-rabbit IgG (Fab fragment) Ab (Jackson ImmunoResearch, product reference 711-007-003)	1:10 000 1:200
Ole e 5	Rabbit anti-Cu/Zn SOD PoAb (Agrisera Prod. No. AS06 170)	1:250	Goat anti-rabbit IgG Ab, Alexa fluor 633-conjugated (Molecular Probes, product reference A21072)	1:10 000
Ole e 9 N-terminal domain	Rabbit anti-olive Ole e 9 PoAb	1:10 000	Goat anti-rabbit IgG Ab, Alexa fluor 633-conjugated (Molecular Probes, product reference A21072)	1:10 000
Olive pollen allergens	Sera from olive allergic patients	1:10	Goat anti-human IgE, HRP-conjugated (Sigma, product reference A9667)	1:10 000

a) mAb, monoclonal antibody; PoAb, polyclonal antibody.

incubation, membranes were rinsed in TBST buffer four times for 5 min each. The different Abs used in this work and their corresponding dilutions are summarized in Table 1. Each experiment described below was repeated in triplicate. Negative controls included preimmune serum in the cases of Ole e 2 and Ole e 9, as well as sera from nonallergic patients.

2.5.1 Individual immunodetection of allergens

The antibodies to Ole e 1, Ole e 2, Cu/Zn SOD (Ole e 5), and Ole e 9, were separately assayed against total protein extracts from “Picual” pollen.

2.5.2 Double immunodetection of allergens

Anti-Ole e 1 and -Ole e 2 antibodies (raised in mouse and rabbit, respectively) were concurrently assayed against total protein extracts from “Picual” pollen. Detection was developed by incubation in a solution containing the anti-mouse IgG-Alexa fluor 488 and anti-rabbit IgG (Fab fragment)-Cy3 secondary Abs.

Similarly, anti-Cu/Zn SOD (Ole e 5) and Ole e 9 Abs (both raised in rabbit) were simultaneously tested against total protein extracts from “Picual” pollen. In this case, the signal was detected using an anti-rabbit IgG, Alexa fluor 633-conjugated as the secondary Ab for both, Cu/Zn SOD (Ole e 5), and Ole e 9 proteins.

2.5.3 Multiple immunodetection of allergens

The multiplex assay was performed against total protein extracts from pollen of five different cultivars and from a

commercial source, according to the flowchart displayed in Fig. 1. Briefly, PVDF membranes containing the different protein samples were first incubated with a mixture of anti-Ole e 1 and Ole e 2 Abs, followed by incubation with a solution containing the anti-mouse IgG-Alexa fluor 488 and anti-rabbit IgG (Fab fragments)-Cy3 secondary Abs. In a second step, the free binding sites of the anti-Ole e 2 Ab were blocked using anti-rabbit IgG Fab fragments. Then, membranes were concurrently treated with the anti-Cu/Zn SOD (Ole e 5) and Ole e 9 Abs followed by incubation with an anti-rabbit IgG-Alexa fluor 633 secondary Ab.

2.5.4 Analysis of the reactivity of patient's sera

The IgE reactivity was assayed on the same blots using a pool of eight olive allergic patients' sera, diluted in blocking solution. A goat anti-human IgE-peroxidase was used as the secondary Ab. Chemiluminiscent detection of Ab binding was carried out with the Immun-StarTM WesternCTM Chemiluminescence Kit (Bio-Rad, product reference 170-5070) according to the manufacturer's instructions.

2.6 Imaging and allergen quantitation

After washing, PVDF membranes were left to dry at room temperature in the dark. Imaging was carried out with a Pharos FX Plus Molecular Imager (Bio-Rad) using the Quantity One v4.6.2 software (Bio-Rad). In multiplex experiments, blots were sequentially scanned at a resolution of 50 μ m with the 488 (blue), 532 (green), and 635 nm (red) lasers using the multichannel function and the appropriate emission filters. The intensity of each fluorescent band was calculated using the quantitation tools of the Quantity One 4.6.2 software

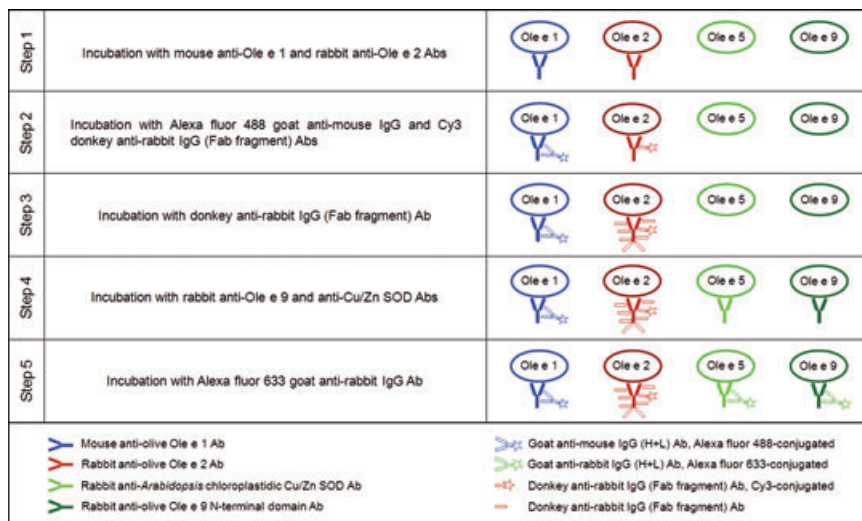


Figure 1. Flowchart of multiplex fluorescent detection of olive pollen allergens Ole e 1, Ole e 2, Ole e 5, and Ole e 9 on Western blots.

(Bio-Rad). The bands reactive to each antibody were identified and defined with the help of specific tools of the program (e.g. “contour”). Quantitation of each band was calculated using the “volume” parameter of the program, defined as the total signal intensity of the pixels $\times$ area of a single pixel, inside the defined boundary drawn for each band. This intensity was compared with that of the background, in a previous calibration procedure. For presentation purposes, we automatically assigned a false color to each channel, which corresponds to the excitation wavelength (e.g. images captured by exciting the Alexa Fluor 488 fluorophore with the blue laser were displayed in blue), to better differentiate images. Chemiluminiscent bands were imaged with a ChemiDoc XRS Molecular Imager (Bio-Rad). Fluorescent and chemiluminiscent images were overlapped using the facilities of the Adobe Photoshop C62 9.0 software (Adobe Systems Inc., USA).

3 Results

3.1 Multiplex detection of olive pollen allergens on Western blots

In this work, we have developed a five-step multiplex method that allows simultaneous detection of four different allergenic proteins, namely Ole e 1, Ole e 2, Ole e 5, and Ole e 9 on Western blots. Firstly, each antibody was assayed individually on blots containing total protein extracts from olive (cv. “Picual”) pollen, in order to demonstrate their specificity (Fig. 2, lanes 2–5). Data showed that the anti-Ole e 1 antibody was able to recognize two major protein species of approximately 18.4 and 20 kDa, which correspond to the nonglycosylated and monoglycosylated forms of the allergen, respectively [23]. A very faint band of about 22 kDa, corresponding to a polyglycosylated variant of Ole e 1 was also distinguishable. Three reactive bands in the range of 14–16 kDa were detected on 1-D blots when using the anti-Ole e 2 Ab, as previously described [24], whereas the corresponding preimmune serum

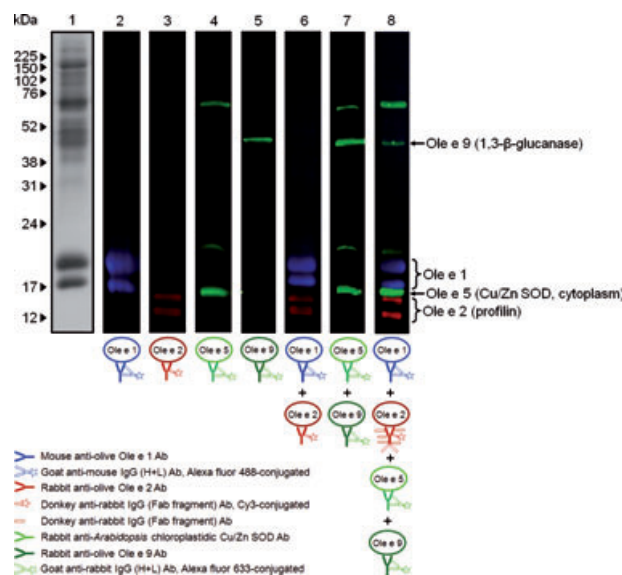


Figure 2. Multiplex fluorescent detection of olive pollen Ole e 1, Ole e 2, Ole e 5, and Ole e 9 allergens on Western blots. To test antibody specificity, olive (cv. “Picual”) pollen total proteins (lane 1, silver staining) were transferred to PVDF membranes and the resulting blots were probed with a mouse anti-Ole e 1 (lane 2), rabbit anti-Ole e 2 (lane 3), rabbit anti-Cu/Zn SOD (Ole e 5) (lane 4) and rabbit anti-Ole e 9 antibody (lane 5). In a second step, both Ole e 1 and Ole e 2 (lane 6), as well as Ole e 5 and Ole e 9 proteins (lane 7) were simultaneously detected on Western blots. Finally, using a multiplex design, the four allergens were simultaneously labeled on the same blot (lane 8). Thirty micrograms of total protein were loaded per lane. Protein markers are displayed on the left.

lacked of reactivity (results not shown). In the bottom end of the blot, the anti-Cu/Zn SOD Ab bound to a protein of approximately 16.4 kDa, which corresponds to a cytosolic form of this enzyme, previously identified as the allergen Ole e 5 [25]. Finally, the anti-Ole e 9 Ab was able to recognize a single protein band of about 46 kDa, which corresponds to a pollen 1,3-β-glucanase [17] not reactive to the

preimmune serum (results not shown). None of the Abs used showed cross-reactivity with the others. However, the anti-SOD Ab also recognized a 22 kDa protein form, and a higher molecular weight band of about 66 kDa, which might correspond to a multimeric form of the enzyme, was also visible. Therefore, a more specific anti-Ole e 5 Ab would be desirable to avoid cross-reactivity with other Cu/Zn-SOD protein forms present in the pollen grain.

In an intermediate step to the final multilabeling procedure, Ole e 1 and Ole e 2 were detected simultaneously on 1-D blots using two specific Abs, raised in different animal hosts. As expected, the resulting blot was a combination of both Ole e 1 and Ole e 2 labeling patterns (Fig. 2, lane 6). Similarly, Ole e 5 and Ole e 9 were also concurrently probed using specific Abs with identical results (Fig. 2, lane 7).

Finally, all the four allergens were tested simultaneously in the same blot (Fig. 2, lane 8), following the five steps method described in Fig. 1. We assigned a false color to each fluorescent labeling, allowing a better visualization and discrimination of allergens on the same blot. Dried blots could be scanned several times and stored at 4°C for at least 1 year without a significant loss of fluorescence.

3.2 Multiplex detection and relative quantitation of olive pollen allergens from different cultivars

We validated the multiplex method by testing total protein extracts from pollen of five olive genotyped varieties, as well as from a commercial pollen sample of uncertain genetic origin. We found that olive cultivars exhibited a remarkable quantitative variability regarding the allergen content (Fig. 3). Thus, “Picual”, “Cornicabra”, and “Lechín” showed high quantities of both 18.4 and 20 kDa species of Ole e 1, whereas levels were lower in the other two cultivars analyzed and almost undetectable in the commercial pollen sample. Moreover, the 22 kDa form of this allergen was only detected as a faint band in “Picual” and “Cornicabra” varieties. Ole e 2 also showed inter-cultivar differences in terms of number and amount of allergen protein species. Indeed, olive cultivars could be grouped into two categories with high (“Arbequina”, “Verdial”, and

“Lechín”) and low (“Picual”, “Cornicabra”, and the commercial sample) Ole e 2 content, respectively. Moreover, they also displayed qualitative differences. Thus, three distinct Ole e 2 protein forms with SDS-PAGE molecular weights of 14.0, 15.1, and 15.6 kDa were detected in “Lechín” and “Picual”. The 15.6 kDa species was absent in “Arbequina”, “Picual”, “Cornicabra”, and the commercial sample, whereas the 15.1 kDa variant was not detected in “Cornicabra” and “Verdial”. Ole e 9 was detected in all cultivars analyzed, and we found significant quantitative differences. An intense fluorescent labeling was observed in “Picual” and the commercial sample, while the allergen remained scarce in the rest of cultivars studied. Finally, Ole e 5 (Cu/Zn SOD) was detected in higher levels in “Arbequina”, “Lechín”, and the commercial sample.

We also measured the intensity of fluorescence of each band detected on the Western blot from Fig. 3B, in order to quantitate the relative amount of Ole e 1, Ole e 2, Ole e 5, and Ole e 9 allergens in the six pollen samples analyzed. The relative quantity of each protein species and the sum of all for the four allergens studied are shown in Fig. 4.

3.3 IgE binding of olive pollen proteins with a pool of sera

The allergenic capacity of olive pollen proteins was assayed by studying their IgE-binding capacity by Western blot. For this purpose, the same blot used above was reprobed with a pool of sera from eight patients hypersensitive to olive pollen and the reaction was detected by chemiluminescence. The IgE-binding pattern of each olive cultivar and the commercial pollen is shown in Fig. 5A. Sera incubation revealed up to seven IgE-reactive bands with molecular weights ranging from 10.3 to 74.2 kDa. However, we found major intercultivar differences in the number and intensity of IgE-binding proteins. The commercial pollen sample did not show any immunoreactivity to sera. In “Picual”, two intense bands of 18.4 and 20 kDa were visible. The spectrum of allergenic proteins in “Arbequina” and “Lechín” extracts included a protein of 10.3 kDa, which showed the highest reactivity to the pool of sera, and four other bands of higher molecular weight but

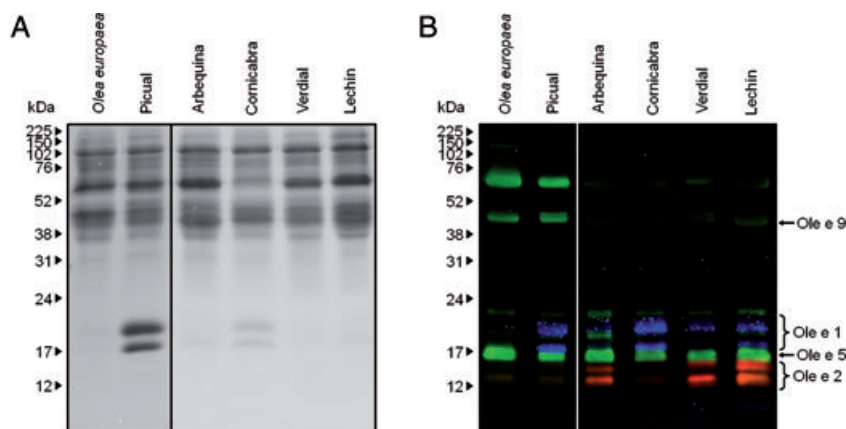


Figure 3. (A) Silver staining of olive pollen total protein extracts from five different olive cultivars and a commercial batch of uncertain varietal origin. (B) Multiplex fluorescent detection of Ole e 1, Ole e 2, Ole e 5, and Ole e 9 allergens on a blot as above. Thirty micrograms of total protein were loaded per lane. Protein markers are displayed on the left of each image.

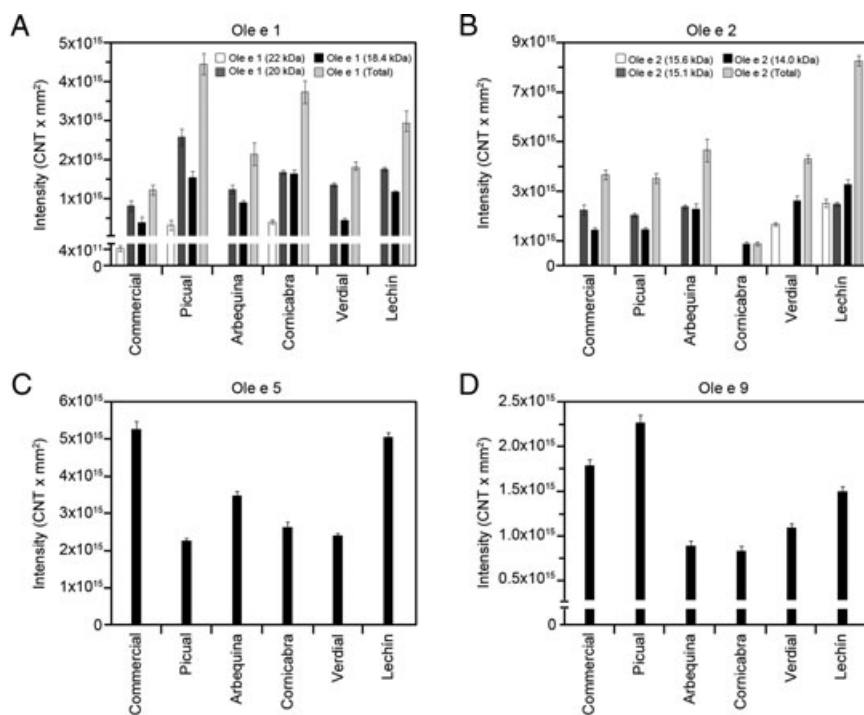


Figure 4. Quantitative analysis of Ole e 1 (A), Ole e 2 (B), Ole e 5 (C), and Ole e 9 (D) allergens detected on the blots corresponding to three independent experiments. The fluorescent labeling intensity was measured in a densitometer and expressed as the average counts (CNT) per surface unit (mm²).

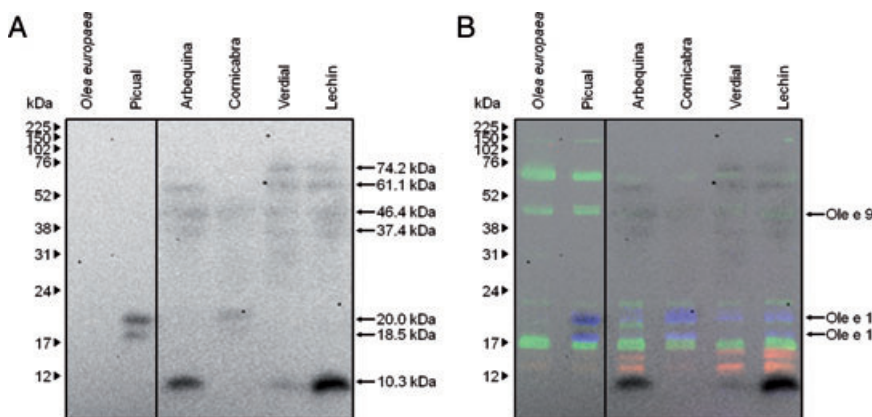


Figure 5. (A) IgE-binding analysis by Western blotting of olive pollen total proteins from five cultivars and a commercial source. The blot used for multiplex detection of allergens was reprobed with a pool of sera from patients allergic to olive pollen. Seven reactive bands with molecular weights ranging from 10.3 to 74.2 kDa were visible on the blot after detection by chemiluminescence. (B) Overlap of IgE-reactive and multiplex protein bands. Three out of seven IgE-reactive proteins were identified as two Ole e 1 species and the Ole e 9 allergen. Protein markers are displayed on the left of each image.

much lower intensity. Finally, “Verdial” showed the highest number of immunoreactive bands but all exhibited a very weak signal.

We could easily confirm the identity of some of the IgE-binding proteins by overlapping both multiplex and chemiluminescence images obtained from the same blot (Fig. 5B). Thus, the 18.4 and 20 kDa bands undoubtedly corresponded to the nonglycosylated and monoglycosylated glycoform of Ole e 1, respectively whereas the 46 kDa band was identified as Ole e 9. The remaining five bands did not match with any of the multiplex bands. We can speculate that the 10.3 kDa band might correspond to either Ole e 7 or Ole e 10, since both allergens have similar molecular masses [26, 27]. In addition, the 37.4 kDa proteins might correspond to the recently characterized Ole e 11 allergen [4]. Two additional bands of higher molecular weight were also visible on Western blots but they did not correspond to any known olive allergen to date. Sera

from non-atopic individuals showed null or very weak reactivity, non-associated to the identified allergens (results not shown).

4 Discussion

In the present work, we have optimized a multiplex method that allows simultaneous detection and relative quantitation of four olive pollen allergenic proteins on a single Western blot. Indirect immunoblotting made unnecessary custom labeling of primary Abs with enzymes, which is time consuming and expensive. Moreover, primary Abs were not chemically modified, so they preserved its antigenic capacity [28–30]. A colorimetric indirect immunoblotting method for multiple antigen detection (MAD) was first developed by Krajewski and coworkers [12]. This procedure allowed the sequential

detection of multiple antigens on a single blot, avoiding additional stripping and reprobing steps or the use of blot replicas. A variation of the MAD method, called “Rainbow Western” has been recently developed by these authors [31], using four different enzyme (i.e. horseradish peroxidase) substrates that produce brown, black, red, and green precipitates at the site of reaction. However, this method has a number of limitations. Thus, enzyme substrates (e.g. 3,3'-diaminobenzidine) often precipitate, so they might block non-overlapping epitopes on the same protein and/or prevent detection of other antigens on the same blot. Repeated treatments with H_2O_2 might also damage some protein epitopes. Moreover, the simultaneous detection of multiple antigens is only possible when the antigenic proteins present different molecular weights and do not overlap on the blot. Finally, relative quantitation between protein extracts is quite challenging owing to the low reproducibility of the enzymatic reactions.

Our modified multiplex protocol is simple and time saving. The main advantages of this method are: (i) relative quantitation of labeled proteins is more accurate, (ii) overlapping antigens are easily discriminated, (iii) image acquisition is carried out by sequential scanning in a single step, (iv) primary Abs raised in the same animal host can be simultaneously probed with others obtained in different hosts, and (v) it requires a lower amount of protein for analysis.

These advantages are mainly derived from the use of fluorescent detection methods, which previous studies have shown may involve in some cases a tenfold greater quantifiable range but with two- to fourfold less sensitivity than the well-known chemiluminescent detection methodologies [32]. These authors provide several examples of analogous experiments showing excellent limits of detection and linear range.

An additional advantage of the use of fluorescent detection as shown here consists in the potential use for allergen quantitation purposes. Fluorescently labeled probes offer ten to thousands times greater quantifiable linear range than other methods like enhanced chemiluminescent substrates. This occurs as the result of their direct detection, which is independent from the enzyme/substrate kinetics associated to other detection systems [32, 33]. Accurate quantitation of allergens using this multiplex method will greatly benefit of their comparison and standardization using alternative methods, considered highly specific, reproducible, and sensitive like ELISA-derived quantitation methods [34].

Two major methodological novelties were tested in this work. First, we used unconjugated Fab fragments for blocking primary Abs from the same host species. In a pioneer study, Martin and coworkers used enzyme-conjugated Fab fragments for simultaneous blocking and detection [35]. However, the use of conjugated Fab fragments in excess might cause smearing, particularly when the antigen is present in high amounts. This undesirable effect might make difficult to distinguish antigens that are sited very close to each other on the blot. This problem was overcome when detection and blocking were carried out sep-

arately using fluorochrome-conjugated and unconjugated Fab fragments, respectively. We found that long incubation times and/or an excess of unconjugated Fab fragments might decrease fluorescent labeling, likely because both Abs compete for binding to the primary Ab. To avoid this effect, it is essential to optimize the concentration of the blocking Fab fragments, as well as the incubation time.

Second, antigens were simultaneously detected using fluorochrome-conjugated secondary Abs, which are commercially available. We minimized fluorescence crosstalk by using several fluorescent probes, which were excited with different laser lines and presented nonoverlapping emission wavelengths. Images were captured using a densitometer with high sensitivity and a broad dynamic range. Since the fluorescent signals can be spectrally separated using the appropriate emission pass filters, integrated intensities and relative quantitation data were obtained by means of volumetric analysis from a single digital image.

The multiplex procedure described in the present work might be a useful tool for a faster and more accurate standardization of protein extracts that are used for olive pollen allergy diagnosis and immunotherapy. Despite we focused on olive pollen allergy, this method is versatile since it can be applied to other plant species and other types of allergies (e.g. food allergy). For simplicity, our studies were limited to four allergenic proteins. However, it is also possible to extend the analysis to a higher number of allergens (or other proteins) using other animal hosts to produce primary antibodies (e.g. chicken, rat, etc.), a higher variety of fluorochromes, and a suitable detection platform equipped with a higher number of lasers and filters. Moreover, this multiplex methodology could be coupled to 2-D electrophoresis in order to increase its resolution and to improve the detection of new allergen forms.

On the other hand, allergens often show distinct IgE-reactivities, so quantitative and/or qualitative differences of individual allergenic molecules with varying IgE-reactivity might also affect the total allergenic potency of the protein extract. In an effort to identify olive cultivars with low and high IgE reactivity, we reprobed the same blot with a pool of sera from eight patients allergic to olive, using a chemiluminescent detection method. This experiment allowed us to easily confirm the identity of some of the IgE-binding proteins by simply overlapping both fluorescent and chemiluminescent images. Some authors have suggested that, for a more efficient immunotherapy, allergic patients should be treated with those allergens for which they produce high levels of IgE [36, 37]. According to this theory, the commercial extract tested here would not be suitable for immunotherapy in these eight olive allergic patients, since it did not show any IgE reactivity. On the contrary, a combination of either “Picual”-“Arbequina” or “Picual”-“Lechín” extracts could be the best choice for vaccination in this particular case. For this purpose, it would be necessary to initially determine the IgE-binding profile of each allergic patient by testing individual sera.

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The authors have declared no conflict of interest.

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