Allergy 1997: 52: 829–835
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ALLERGY
ISSN 0105-4538

Cross-reactivity of *Olea europaea* with other Oleaceae species in allergic rhinitis and bronchial asthma

Pajarón MJ, Vila L, Prieto I, Resano A, Sanz ML, Oehling AK. Cross-reactivity of *Olea europaea* with other Oleaceae species in allergic rhinitis and bronchial asthma.
Allergy 1997: 52: 829–835. © Munksgaard 1997.

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Cross-reactivity between pollen extracts of four species of Oleaceae was studied: olive (*Olea europaea*), ash (*Fraxinus excelsior*), privet (*Ligustrum vulgare*), and lilac (*Syringa vulgaris*). Thus, 51 patients and 13 atopic controls were studied, by means of intracutaneous skin tests, histamine-release tests against the four extracts, and specific IgE to *O. europaea*. The proteic content of the four extracts was assessed by SDS–PAGE and immunoblotting, and similarity of all the extracts studied was observed after electrophoresis and immunodetection. Six common bands were found to be responsible for the cross-reactivity, with apparent molecular weights of 49.6, 40, 36.7, 19.7, 16.7, and 14 kDa, respectively. The cross-reactivity was also corroborated by immunoblotting inhibition and FEIA inhibition. The patients had a similar response to the four allergenic extracts used, although the response to *Olea* was greatest. When the patients were compared by their geographic origin (northern or southern Spain, according to the distribution of areas of olive pollen influence), there were no significant differences between the two groups in skin reactivity, but a higher histamine release was observed for the four extracts in the southern group, although it was significant only for *Fraxinus* and *Ligustrum*. This work corroborated the practicality of the diagnostic methods used and the cross-reactivity between the four species studied, as demonstrated by the different methods used. Therefore, we suggest that only *O. europaea* extract be used in diagnosis and immunotherapy in Oleaceae pollen allergy.

Key words: cross-reactivity; *Fraxinus*; histamine-release test; immunoblotting inhibition; intracutaneous tests; *Ligustrum*; *Olea*; specific IgE; *Syringa*.

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Accepted for publication 11 March 1997

Olea europaea belongs to the Oleaceae family, and is widely distributed in all Mediterranean countries (1–7), where at least 21 different varieties of this species have been described (8), with important differences in the immunogenic and allergenic potential of their pollens (9, 10). Although *O. europaea* is rare in the rest of Europe, other species of the Oleaceae family are more common. Among them are the ash tree (*Fraxinus excelsior*), the privet (*Ligustrum vulgare*), and the lilac (*Syringa vulgaris*) (9, 11).

Several studies have demonstrated the existence of cross-reactivity between the various species of the Oleaceae family (11–18).

The major antigen of olive pollen (Ole e 1) has been identified as the main cause of cross-reactivity between the Oleaceae pollens (16) after its purification by means of monoclonal antibodies, and the observation that it shares similar molecular properties with the major antigens of the rest of the family (13, 15, 19, 20). Ole e 1 is defined as a glycoprotein of 145 amino acids, with a weight of 19 kDa in its glycosylated form, whose sequence has been determined (21–23). The presence of *Olea* pollen in the various regions of Spain is very irregular. The existence of olive groves in the southern region makes *Olea* pollen the one most frequently found in the pollination season in this area, whereas in the north other species of the Oleaceae family, such as ash, privet, and lilac, are found.

The aims of this study were as follows:

1) to prove the existence of cross-reactivity between

extracts of olive, ash, privet, and lilac pollens by different methods of allergologic clinical diagnosis, and their capacity to induce histamine release *in vitro*
2) to use electrophoresis and immunoblotting to determine which proteins cause this cross-reactivity
3) to corroborate this by blotting inhibition and inhibition FEIA
4) to study the differences between *Olea*-sensitive patients sensitized to olive pollen in the two regions of different influence of this pollen.

Material and methods

We studied 51 patients (age: 29.5±2.3 years) with clinical manifestations of pollinosis and diagnosed as having *Olea* pollen sensitization, by means of intracutaneous skin test, specific IgE determination, and histamine-release test, without previous treatment or immunotherapy. We divided the patients into two groups, one comprising patients coming from a region where olive pollen is important (designated the "south area"), totaling 37 patients, and the other group comprising patients coming from regions with a lesser influence ("north area"), totaling 14 patients. We made this division according to a scheme suggested by Subiza et al. and modified by us, which refers to the "south area" as that where the olive is cultivated and to the "north area" as that where it does not usually grow (24). Six of these patients suffered only from rhinoconjunctivitis. The rest had been diagnosed as having bronchial asthma, as well as rhinoconjunctivitis. As a control group, 13 atopic patients, with no sensitization to pollens, were studied (age: 37.8±3.6 years).

Skin tests

Skin tests were performed, with intradermal injection of 50 µl of the following pollen extracts from IFIDESA-ARÍSTEGUI (Bilbao, Spain): *O. europaea* (190 UBE/ml), *F. excelsior* (200 E/ml), *L. vulgare* (200 E/ml), and *S. vulgaris* (200 E/ml). Papules were read 20 min after injection.

Histamine-release test

We used the fluorometric method of Shore et al. (25), subsequently automated by Siraganian et al. (26–29), and modified by us (30, 31). We used the following antigen concentrations: *O. europaea* (9770 UBE/ml), *F. excelsior* (10 000 E/ml), and *S. vulgaris* (10 000 E/ml) (IFIDESA-ARÍSTEGUI, Bilbao, Spain).

IgE determination

Total and specific IgE determinations against *O. europaea* were performed by the fluoroenzymoimmunoassay (FEIA) (Pharmacia CAP System, Uppsala, Sweden), according to the instructions of the manufacturer. Values greater than 0.7 kU/l (32) were considered positive.

Inhibition FEIA

A pool formed by six sera of *O. europaea* pollen-sensitive patients, selected by means of intracutaneous tests and *O. europaea*-specific IgE greater than 17.5 kU/l (class 4), was used. The sera were stored at −80°C until further use.

The different lyophilized pollen extracts used were supplied by IFIDESA-ARÍSTEGUI (Bilbao, Spain), with a total proteic content of 0.397 mg, as determined by the Lowry et al. (33) and Bradford (34) methods, and were reconstituted in 1 ml of 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS).

An inhibition FEIA was performed, with the extracts of *Fraxinus*, *Ligustrum*, and *Syringa* reconstituted previously. Specific IgE determination to *Olea* (FEIA, Pharmacia CAP System, Uppsala, Sweden) was performed, after incubation of the pool of sera with the extracts at concentrations 0.397, 0.189, 0.099, and 0.049 mg/ml for 2 h at room temperature with gentle shaking. The results were contrasted with those obtained with the pool of sera diluted in PBS, as a control.

Electrophoresis

Each extract was reconstituted in PBS, and 40 µg/well was used to run the electrophoresis. The allergenic extracts were the same as used in FEIA inhibition, reduced by incubation at 95°C for 3 min with sample buffer (6% SDS, 30% glycerol, 0.15% bromophenol blue, and 15% β-mercaptoethanol in 220 mM Tris-HCl, pH 6.8) before their application to the gel. A solution of low-molecular-weight markers (Bio-Rad) was used as reference.

Discontinuous electrophoresis was performed in polyacrylamide gels (SDS–PAGE) (35) in a Bio-Rad Protean system, using 12% (w/v) polyacrylamide separating gels in 0.375 M Tris-HCl, pH 8.8, with 3% (w/v) superior gels. Electrophoresis was performed for 45 min at room temperature, at 200 V and 50 mA. Once it was finished, one part of the gel was dyed with Coomassie brilliant blue, and the rest was used for transfer to nitrocellulose membrane.

Transfer and immunodetection

Transfer of proteic bands to the nitrocellulose membranes was made in a Bio-Rad transfer tray for 1 h at 200 V and 0.8 mA/cm^2. For the immunodetection, the nitrocellulose membrane was washed for 10 min in PBS, pH 6.8, and incubated for 2 h in 1% BSA in PBS, pH 6.8, with continuous shaking, to block unspecific binding. Once the incubation was finished, four 10-min washes with 0.1% Tween 20 in PBS were performed. Then the membrane was incubated in the sera pool detailed before, for 16 h. After another four 10-min washes, it was incubated for 4 h with a 1/50 solution of anti-IgE antibodies conjugated to horseradish peroxidase (Dakopatts) in PBS. Finally, after another four 10-min washes, the bands were stained with 0.06% 4-chloro-1-naphthol in PBS, with 0.01% H_2O_2.

As a control, the same experiment was performed with a pool of six sera from patients not sensitized to *O. europaea*, as verified by IgE determination, histamine release, and intracutaneous tests.

Blotting inhibition

After incubation of the pool sera with *O. europaea* pollen extract (9.925 µg/ml) for 2 h with continuous and gentle shaking, the immunodetection was performed as described before.

Statistical analysis

The Pearson correlation coefficient for normal variables was used. When at least one nonnormal variable was involved (e.g., skin tests), the Spearman coefficient was used. For detection of differences between the means of the different groups, one-way ANOVA, followed by the Student's-Newman-Keuls test *a posteriori*, was used (or their nonparametric equivalents; for skin tests, Kruskal-Wallis ANOVA, followed by Mann-Whitney U tests).

Results

A highly significant correlation was found between the different allergologic diagnostic methods available for *O. europaea* pollen (Table 1), the most remarkable being the correlation between histamine release and specific IgE (r=0.52, P=0.001), followed by the correlation between skin test and specific IgE results (r_s=0.41, P=0.002). The correlation of intracutaneous tests with histamine release was lower for *Olea* (r_s=0.30, P=0.018) than for the other three pollens. The most important correlation was obtained for *Syringa* (r=0.57, P<0.001), followed by *Ligustrum* (r_s=0.053, P=0.001) and *Fraxinus* (r_s=0.46, P=0.005).

Table 1. Correlation between histamine release test results from the different studied allergenic pollens

	n	*r**	*P*
Olea-Fraxinus	63	0.82	<0.001
Olea-Ligustrum	53	0.75	<0.001
Olea-Syringa	63	0.87	<0.001
Fraxinus-Ligustrum	53	0.90	<0.001
Fraxinus-Syringa	63	0.93	<0.001
Ligustrum-Syringa	53	0.90	<0.001

(*) Pearson.

Table 2 shows the high correlation between skin tests and histamine release for every extract, and also between *Olea*-specific IgE and antigen-specific histamine release with the different extracts.

After performance of inhibition FEIA, the pool of sera being incubated with the four different extracts, inhibition of binding of specific IgE to *Olea* with values of 75–90% was observed (Fig. 1).

After electrophoresis and transfer to the nitrocellulose membrane, more than 12 proteic bands appeared in the four pollen extracts. In immunodetection, several of these proteic bands described for the four antigenic extracts were observed to react with the IgE of the pool of sera of *Olea*-sensitive patients. These bands had apparent masses of 49.6, 40, 36.7, 19.7, 16.7, and 14 kDa (Fig. 2). The *Olea* extract showed a larger number of proteic bands that were not common to the other extracts (32.3, 29.7, and 26.2 kDa). *Olea* and *Fraxinus* contained more common bands than the other two extracts, while *Ligustrum* had fewer common bands than the rest of the extracts.

No bands appeared when the immunoblot was performed with the pool sera from unsensitized patients (Fig. 2). After blotting inhibition of the pool of sera with *O. europaea* pollen extract, almost all the bands disappeared. This assay was performed three consecutive times, and the same result was always obtained, demonstrating the antigenic community of the four extracts studied.

Table 2. Correlation between *Olea*-specific IgE (sIgE) and the other two diagnostic methods: skin test (ST) and histamine release test (HRT), with the four studied extracts

	sIgE/ST			sIgE/HRT		
	n	*r**	*P*	*n*	*r**	*P*
Olea	57	0.41	0.002	57	0.52	<0.001
Fraxinus	35	0.62	<0.001	57	0.63	<0.001
Ligustrum	32	0.61	<0.001	48	0.61	<0.001
Syringa	57	0.72	<0.001	57	0.56	<0.001

(*) Spearman. (**) Pearson.

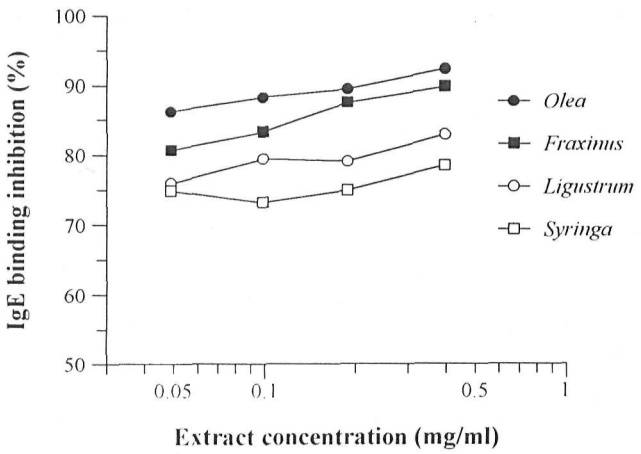

Fig. 1. Inhibition FEIA results of recognition of *O. europaea* pollen extract after incubation of pool sera with each extract.

Fig. 3A shows the similarity of the skin test results of the four extracts. We found significant differences only between the controls and the positive patients. There were no significant differences between the "north" and "south" areas, but differences between controls and patients were found between the skin test with *Olea* and the other pollens. For *Olea*, there seemed to be a higher sensitization, as proven by some false-positives found in the control group.

Fig. 3B shows that patients from the "south area" showed a greater histamine release induced by all the extracts than those from northern regions, but we did not find a significant difference between the response to *Olea* and *Syringa*, which seem to behave very similarly. Differences between *O. europaea*-specific IgE levels of patients from both groups were not found, but differences were found when these levels were compared with those from the control group (Fig. 3C).

Discussion

Because of its clinical importance, *O. europaea* pollen is the most studied of all pollens of the Oleaceae family (36). The major antigens of this pollen, Ole e 1 and Ole e 2, have been described (16, 19). Therefore, the *Olea* extract is the best standardized of all Oleaceae pollens (37), as proven in our work, where high levels of correlation were reached for all the diagnostic tests. Especially remarkable was the good correlation found between specific IgE and the histamine-release test, confirming this test's diagnostic usefulness and efficiency (38, 39).

We observed a high degree of FEIA inhibition in specific IgE binding to *O. europaea* for all the extracts studied. The inhibition reached by *F. excelsior* (90%) was especially remarkable, being almost the same as that produced by the extract of *O. europaea*. Less important, but also very high were the inhibition levels reached by the two entomophilous species of the family, *L. vulgare* (85%) and *S. vulgaris* (75%), suggesting that the greatest antigenic community would be found between the first two anemophilous pollens indicated (15, 16, 40, 41).

Among all the bands found to be common to the four extracts (49.6, 40, 36.7, 19.7, 16.7, and 14 kDa), the 19.7- and 16.7-kDa bands have been previously described, corresponding to Ole e 1. The 40-kDa band corresponds to Ole e 2 (42 kDa) in the immunodetection for the four species of Oleaceae studied (14–23, 42). The *Olea* extract bands which were not common to the rest of the other extracts

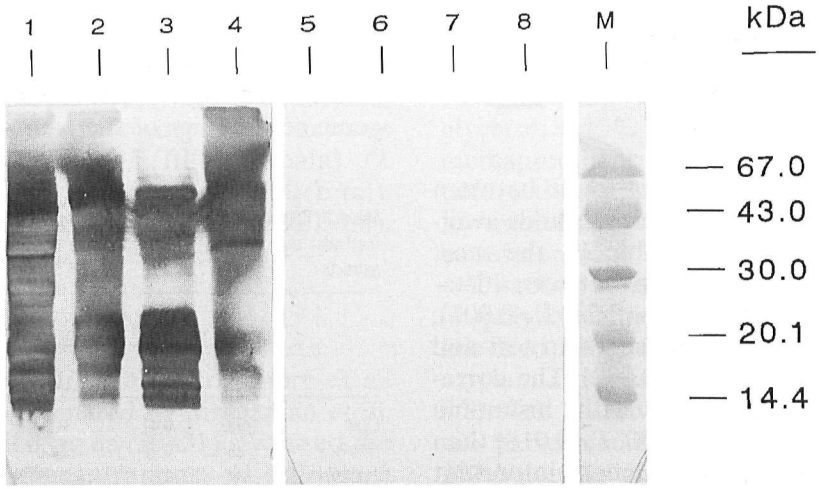

Fig. 2. Immunodetection of allergenic bands of *Olea* (lanes 1 and 5), *Fraxinus* (lanes 2 and 6), *Ligustrum* (lanes 3 and 7), and *Syringa* (lanes 4 and 8) extracts, after treatment with pool sera from sensitized patients (left, lanes 1–4) and nonsensitized patients (center, lanes 5–8). Lane M (right) shows molecular weight markers.

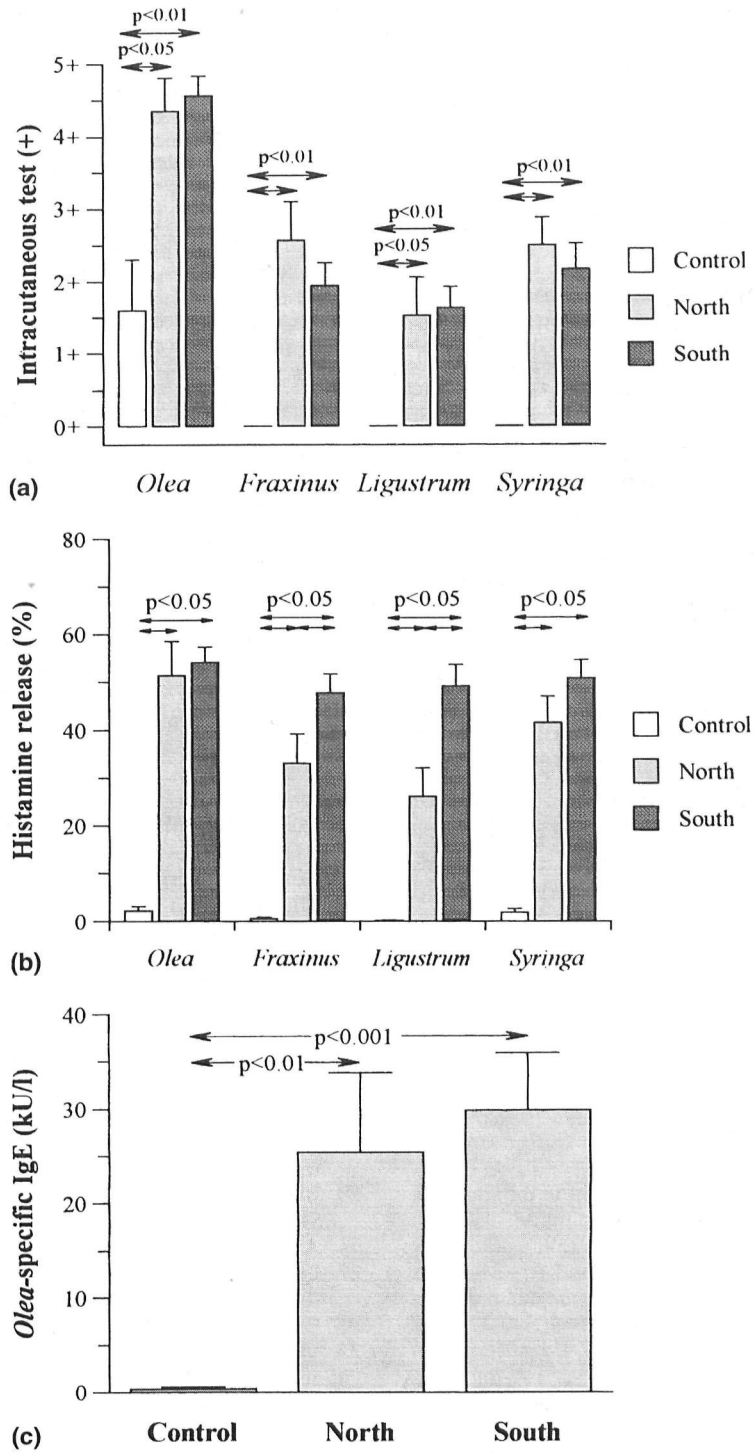

Fig. 3. A) Results of intracutaneous skin tests with *Olea*, *Fraxinus*, *Ligustrum*, and *Syringa* extracts. B) Histamine release provoked by *Olea*, *Fraxinus*, *Ligustrum*, and *Syringa* extracts. C) *O. europaea*-specific IgE results.

(32.3, 29.7 and 26.2 kDa) have also been previously described, but IgE antibodies against these bands did not seem to be present in all the patients studied (14).

Ligustrum and *Syringa* had fewer majority antigens common to the four species, especially *Syringa*. This fact would explain the weaker FEIA inhibition against *Olea* of both of them. The antigenic community of the four extracts, as well as their activity, was demonstrated by blotting inhibition with *O. europaea* pollen extract, with negative immunodetection in all the cases.

We did not find significant differences in the results of the skin tests (Fig. 3A) and specific IgE depending on whether patients were from the "north" or "south" areas (Fig. 3C). Only *Fraxinus*- and *Ligustrum*-specific histamine releases were lower in patients from the "north area", similar to the behavior of *Olea* and *Syringa*, although the interpretation of these differences is not very clear (Fig. 3B).

We found no differences between the four extracts in the diagnosis by skin tests and histamine-release tests. Something similar happens with grass pollen, where it has been observed that the use of a unique species is sufficient for the diagnosis of sensitization to Poaceae (43). Therefore, we may assume that there is no need for specific diagnosis or immunotherapy different from that related to *O. europaea*, since olive pollen contains all the antigens triggering the clinical response in patients whose primary sensitization is to *Olea* or other Oleaceae species (*Fraxinus*, *Ligustrum*, and *Syringa*).

From a practical point of view, we consider that, for the diagnosis of patients with sensitization to Oleaceae in areas where there is no olive pollen influence, it is necessary to include in the routine diagnostic tests a pollen extract of one species of the Oleaceae family (37). In our case, the *Olea* extract is the most appropriate for both areas, "north" and "south", but *Fraxinus* extract could also be used, as it has the most antigenic community with *Olea*.

Acknowledgment

We thank Dr Alberto Martínez, of IFIDESA-ARÍSTEGUI (Bilbao, Spain) for technical support.

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