

ORIGINAL ARTICLE

BIOTECHNOLOGY AND IN VITRO DIAGNOSTICS

Component-resolved diagnosis of vespid venom-allergic individuals: phospholipases and antigen 5s are necessary to identify *Vespula* or *Polistes* sensitization

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Abstract

Background: Cross-reactivity between hymenoptera species varies according to the different allergenic components of the venom. The true source of sensitization must therefore be established to ensure the efficacy of venom immunotherapy.

Objective: In the Mediterranean region, *Polistes dominulus* and *Vespula* spp. are clinically relevant cohabitating wasps. A panel of major vespid venom allergens was used to investigate whether serum-specific IgE (sIgE) could be used to distinguish sensitization to either vespid.

Methods: Fifty-nine individuals with allergic reactions to vespid stings and positive ImmunoCAP and/or intradermal tests to vespid venoms were studied. sIgE against recombinant and natural venom components from each wasp species was determined using the ADVIA Centaur® system.

Results: sIgE against recombinant antigen 5s sensitization to be detected in 52% of the patients tested (13/25). The sensitivity increased to 80% (20/25), when using natural antigen 5s, and to 100% with the complete panel of purified natural components, because the sIgE was positive to either the antigen 5s (Pol d 5/Ves v 5) or to the phospholipases (Pol d 1/Ves v 1) of the two vespids, or to both components at the same time. In 69% of cases, it was possible to define the most probable sensitizing insect, and in the rest, possible double sensitization could not be excluded. *Vespula* hyaluronidase was shown to have no additional value as regards the specificity of the assay.

Conclusions: The major allergens of *P. dominulus* and *Vespula vulgaris* venom, namely phospholipases and antigen 5s, are required to discriminate the probable sensitizing species in vespid-allergic patients.

Venom immunotherapy (VIT) is performed by repeated subcutaneous injections of Hymenoptera venoms, most often from vespids (species of *Vespula* or *Polistes*) and honey bee (*Apis mellifera*) (1). Identification of the species that provoke the allergy is therefore essential for the success of this treatment (2–4). Previous studies have suggested that recombinant technology and/or component-based determination of serum-specific IgE antibodies to major venom allergens could

improve the diagnosis of hymenoptera-allergic patients, particularly to overcome the indication for double sensitization between *Apis* and *Vespula* (5–8). In this respect, Müller et al. (7) demonstrated that a true double sensitization could be established *in vitro* using the major bee venom allergen Api m 1 and the major vespid venom (*Vespula vulgaris*) allergen Ves v 5. These findings were further confirmed when cross-reactivity within the hymenoptera hyaluronidases was shown

to be mainly dependent on cross-reactive IgE antibodies directed to carbohydrate determinants (9–11). Cross-reactive IgE antibodies against specific core α 1,3-fucose and/or α 1,6-fucose structures are known to affect routine *in vitro* allergy diagnosis, thereby resulting in double positivity in venom-allergic individuals. However, discrimination between true double sensitization and the identification of primary and/or cross-sensitization has normally been assessed by inhibition experiments, which cannot be used as common diagnostic tools (7, 12–14).

The genera *Vespula*, *Dolichovespula* and *Vespa* are found all over Europe, whereas the genus *Polistes* represents a specific clinical problem in the Mediterranean region (2, 4, 15). Although *Polistes* are found in both North America and Europe, these species have different geographical distributions. Thus, the prevalent species in Europe are *Polistes dominulus* and *Polistes gallicus*, whereas in North America, they are *Polistes annularis*, *Polistes fuscatus*, *Polistes metricus* and *Polistes exclamans* (15, 16).

Despite their different geographical distribution, the overall composition of allergenic compounds is similar in all vespids venoms (17, 18), with the most common allergens being phospholipase A1, hyaluronidase and antigen 5. In addition, serine-protease has been identified as an important allergen for vespid-allergic individuals in European *Polistes* (19) venom and dipeptidylpeptidase IV (20) in *V. vulgaris* venom. Although antigen 5 and phospholipases show up to 95% sequence identity between *Vespula* species (e.g. *V. vulgaris*, *Vespula germanica*), their sequence identity as regards the corresponding proteins in *Polistes* species is only around 50%. Furthermore, the sequence identity between antigen 5s in *P. dominulus* and its major American counterpart *P. annularis* is up to 85% (84% to phospholipases) (3). The incomplete cross-reactivity between North American and European *Polistes* species has been suggested to result from differences in their surface structures, which lead to variations in their relative immunogenicity. Furthermore, such surface variation was also considered to be sufficient to distinguish the origin of sensitization between *Polistes* and *Vespula* species (3, 19). The aim of our study was to find the most appropriate allergenic components to be used *in vitro* as possible markers for sensitization of patients to either of the wasp species, which is a very important clinical issue in Southern European countries (4, 21, 22).

Materials and methods

Study design

A total of 59 allergic individuals (aged 11–72; average age, 42.4 years) were included in this study. All had been diagnosed with vespid allergy as a result of either a clinical reaction after a vespid sting and positive skin test and/or specific IgE (determined by ImmunoCAP; Phadia, Uppsala, Sweden) to *Vespula* or *Polistes* (Table 1). To distinguish *Polistes*- and/or *Vespula*-sensitized individuals amongst those showing double positivity or with an uncertain diagnosis, serum-specific (sIgE) IgE against these two vespid

species was assessed using the ADVIA Centaur® (ADVIA) system.

Patient's clinical evaluation

Patients who suffered a large local or a systemic reaction after a vespid sting were included in the study. Informed consent was obtained. Table 1 contains the relevant clinical data for the venom-allergic patients included in this study. Clinical reactions were classified according to Mueller (23).

sIgE to bee (*A. mellifera*), *Vespula* (*Vespula* spp.) and *Polistes* (*Polistes* spp.) venoms was determined by ImmunoCAP (Phadia). A value >0.35 kU/l was considered to be positive.

Stepwise incremental intradermal skin tests were performed using venom extracts from *Polistes* spp., *Vespula* spp. and *A. mellifera* (ALK-Abelló, Madrid, Spain), following the recommendations of the European Academy of Allergy and Clinical Immunology. When specified, venom extract from *P. dominulus* (ALK-Abelló) was also used. The results were considered positive when a concentration of ≤ 1 μ g/ml resulted in a wheal >3 mm in diameter accompanied by erythema.

Purification of the allergenic components used in the study

Expression and purification of recombinant (r) rPol a 5 and rVes v 5 were achieved with the *Pichia pastoris* expression system using the clones and conditions described by Monsalve et al. (24). *Vespula vulgaris* and *P. dominulus* extracts were obtained from Vespa Laboratories (ALK-Abelló Source Materials, Inc., Spring Mills, PA, USA). The natural venom allergens Ves v 1, Ves v 2 and Ves v 5 and Pol d 1, Pol d 2 and Pol d 5 were purified from 200 mg of pure venom as previously described by King et al. (25, 26). The purified allergens were analysed by SDS-PAGE (Novex-Tricine, Invitrogen, UK) and silver stained. The enzymatic activity of each of the purified components was verified by Linker (27) and Richman and Baer (28) assays. The identity and concentration were confirmed by mass spectrometry and amino acid analysis. Purified nApi m 1, which was used as reference for possible cross-reactivity with *Apis* (7, 29), was obtained commercially (SIGMA, St Louis, MO, USA). Nomenclature used for allergenic molecules follow the rules recommended in the study of King et al. (30).

Serum-specific IgE antibodies (sIgEs)

sIgE to biotinylated rVes v 5, rPol a 5, nVes v 1, nVes v 2, nVes v 5, nPol d 1, nPol d 5 and nApi m 1 was detected using the ADVIA Centaur® (ADVIA) system (Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA) (7, 31).

These assays were initially performed using rVes v 5 and rPol a 5 together with their natural counterparts (Table 2). To enhance the selectivity and sensitivity of the assay, other major allergens from *P. dominulus* and *V. vulgaris* venom were isolated and the sIgE of vespid-allergic individuals was analysed against the panel of natural (n) allergens (Table 3). As a result of serum availability, Table 3 includes only 11 sera out of the 25 patients included in Table 2.

Table 1 Clinical data for the patients ($N = 59$) included in the studies presented in Tables 2 and 3 (where the same serum nomenclature is used). CAP for *Vespula*, *Polistes* spp. and *Apis* is shown, as well as intradermal (ID) test results with *Vespula* (V), *Polistes* spp. (P) or *Polistes dominulus* (PD) extract, showing the quantity that gave a positive result. The degree of severity is given according to Mueller (23)

Serum Nomenclature	CAP Ves	CAP Pol	CAP Apis	ID (Conc.+)	Mueller reaction	Age	Gender (female/male)	Occupation
LHSM1	23.5	9.77	<0.35	V, P (0.1)	2	67	F	Housewife
LHSM2	8.87	13.6	<0.35	V, P (0.001)	3	44	M	Beekeeper (hobby)
LHSM03	10.1	18.6	<0.35	P (0.1)	4	48	M	Glass Factory
LHSM05	0.9	0.58	<0.35	V, P (0.1)	3	44	M	Shutterer, Hunter
LHSM06	10.6	nd	15.0	A (1)	4	20	M	Student
LHSM07	3.04	5.95	<0.35	A,V (0.01), P (0.1)	2	39	M	Postman
V04HT17	<0.35	0.82	<0.35	P (1)	3	30	M	Mechanic
V04HT18	0.83	0.98	<0.35	V (0.01) P (1)	4	49	M	Driver
V04HT19	1.98	2.0	1.34	V (0.1) P (0.01)	2	52	M	Maintenance
V04HT20	<0.35	<0.35	<0.35	V (1)	4	52	F	Housewife
V04HT22	<0.35	4.06	<0.35	P (1)	LLR	54	F	Housewife
V04HT23	4.66	7.78	<0.35	P (1)	3	49	M	Construction
V04HT24	15.7	<0.35	<0.35	V (0.01)	4	41	M	Driver
V04HT25	4.97	6.76	0.71	V, P (0.01)	4	16	F	Student
ALHT02	0.36	0.43	<0.35	V, P (1)	4	37	M	Construction
ALHT03	8.54	0.85	<0.35	P (0.01)	3	61	F	Housewife
THTVC01	11.0	11.3	0.78	V (0.01), P (1)	3	58	M	Woodcrafter
THTVC02	0.83	1.46	<0.35	V (1), P (0.1)	2	46	F	Teacher
V15CHUS01	7.56	0.35	nd	V (0.01)	3	48	M	Bricklayer
V15CHUS02	87.7	0.78	nd	V (0.01)	4	63	M	Retired
V15CHUS03	14.7	nd	nd	V (0.01)	3	57	M	Gardener
V15CHUS04	2.42	7.08	0	V, P (0.01)	3	15	M	Student
GU001	16.6	<0.35	<0.35	V (0.1)	4	38	M	Unknown
GU003	<0.35	12.9	<0.35	Negative	1	32	F	Housewife
GU004	<0.35	<0.35	<0.35	V, P (1)	3	38	M	Office work
GU005	0.43	0.46	<0.35	V (0.01)	1	61	M	Retired
GU007	4.73	2.4	<0.35	P (0.01), V (1)	4	24	F	Student
GU008	95.9	2.41	0.84	V (0.001)	1	42	F	Housewife
GU009	9.53	3.71	2.77	V (0.001) P (0.1)	2	21	M	Construction
GU010	3.97	<0.35	<0.35	V (0.1)	3	45	F	Agriculture
GU011	9.91	13.4	<0.35	V, P (0.001)	3	35	M	Ceramics Factory
GU012	6.53	nd	nd	V (0.1)	2	20	M	Student
GU013	<0.35	<0.35	<0.35	Negative	LLR	72	F	Housewife
GU014	1.94	<0.35	<0.35	nd	LLR	11	M	Student
GU015	<0.35	0.37	<0.35	Negative	1	35	M	Trucks Driver
GU016	30.3	27.9	<0.35	Negative	LLR	18	F	Student
GU017	4.15	10	<0.35	Negative	2	37	F	Gardener
GU018	18.9	29.4	nd	Negative	LLR	47	F	Housewife
GU019	0.88	2.17	<0.35	Negative	LLR	26	F	Office work
GU020	10.2	19.4	1.8	nd	LLR	46	M	Trucks Driver
GU021	4.0	8.09	nd	P (0.1)	LLR	19	M	Office work
GU024	6.36	6.03	nd	Negative	4	40	F	Housewife
GU025	0.38	<0.35	<0.35	V (0.1) P (0.01)	3	45	M	Meat Transporter
GU031	4.53	3.54	1.03	Negative	3	62	M	Logistics
GU036	3.75	2.22	1.24	Negative	LLR	62	M	Scrap merchant
GU039	0.39	4.7	<0.35	nd	4	S3	M	Office work
GU041	0.4	0.77	1.7	P, V (0.1)	4	55	M	Gardener
SAN07	3.47	4.41	<0.35	V (1), PD (0.1)	4	28	M	Local Council
LAG01	2.21	4.45	0.98	V, PD (0.1)	1	44	M	Local Police

(continued)

Table 1 (continued)

Serum Nomenclature	CAP Ves	CAP Pol	CAP Apis	ID (Conc.+)	Mueller reaction	Age	Gender (female/male)	Occupation
LAG11	43.8	29.7	<0.35	V, P (1)	1	48	F	Housewife
LAG10	46.5	23.3	1.17	V, P (0.1)	1	26	M	Underground Driver
LAG02	14.6	22.3	2.19	V (1), PD (1)	2	70	M	Retired
LAG05	1.65	1.98	<0.35	V, P (1)	4	72	M	Retired
LAG04	3.6	4.4	0.74	V, PD (0.01)	4	44	M	Lead Factory
LAG03	40.9	11.1	2.35	V, PD, A (0.1)	1	50	M	Construction
LAG08	2.65	1.46	<0.35	V, PD (0.1)	1	40	F	Cooker
LAG09	1.76	0.45	<0.35	V, PD (1)	1	23	M	Teacher
VSOR90	2.01	<0.35	<0.35	V (0.0001), P (0.1)	2	Unknown	Unknown	Unknown
VSOR57	81.5	66.8	<0.35	V (1)	4	Unknown	Unknown	Unknown

LLR, large local reaction; nd, not determined.

Table 2 sIgE to rVes v 5/nVes v 5 and rPol a 5/nPol d 5 in wasp-allergic individuals ($N = 25$) determined using the ADVIA system. ImmunoCAP data are also shown for comparison as well as the degree of severity, according to Mueller (23).

Serum Nomenclature	ADVIA (kU/L)				ImmunoCAP (kU/L)			Degree of severity Grade
	rVes v 5	nVes v 5	rPol a 5	nPol d 5	Ves spp	Pol spp	Apis	
LHSM1	10.95	11.7	0.42	29.31	23.5	9.77	<0.35	2
LHSM2	58.7	25.51	6.57	539.32	8.87	13.6	<0.35	3
V04HT17	0.01	0.05	0.02	0.45	<0.35	0.82	<0.35	3
V04HT18	0.10	0.17	0.02	0.03	0.83	0.98	<0.35	4
V04HT19	0.15	0.31	0.01	0.20	1.98	2.0	1.34	2
V04HT20	0.00	0.02	0.00	0.00	<0.35	<0.35	<0.35	4
V04HT22	0.01	0.03	0.07	0.42	<0.35	4.06	<0.35	LLR
V04HT23	0.02	0.14	0.76	6.12	4.66	7.78	<0.35	3
V04HT24	2.34	5.12	0.01	0.05	15.7	<0.35	<0.35	4
V04HT25	1.70	3.45	1.71	9.39	4.97	6.76	0.71	4
ALHT02	0.19	0.93	0.29	3.72	0.36	0.43	<0.35	4
ALHT03	0.31	2.18	0.37	1.29	8.54	0.85	<0.35	3
THTVC01	1.03	1.76	0.44	2.86	11.0	14.3	0.78	3
THTVC02	0.05	0.21	0.06	0.22	0.83	1.46	nd	2
V15CHUS01	8.1	12.03	0.2	2.76	7.56	0.35	nd	3
V15CHUS02	0.1	0.88	0.19	2.25	87.7	0.78	nd	4
V15CHUS03	0.33	1.27	0.39	2.96	14.7	nd	nd	3
V15CHUS04	1.28	3.75	4.85	56.8	2.42	7.08	nd	3
GU015	0.02	0.15	0.11	0.33	<0.35	0.37	<0.35	1
GU016	0.06	0.38	0.41	29.69	30.9	27.9	<0.35	LLR
GU017	0.26	2.83	0.34	2.15	4.15	10	<0.35	2
GU018	0.05	0.22	0.30	5.61	18.9	29.4	nd	LLR
GU019	0.02	0.05	0.08	1.10	0.88	2.17	<0.35	LLR
VSOR90	6.74	9.56	0.01	0.00	2.01	<0.35	<0.35	2
VSOR57	4.46	2.03	0.38	2.5	81.5	66.8	<0.35	4

LLR, large local reaction; nd, not determined.

Complete clinical data for the patients are provided in Table 1. The values in bold indicate clearly positive values.

Results

Purification of recombinant and natural vespid allergens for ADVIA assays

The allergens rPol a 5, rVes v 5, nPol d 1/nVes v 1, nPol d 2/nVes v 2 and nPol d 5/nVes v 5 were purified by standard

chromatographic methods as described previously (24–26). The purity of the isolated natural allergens is shown in Fig. 1. A total of 830 µg of nVes v 5, 1.32 mg of nVes v 2 and 2.55 mg of nVes v 1 were obtained from 200 mg of *V. vulgaris* extract. The yield of nPol a 5 from *P. dominulus* extract was 530 µg (70 µg for nPol d 2 and 5.39 mg for nPol

Table 3 IgE to panel of major *Polistes dominulus* and *Vespula vulgaris* allergens in wasp-allergic individuals ($N = 45$) determined using the ADVIA Centaur® system. The values in bold indicate clearly positive values. 'Suggested Diagnosis' indicates the possible sensitizing species on the basis of these *in vitro* data (V, P and A stand for *Vespula*, *Polistes* and *Apis*, respectively). The ImmunoCAP data are also included for comparison (nd, not determined). Complete clinical data for the patients are provided in Table 1

Serum Nomenclature	ADVIA (kU/L)						Suggested Diagnosis	ImmunoCAP (kU/L)		
	nPol d 5	nPol d 1	nVes v 5	nVes v 1	nVes v 2	nApi m 1		CAP Ves	CAP Pol	CAP Apis
LHSM1	29.31	28.74	11.7	81.21	1.17	1.02	P, V	23.5	9.77	<0.35
LHSM2	539.32	12.29	25.51	5.98	0.00	1.04	P	8.87	13.6	<0.35
LHSM03	8.95	129.20	0.15	0.14	0.00	0.02	P	10.1	18.6	<0.35
LHSM05	1.41	0.00	3.29	0.00	0.00	0.00	V	0.9	0.58	<0.35
LHSM06	0.05	0.76	0.22	0.27	108.00	3.22	P, V, A	10.6	nd	15
LHSM07	3.09	7.72	0.00	0.51	0.39	0.04	P	3.04	5.95	<0.35
ALHT02	3.72	0.32	0.93	0.85	0.12	0.35	P	0.36	0.43	<0.35
A1HT03	1.29	3.95	2.18	2.58	0.54	0.00	P, V	8.54	0.85	<0.35
THTVCO1	2.86	5.92	1.76	56.69	2.65	0.01	P, V	11	14.3	0.78
V15CHUS04	56.8	1.64	3.75	3.69	1.41	0.06	P, V	2.42	7.08	<0.35
GU001	0.39	1.30	0.74	3.88	0.14	0.00	V	16.6	<0.35	<0.35
GU003	4.54	20.91	0.18	0.02	0.07	0.00	P	<0.35	12.9	<0.35
GU004	1.33	0.19	0.65	0.65	0.67	0.04	P, V	<0.35	<0.35	< 0.35
GU005	3.16	1.00	0.62	1.10	0.08	0.00	P	0.43	0.46	<0.35
GU007	1.74	2.67	0.90	9.01	0.49	0.02	P, V	4.73	2.4	nd
GU008	0.88	0.17	44.77	41.63	3.74	0.06	V	95.9	2.41	0.84
GU009	0.10	0.44	1.36	3.67	12.88	0.03	V	9.53	3.71	2.77
GU010	5.02	0.10	2.52	10.15	1.75	0.07	P, V	3.97	<0.35	<0.35
GU011	6.32	51.86	2.11	11.67	0.55	nd	P	9.91	13.4	<0.35
GU012	0.26	11.94	0.18	13.65	1.04	0.01	P, V	6.53	nd	nd
GU013	0.00	3.67	0.00	0.68	0.77	0.00	P	<0.35	<0.35	<0.35
GU014	0.27	83.06	0.09	34.30	2.00	0.00	P	1.94	<0.35	<0.35
GU015	0.33	1.69	0.15	0.31	0.00	0.00	P	<0.35	0.37	<0.35
GU016	29.69	288.2	0.37	75.9	0.23	0.00	P	30.3	27.9	<0.35
GU017	2.15	74.73	2.83	8.19	0.05	0.00	P, V	4.15	10	<0.35
GU018	5.61	14.9	0.22	33.33	0.00	0.00	P, V	18.9	29.4	nd
GU019	1.1	16.31	0.05	0.33	0.08	0.00	P	0.88	2.17	<0.35
GU020	0.00	46.62	0.00	10.64	0.00	0.00	P	10.2	19.4	1.8
GU021	0.95	41.30	0.12	10.35	0.00	0.00	P	4.0	8.09	nd
GU024	0.32	20.00	0.04	23.11	0.00	0.00	P, V	6.36	6.03	nd
GU025	0.55	0.44	0.18	0.09	0.00	0.00	P	0.38	<0.35	<0.35
GU031	1.26	7.87	0.00	6.42	1.09	0.00	P	4.53	3.54	1.03
GU036	0.17	4.72	0.03	13.90	nd	1.23	V	3.75	2.22	1.24
GU039	0.00	12.10	0.00	0.00	nd	0.00	P	0.39	4.7	<0.35
GU041	0.00	1.26	0.00	0.71	nd	1.38	P, A	0.4	0.77	1.7
SAN07	0.00	15.52	0.00	10.39	0.00	0.00	P, V	3.47	4.41	<0.35
LAG01	2.63	0.38	0.10	0.40	3.73	0.19	P	2.21	4.45	0.98
LAG11	230.74	7.29	33.35	17.46	0.04	0.00	P, V	43.8	29.7	<0.35
LAG10	7.72	102.75	85.81	148.35	5.24	0.19	V	46.5	23.3	1.17
LAG02	0.00	118.33	0.00	6.88	0.38	0.00	P	14.6	22.3	2.19
LAG05	2.44	4.73	0.09	0.62	0.00	0.00	P	1.65	1.98	<0.35
LAG04	18.66	2.37	2.73	0.27	9.65	0.89	P	3.6	4.4	0.74
LAG03	6.04	21.20	108.9	83.20	14.6	1.93	V	40.9	11.1	2.35
LAG08	0.13	2.25	0.64	8.44	0.00	0.00	V	2.65	1.46	<0.35
LAG09	0.37	0.00	1.49	0.00	0.00	0.00	V	1.76	0.45	<0.35

d 1). nVes v 2 was shown to be composed of two isoallergens, namely Ves v 2.01 and Ves v 2.02, by mass fingerprinting (11). All the enzymes (nPol d 1, nVes v 1 and nVes v 2) were shown to be enzymatically active (data not shown). No

enzymatic activity was detected in group 5 antigens, thus demonstrating that the preparations were not contaminated by phospholipase and/or hyaluronidase. The purified allergen components were subsequently biotinylated and subjected to

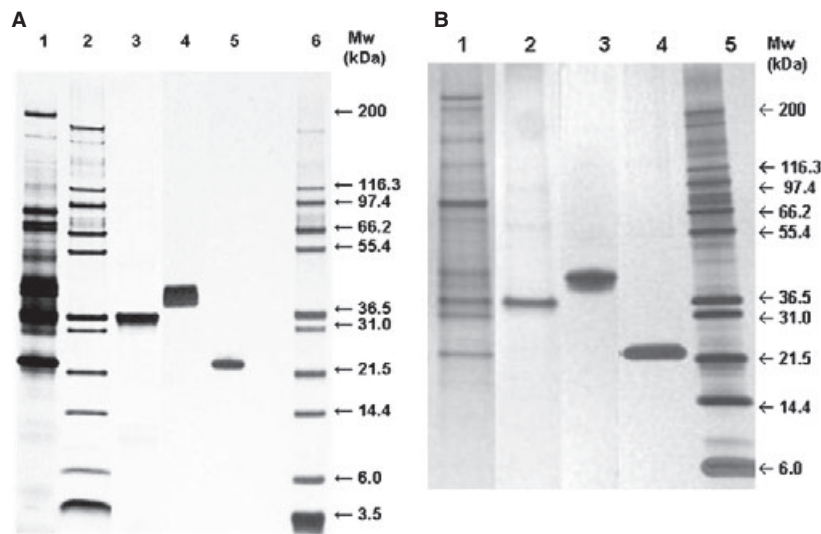


Figure 1 Purity analysis of *Vespula vulgaris* and *Polistes dominulus* venom allergens by silver-stained SDS-PAGE. Panel (A) shows *V. vulgaris* venom extract (lane 1) and its purified allergens (0.25 µg per lane): Ves v 1 (lane 3); Ves v 2 (lane 4), and Ves v 5 (lane 5).

Mw markers are shown in lanes 2 and 6 (Mark12, Invitrogen). Panel (B) shows *P. dominulus* extract (lane 1), and 0.25 µg of Pol d 1 (lane 2), Pol d 2 (lane 3) and Pol d 5 (lane 4), Mw markers are applied in lane 5.

ADVIA analyses, as described previously (31). Figure 2 summarizes all the individual components and extracts used in the different IgE determination methods mentioned in this study, as well as in the intradermal tests performed.

Specific IgE to natural and recombinant antigen 5s

Recombinant antigen 5s from *V. vulgaris* (rVes v 5) and *P. annularis* (rPol a 5) were initially used to test the sensitization in a set of 25 individuals suffering from allergic reactions to vespids (Table 2), and the results were compared with those obtained by ImmunoCAP and skin testing. Very low sIgE levels [below the limit of quantification (LOQ) specified in (30)] to either or both of the recombinant antigen 5s (rVes v 5 and rPol a 5) were observed in about 48% (12/25) of cases. The remaining patients (13/25) showed clearly positive sIgE values, which were quantitatively higher for one or the other recombinant component in ten cases and almost equal in a further three, therefore not allowing for discriminating between the sensitization against either of the two vespid species.

The sIgE reactivity was found to be higher when using the natural counterparts nVes v 5 and nPol d 5 (Table 2). Thus, only 4/25 individuals (16%) did not exhibit IgE values above the LOQ, thereby showing that these components significantly increased the sensitivity of the assay. The difference between natural/recombinant components differed for the *Polistes* and *Vespula* antigen 5s. Thus, in the case of nPol d 5 vs rPol a 5, an approximately 26.0-fold higher average IgE ratio was found for the natural counterpart (comparison made with data in Table 2 from the 11 patients exhibiting

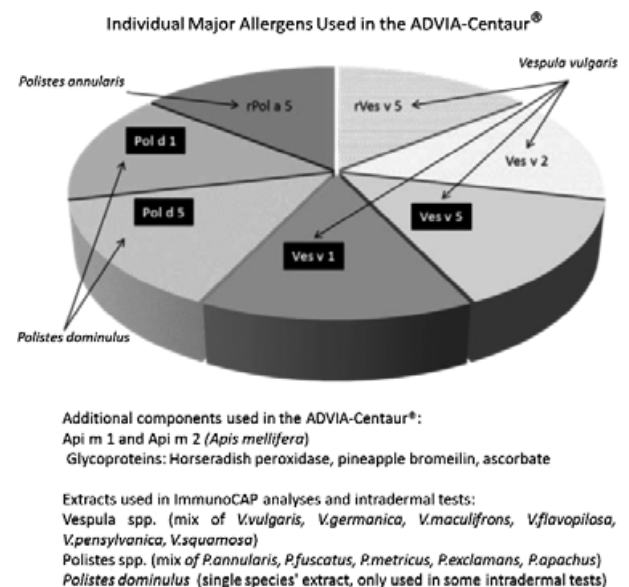


Figure 2 Summary of the individual components and extracts used in the different *in vitro* and *in vivo* clinical determinations. The upper scheme shows the individual major allergens used in the ADVIA Centaur® for sIgE determinations and generally correspond to the purified natural molecule (in the case of recombinant components allergenic molecules appear with the prefix 'r': rVes v 5 and rPol a 5). Individual components included in boxes are suggested to be the most relevant in differentiating sensitization amongst vespid-allergic patients. Other components and extracts used in ImmunoCAP and skin tests are shown below, describing the species' composition of the common mixtures generally used.

values above the LOQ for both nPol d 5 and rPol a 5; seven patients with positive nPol d 5 values showed rPol a 5 values below LOQ).

In contrast, a higher response was found for the natural component of Ves v 5, although the average difference was only 1.5-fold in the nine patients who exhibited both nVes v 5 and rVes v 5 values above the LOQ. Furthermore, the response was higher for the recombinant form in two cases (LHSM2 and VSOR57, Table 2). The sIgE was clearly positive for nVes v 5 in six further cases, although the values for the recombinant counterpart were below the LOQ.

A comparison of the component-based results with CAP data for the natural extract and the clinical data (Tables 1 and 2) showed that some patients (about 16% of those listed in Table 2) still did not show clearly measurable sIgE levels, even with the natural counterparts. To improve the sensitivity and specificity of the ADVIA assay, other natural major allergens from *V. vulgaris* and *P. dominulus* were isolated and used to assess the individual sensitizations.

Specific IgE to the complete panel of major *Polistes dominulus* and *Vespula vulgaris* allergens

The panel of natural components from *V. vulgaris* and *P. dominulus* included nVes v 1, nVes v 2 and nVes v 5, nPol d 1 and nPol d 5 (Fig. 2 and Table 3). Unfortunately, the quantity of nPol d 2 was insufficient for its use in the ADVIA system because the level of hyaluronidases in *Polistes* is considerably lower than that in the other species, as reported previously by Pantera et al. (3), and confirmed in this study (Fig. 1). By using these major vespid venom components in the ADVIA Centaur® system, all the sera tested showed IgE reactivity to the major components from *Polistes* and *Vespula*, and the quantitative responses allowed a possible sensitization profile to be proposed (Table 3). Around 31% (14/45) of individuals could not be clearly defined as being sensitized to *Vespula* or *Polistes* only, thus indicating that double sensitization could be possible, whereas the remaining 69% had markedly higher quantitative sIgE values for either the phospholipase or the antigen 5 of only one of the sensitizing species. Possible *Vespula* sensitization could be determined in 9/45 individuals and 22/45 seemed to be mono sensitized to *Polistes* (Table 3).

In addition, the sIgE values exhibited by Ves v 2 were less prevalent and generally much lower than those shown by the other components (in only 3/45 showed higher values than those of antigen 5s or phospholipases: LHSM06, GU009, LAG01 in Table 3). These data confirm the importance of both phospholipases and antigen 5s as the most significant allergens when attempting to determine sensitization to either of the vespid species. Furthermore, although only vespid venom-sensitized patients were included in our study, a possible double sensitization between *Apis* and vespids venoms was found in 2/45 cases (LHSM06 and GU041, Table 3), as suggested by their IgE reactivity to nApi m 1 [as shown also by Muller et al. (7)]. In the case of sample LHSM06, additional allergens and glycoproteins,

namely Api m 2, ascorbate, horseradish peroxidase and pineapple bromelin, were tested on the ADVIA Centaur® [samples prepared as in (7, 29)]: the values obtained (57.3, 15.0, 11.60 and 0.91 kU/l, respectively) indicate the likely relevance of cross-reacting carbohydrates (CCDs) in this patient, but do not rule out a possible sensitization to *Apis*. Apart from this particular case, the carbohydrate determinants did not appear to play a significant role in this group of patients because antigen 5s and phospholipases are not affected by these CCDs (7).

Discussion

Previous studies have indicated that the major Hymenoptera venom components can be used to discriminate between true double sensitization and cross-reactive sensitization in allergic individuals. The present study uses a component-resolved diagnostic approach to establish the possible source of sensitization in Spanish vespid-allergic individuals.

Sera from 59 patients with large local or systemic reaction after being stung by either *Polistes* or *Vespula* were analysed in this study. Initially, the sera from 25 patients were tested with recombinant antigen 5s (rPol a 5 and rVes v 5) and with natural antigen 5s (nPol d 5 and nVes v 5), to assess whether these components could be used to discriminate the individual sensitization profiles for the two species (Table 2). Although the results were promising (84% of patients had positive IgE values), other natural major *V. vulgaris* and *P. dominulus* allergens were isolated, and most of the sera ($N = 45$) were tested with a more complete panel of allergenic components (Table 3) to increase the sensitivity and specificity of the ADVIA assay. Thus, the molecular sensitization profiles of vespid-allergic individuals were investigated using a panel including natural (n) major *P. dominulus* and *V. vulgaris* allergens: nPol d 1/nVes v 1, nVes v 2 and nVes v 5/nPol d 5 (Fig. 2).

Sensitivity towards the natural antigen 5s was considerably higher than those of rVes v 5 and rPol a 5 (Table 2). Furthermore, a marked difference in IgE binding was demonstrated between nPol d 5 and rPol a 5. In the light of the fact that the recombinant antigen 5 from *P. annularis* has been found to be structurally and immunologically equivalent to its natural counterpart (24), and the purity analysis of the components ruled out any effect of contaminating components on this huge difference in IgE binding, we interpret that the difference between the two antigen 5s of the *Polistes* species might be mainly species-related, because *P. annularis* is present only in North America whereas *P. dominulus* is the prevalent species in the Mediterranean region, and they have been shown to present components with significant antigenic differences (3, 4). It is also possible that the rPol a 5 used represents a less-reactive isoform, which would also confirm that the natural components are more reliable for this type of determination, unless the recombinant component has been exhaustively characterized. In this respect, it should also be noted that Pharmed®-*P. dominulus* was launched on the European market by ALK-Abelló as far back as June 2006 to cor-

rectly diagnose and treat *Polistes*-sensitized patients, and at around the same time Severino et al. (4) suggested that *P. dominulus* should be used for both the diagnostics and VIT. Indeed, several cases that confirm the importance of correctly diagnosing and treating *Polistes*-sensitive patients have been reported (21, 22). In the case of *Vespula*'s antigen 5s, where the components used were both from the same species (*V. vulgaris*), the differences observed were much lower (an average of 1.5-fold stronger response towards the natural component, but with some patients exhibiting a higher response towards the recombinant protein). In the case of Ves v 5, the differences observed between the natural and recombinant components may also be due to the specific isoform used or to slight differences in the structure of both proteins, even though they have been shown to be immunologically equivalent (24).

The larger panel of isolated vespid allergens allowed the sIgE to be measured quantitatively, in other words, individual sensitivity to the different allergens tested to be determined (Table 3). In all cases (45/45), the IgE was specific to either both the antigen 5s (nPol d 5 or nVes v 5) and the phospholipases (nPol d 1 or nVes v 1) of one species or to only one of these two main allergenic components. Nine of these patients showed clearly higher IgE values to nVes v 1 or nVes v 5 or both, thus indicating that *Vespula* was most probably the sensitizing species for these patients. Likewise, the probable sensitization could be clearly assigned to *P. dominulus* in 22/45 cases, because of the higher values of Pol d 1 and/or Pol d 5, whereas in 14/45, the quantitative response did not allow the possible sensitizing species to be determined. In this case, unless complex inhibition studies (12–14) or sting challenges (21, 22) are performed to confirm the sensitizing species, double sensitization should be considered prior to entering into VIT.

The hyaluronidase from *V. vulgaris* (nVes v 2) was not found to be useful for the discrimination of vespid-allergic individuals because of its lower prevalence and considering the fact that it only exhibited high sIgE values (>1.0 kU/l) in $<32\%$ (14/45) of cases, with the other components having a similar or higher reactivity in the same samples. Our results are in agreement with the low allergenicity and relevance reported for this protein in vespid-sensitive patients (3, 9, 32). These results confirm previous findings, which showed that the ADVIA-Cenatur[®] system is both highly specific and highly sensitive and is not influenced by cross-reactivity mediated by low affinity-IgE (31, 33). Therefore, based on our results, the determination of sIgE against phospholipases and antigen 5s seems to be sufficient to discriminate the source of sensitization in vespid-allergic individuals (Fig. 2), which is a critical issue when it comes to differentiating sensitization to *Polistes* or *Vespula* in European patients, especially in the Mediterranean region (4, 15).

Component-resolved diagnosis is an extremely useful research tool that has rapidly generated a large amount of data on individual sensitization. However, a strategy on how best to use it this system as a robust clinical tool needs to be

established and the inclusion of various components need to be rigorously evaluated. In the case of Hymenoptera allergy, it is crucial to identify the source of sensitization and, as a result, adapt the composition of the extract to be used in VIT. This has also been pointed out by Severino et al. (4) and has been demonstrated in the case of re-stings or controlled sting challenges (21, 22), where protection was shown to be insufficient when the incorrect venom had been used in VIT.

Based on our current understanding, we propose that the antigen 5s and the phospholipases of both *V. vulgaris* and *P. dominulus* should be used in any component-resolved platform as a diagnostic tool to attempt to discriminate the sensitizations in vespid venom-allergic individuals. This proposal does not exclude the use of other components (e.g. hyaluronidases and/or other venom allergens, proteins containing CCDs, as shown for patient LHSM06) for particular patients who have multiple sensitizations. To confirm the sensitizing species, all the clinical data available must be taken into account, and other specific tests, such as inhibition studies of *in vitro* tests or controlled sting challenges (13, 14, 21, 22), should also be considered.

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Conflict of interest

Disclosure of potential conflict of interest of the authors: Dr Lluís Marqués has been a consultant for Stallergenes Iberica, has provided expert testimony for ALK-Abelló and has been paid for lectures by Allergy Therapeutics. Dr Arantza Vega has been paid for lectures by ALK-Abelló and Laboratorios Leti. Rafael I. Monsalve and Domingo Barber work for ALK-Abelló A/S. The remaining authors have declared that they have no conflict of interest.

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