Application of In Vitro Tests to Establish an Accurate Diagnosis of Double Sensitization to *Vespula* and *Polistes* Species

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The identification of the culprit insect in double sensitization (DS) to *Vespula* and *Polistes* species is a complex challenge. In Spain, the frequency of DS to *Vespula* and Polistes has been found to range from 50.5% to 61.5% [1].

The principal allergenic proteins of *Vespula* and *Polistes* species are phospholipase A1 (Ves v 1/Pol d 1), hyaluronidases (Ves v 2/Pol d 2), dipeptidyl peptidases IV (Ves v 3/Pol d 3), and antigen 5 (Ves v 5/Pol d 5) [2].

A 68-year-old woman developed repeated and extended local reactions with progressive local extension of the sting while she was at her summer home in Segovia (Spain). The last reaction affected the leg, extending to the ankle without systemic symptoms. The basal tryptase value (ImmunoCAP, Thermo Fisher Scientific) was 4.67 µg/L. DS was assessed based on intradermal skin testing (IST) and specific IgE (sIgE) levels. IST was performed with commercial lyophilized venoms (*Polistes dominula* and *Vespula* species, Pharmalgen, ALK-Abelló SA), which were diluted with albumin-based specific solvent according to the manufacturer's instructions (Pharmalgen, ALK-Abelló SA), reaching a concentration of 100 µg/mL. The venom was prepared in the same way for sIgE-INH/CAP inhibition (sIgE-INH) and the basophil activation test (BAT). For the remaining techniques, phosphate-buffered saline was used as a reconstituent. IST performed with *Vespula* and *Polistes* venoms was positive for both allergens

at 0.1 µg/mL and 1 and 3 months after the sting reaction. sIgE levels (ImmunoCAP) were as follows: total IgE, 984 kU/L; *Vespula* species, 88.60 kU_A/L; *Polistes* species, >100 kU_A/L; *Apis*, 0.31 kU_A/L; rPol d 5, 0.86 kU_A/L; rVes v 5, 2.86 kU_A/L; and rVes v 1 >100 kU_A/L. A 1:2 dilution of the patient's serum revealed sIgE for *Polistes* to be 192 kU_A/L and for Ves v 1 to be 110 kU_A/L. In addition, after obtaining the patient's informed consent, we determined sIgG4 to be 0.86 mgA/L for *Polistes* species and 0.30 mgA/L for *Vespula* species.

Subsequently, it was decided to carry out sIgE-INH and BAT as complementary tests in order to identify the primary sensitizer.

sIgE-INH was carried out by incubating separately two 50-µL aliquots of the patient's serum at room temperature for 1 hour with 100 µL of 100 µg/mL *Vespula* species and *Polistes dominula* venom (ALK Pharmalgen) [adapted from 3-5]. Heterologous inhibition of 70%-75% is considered strongly suggestive of sequence identity [4,5]. Inhibition higher than 70% was detected in the sera that had been preincubated with the venom of *Polistes dominula* (98% homologous inhibition and 97% heterologous inhibition). In the case of inhibition with *Vespula* species venom, the patient presented homologous and heterologous inhibition of 64% in both cases; this result, less than 70% but very close to it, raises suspicions of possible cross-reactivity between *Vespula* and *Polistes* [5].

BAT was performed with 3 concentrations of *Vespula* and *Polistes* venoms. A percentage of CD63-positive basophils was obtained from 5 µg/mL of both venoms [6]. Degranulation for each concentration was almost double after stimulation with *Polistes* than after stimulation with *Vespula* (Supplementary figure 1).

As routine techniques were not sufficient for an accurate diagnosis, we performed nonconventional tests: protein slot blotting (PSB), Western blot, and peptide microarray immunoassays (PMI).

PSB was performed using 50 µL of venom at different concentrations and the patient's serum diluted 1:10 [7]. The protein was revealed using α-hIgE-HRP (Southern Biotech) and visualized using chemiluminescence with the Clarity Western ECL Substrate (Bio-Rad). IgE-binding to *Polistes* was higher than to *Vespula* at all concentrations (Figure, A). A slight reduction in *Vespula* recognition was observed when the serum was incubated in the membrane with both venoms simultaneously (Figure, AI).

Vespula and *Polistes* extracts (5.6 µg protein/lane) were resolved in a 10%-15% SDS-PAGE gel under denaturing conditions. Western blot showed recognition of a ~33-kDa protein by IgE in *Polistes*, probably corresponding to Pol d 1 (Figure, B). A much less intense band recognized in *Vespula* was probably Ves v 1 [1].

We performed an IgE and IgG4 binding analysis using PMI as described by Martinez-Botas et al [8]. Different concentrations of venom extracts were printed on sciCHIP EPOXY slides, each feature in triplicate. Microarrays were

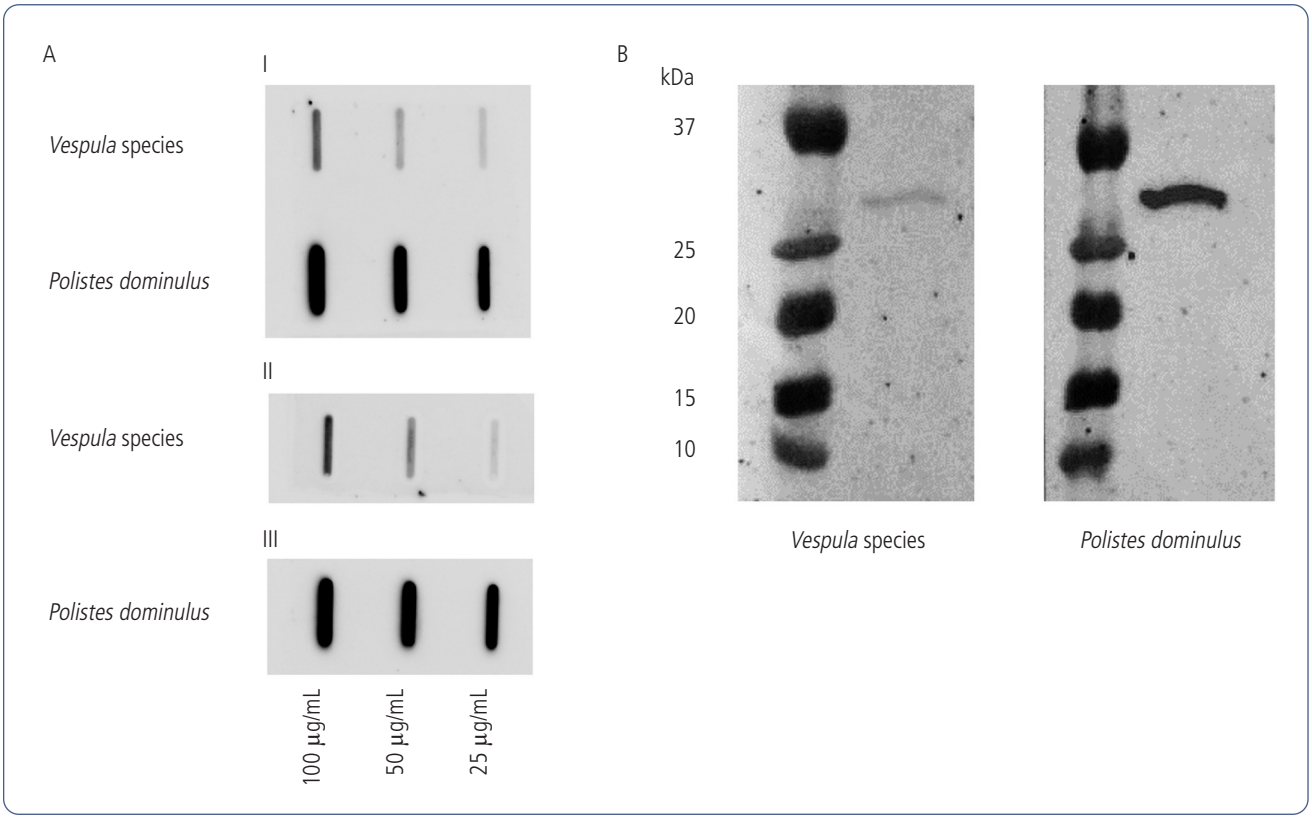

Figure. Recognition of the sIgE. A, Slot blot. Patient serum incubation with *Vespula* species and *Polistes dominula* (I), only with *Vespula* species (II), and only with *Polistes dominula* (III), at different concentrations (100, 50, and 25 µg/mL). B, Western blot. Incubation of the patient's serum with the proteins obtained by acrylamide/bisacrylamide gel for *Vespula* species and *Polistes dominula*.

hybridized with 100 µL of the patient's serum diluted 1:10. Quantification analysis revealed a positive correlation between the fluorescent signal obtained and the venom concentration in both IgE and IgG4 (Supplementary figure 2). IgE recognition was slightly higher in *Vespula* than in *Polistes* at all concentrations studied. However, for IgG4, higher values were obtained for *Polistes*. These were clearly visible, even at 0.125 µg/µL.

sIgE showed DS for the complete extracts of both venoms, with values being higher in *Polistes*. However, considering that phospholipase A1 (Ves v 1) is a major allergen in vespids and with no information available for Pol d 1, it was not possible to accurately determine the most relevant allergen.

sIgE-INH indicates that *Polistes* is dominant over *Vespula*, although this cannot be definitively established owing to the indeterminate value of the percentage of inhibition obtained for *Vespula*. However, it can be explained by the fact that there is only 52% sequence identity between Pol d 1 and Ves v 1, suggesting low cross-reactivity.

BAT was useful in the identification of the dominant allergen [9]. BAT with complete venom extracts resulted in considerable activation of basophils to *Polistes* at all concentrations while remaining positive to *Vespula* at 5 and 10 µg/mL.

Because of the increasingly polarized results for *Polistes* and the very low Pol d 5 value with respect to the total extract, we performed PSB and Western blot as alternative methods for assessing IgE. These enabled us to identify higher sIgE binding to the *Polistes* component Pol d 1.

PMI could not identify primary sensitization, since there was no significant difference between the signal obtained for sIgE in both extracts, although it did reveal greater binding of IgG4 to *Polistes*, possibly owing to greater exposure to that species. This is consistent with the sIgG4 ImmunoCAP result.

sIgE-INH and BAT suggested *Polistes* as the primary allergen, although PSB and Western blot pointed to *Polistes* and Pol d 1 as the primary sensitizers, respectively. We report a complex case of hymenoptera allergy in which conventional techniques cannot identify the clinically relevant allergen. The combined application of unusual in vitro techniques enabled us to achieve an accurate diagnosis for Polistes.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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