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Api m 6 and Api m 10 as Major Allergens in Patients With Honeybee Venom Allergy

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Abstract

Background: Component-resolved diagnosis plays a key role in the diagnosis and treatment of honeybee venom allergy (HVA). Our aim was to study whether any of the allergens not included in the usual diagnostic platforms are relevant in our population.

Patients and Methods: The allergenic sensitization profile of Spanish patients who experienced a systemic reaction after a honeybee sting and were diagnosed with HVA was studied by immunoblotting based on raw autochthonous Apis mellifera venom characterized using SDS-PAGE and mass spectrometry and a commercial assay (ImmunoCAP).

Results: Allergens in the International Union of Immunological Societies database were detected in the raw A mellifera venom extract used, except Api m 12. Sera from 51 patients with a median (IQR) age of 46.2 years (35.6-54.6) were analyzed. ImmunoCAP revealed Api m 1 and Api m 10 to be major allergens (88.2% and 74.5%, respectively). Moreover, Api m 6 (85.4%) was detected by immunoblotting.

Conclusion: Api m 1, Api m 6, and Api m 10 are major A mellifera venom allergens in our population.

Key words: Bee venom allergy. Component-resolved diagnostics. Venom sensitization profile. Api m 6. Api m 10.

Resumen

Antecedentes: El diagnóstico molecular puede ser una herramienta valiosa en el diagnóstico y el tratamiento de la alergia al veneno de abeja. Este estudio investiga si alguno de los alérgenos no incluidos en las plataformas diagnósticas habituales son relevantes en nuestra población.

Pacientes y métodos: Estudiamos mediante immunoblotting el perfil de sensibilización alergénica en pacientes españoles diagnosticados de alergia al veneno de abeja. Los resultados se compararon con los obtenidos usando un ensayo comercial (ImmunoCAP). El veneno crudo de Apis mellifera autóctona se obtuvo y caracterizó mediante SDS-PAGE y espectrometría de masas.

Resultados: Los alérgenos descritos en la base de datos International Union of Immunological Societies (IUIS) fueron detectados en el extracto crudo de veneno de A. mellifera utilizado. Se analizaron sueros de 51 pacientes con una edad media de 46,2 años (rango intercuartil 35,6-54,6). Api m 1 y Api m 10 fueron detectados como alérgenos mayoritarios (88,2% y 74,5%, respectivamente) usando ImmunoCAP. Además, se encontró Api m 6 (85,4%) mediante immunoblotting.

Conclusión: Nuestra población reconoce Api m 1, Api m 6 y Api m 10 como alérgenos mayoritarios del veneno de A. mellifera.

Palabras clave: Alergia a veneno de abeja. Diagnóstico por componentes. Perfil de sensibilización a veneno. Api m 6. Api m 10.
Introduction

Component-resolved diagnosis (CRD) is an extremely useful diagnostic tool that has rapidly generated a large amount of data on individual sensitization. *Apis mellifera* venom is the best characterized hymenoptera venom to date. Phospholipase A2 (Api m 1) and hyaluronidase (Api m 2) were traditionally considered the main allergens [1], although in recent years, Api m 3, Api m 5, and Api m 10 have also been described as major allergens in patients diagnosed with honeybee venom allergy (HVA) [2]. Api m 6 is an 8-kDa serine protease inhibitor with 4 isoforms. Since Api m 6 contains no putative N-glycosylation sites, it is devoid of any cross-reactive carbohydrate determinant (CCD)–based cross-reactivity.

Honeybee venom immunotherapy protects 77% to 84% of patients against new sting–induced anaphylaxis [3]. To reach these levels of effectiveness, it is important that all the allergens in the databases for bee venom are present in the therapeutic extracts [4]. While all the allergens are present in raw venom, they are not always present in the extracts available for immunotherapy.

HVA patients often have a broad sensitization spectrum. CRD can improve immunotherapeutic approaches in this type of allergy [2]. Some specific IgE (sIgE) sensitization profiles seem to behave as biomarkers and include sensitization to melittin (Api m 4), which is associated with poor tolerance to initiation of honeybee venom immunotherapy [5], and sIgE to icarapin (Api m 10), which is associated with treatment failure [6].

This study used immunoblotting with raw autochthonous honeybee venom to analyze the *A. mellifera* allergen sensitization profile in a group of Spanish patients diagnosed with HVA. We also determined whether any of the allergens not included in the usual diagnostic platforms are relevant in our population. These results were compared with those of ImmunoCAP sIgE.

Patients and Methods

Patients

The study population comprised patients who experienced a systemic reaction after a honeybee sting and were subsequently diagnosed with HVA in the Allergy Department of University Hospital of Guadalajara, Guadalajara, Spain. All patients had been diagnosed according to the recommendations of the European Academy of Allergy and Clinical Immunology (EAACI) [7] with a clinical history of a systemic allergic reaction after a bee sting, positive skin test results (ALK-Abell6), and total IgE and sIgE to *A. mellifera* venom in vitro. The patients’ clinical and demographic characteristics were collected. The study was approved by the local ethics committee, and patients gave their written informed consent to participate.

Serum sIgE

sIgE against *A. mellifera* venom and rApi m 1, rApi m 2, rApi m 3, rApi m 5, and rApi m 10 was measured using the ImmunoCAP assay system (Thermo Fisher Scientific) according to the manufacturer’s instructions. sIgE values greater than 0.35 kU/L were considered positive.

Venom Collection

Venom samples were collected from 3 different Spanish honeybee hives (*A. mellifera*) located in the province of León using electrical stimulation [8] and without affecting honey production or bee aggressiveness. Devices producing a mild electric shock through wire were located at the entrance of each hive. This wire was placed on top of the collecting glass tray and was active for 45 minutes. The collecting trays were then cleaned, and the dry venom was scraped off. The venom was kept at –20°C until it was freeze-dried for stabilization within 48 hours of collection. It is then further stored at –20°C until use.

SDS-PAGE and In-Gel Digestion

Protein from autochthonous venom was measured using the Bradford method (BioRad). Five micrograms of venom was dissolved in a 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) solution under reducing conditions and stained with colloidal Coomassie blue. Visible bands were cut and digested [9]. Extracted peptides were identified using a 4800 matrix-assisted laser desorption/ionization–tandem time-of-flight (MALDI-TOF/TOF) mass spectrometer plus a Proteomics Analyzer (Applied Biosystems, MDS Sciex) and 4000 Series Explorer v 3.5 Software (AB Sciex).

Shotgun LC-MS Analysis

A total of 350 µg of extract venom protein was digested in solution [10,11]. The desalted peptides were analyzed using reverse-phase liquid chromatography coupled to ion electrospray ionization tandem mass spectrometry and liquid chromatography mass spectrometry (LC-MS) in an EASY-nLC 1000 System coupled to the Q-Exactive HF mass spectrometer and Protein Discoverer 2.2 Software (Thermo Scientific, USA). Information about nano-liquid chromatography and data acquisition on Q-Exactive can be found in the Appendix.

Protein Identification

Peptides were identified from raw data using the Mascot v. 2.6.1 search engine. The database search was performed against SwissProt without taxonomic restriction (553089 sequences, 2017/02/21), NCBIprot with taxonomy restricted to metazoans (14836660 sequences), and an in-house contaminant database (247 sequences). The parameters used for the searches were tryptic cleavage after arginine and lysine, up to 2 missed cleavage sites allowed, and tolerances of 10 ppm for precursor ions and 0.1 Da for tandem mass spectrometry (MS/MS) fragment ions. The searches were performed allowing optional methionine oxidation and fixed cysteine carbamidomethylation.

The false discovery rate was calculated by means of a search against a decoy database (integrated decoy approach). The acceptance criteria for protein identification were a false discovery rate <1% and at least 1 peptide identified with a high degree of confidence (>95%CI).
Estimated protein abundance was calculated using peptide-spectrum match normalized for the molecular weight of the protein [12].

**Immunoblotting**

Serum samples from nontreated patients were used for the immunoblotting assay. Equal amounts of venom protein were resolved in a 15% SDS-PAGE analysis under reducing conditions and transferred onto a polyvinylidene difluoride membrane for immunodetection after blocking with 3% nonfat milk in 0.5% Tween-20 phosphate-buffered saline. A 1/100 dilution of each patient’s serum was incubated overnight at 4°C. After washing, the membrane was incubated with the human anti-IgE antibody (1:10 000, SouthernBiotech) coupled to horseradish peroxidase for 2 hours. Proteins recognized by IgE were detected by chemiluminescence (Plus-ECL, PerkinElmer). The sample band intensity had to be 2 times greater than that of the negative control (immunoblotting without sera) to be considered positive.

In order to assign the identity of the IgE-binding protein, we took into account the identification of the protein by mass spectrometry, the molecular weight of the protein, the presence of sIgE to that allergen in the serum sample, and, in some cases, the use of high-sensitivity silver staining to identify the exact band with specific marking.

**Statistical Analysis**

Quantitative variables are presented as median (IQR). Categorical variables are shown as percentages. Bivariate analyses were performed using the \( \chi^2 \) test, and the Mann-Whitney test was used to assess differences between groups.

**Results**

A total of 51 Spanish patients were included (43 men and 8 women), with a median age of 46.2 (35.6-54.6) years. Beekeepers accounted for 68.6% of the patients. Patients’ clinical data are summarized in Table 1.
### Table 2. Presence of Allergens in Immunoblotting and Specific IgE Values for Each Patient

| ID | Presence of allergen in immunoblotting | Specific IgE values, kU/L |
|----|---------------------------------------|--------------------------|
|    | Api m 1 | Api m 2 | Api m 3 | Api m 4 | Api m 5 | Api m 6 | Api m 10 | Api m 1 | Api m 2 | Api m 3 | Api m 5 | Api m 10 |
| 1  | +       | -       | -       | +       | -       | +       | -       | 0.52    | 0.01    | 0.01    | 0.05    |           |
| 2  | +       | -       | -       | -       | -       | +       | -       | 2.05    | 2.31    | 0.3     | 0.57    | 4.58     |
| 3  | +       | -       | -       | -       | -       | +       | -       | 0.34    | 0.001   | 0.03    | 0.03    | 0.01     |
| 4  | +       | -       | -       | -       | -       | -       | +       | 0.51    | 0.01    | 0.01    | 0.07    | 0.52     |
| 5  | +       | -       | -       | -       | -       | +       | -       | 0.52    | 0.12    | 0.16    | 0.25    | 1.84     |
| 6  | +       | -       | -       | -       | -       | +       | -       | 0.98    | 0.003   | 0.002   | 2.36    | 1.69     |
| 7  | +       | -       | -       | -       | -       | -       | +       | 1.82    | 0.47    | 0.02    | 0.01    | 0.21     |
| 8  | +       | -       | -       | -       | -       | -       | +       | 0.43    | 2.42    | 0.26    | 0.94    | 1.84     |
| 9  | +       | -       | -       | -       | -       | -       | +       | 2.97    | 0.26    | 0.09    | 0.93    | 5.62     |
| 10 | +       | -       | -       | -       | -       | +       | +       | 1.22    | 1.46    | 0.01    | 0.09    | 0.1      |
| 11 | +       | -       | -       | -       | -       | +       | +       | 1.98    | 0.002   | 2.59    | 0.87    | 13       |
| 12 | +       | -       | -       | -       | -       | -       | +       | 0.42    | 0.003   | 0.03    | 0      | 3.89     |
| 13 | +       | +       | -       | -       | -       | +       | +       | 19.4    | 3.08    | 3.53    | 0.93    | 5.09     |
| 14 | +       | -       | -       | -       | -       | +       | +       | 0.68    | 0.09    | 0.14    | 0.03    | 2.09     |
| 15 | +       | -       | -       | -       | -       | +       | +       | 3.99    | 0       | 0.03    | 0.003   | 0.24     |
| 16 | +       | -       | -       | -       | -       | +       | -       | 0.01    | 0.01    | 0.23    | 0.96    | 2.89     |
| 17 | +       | -       | -       | -       | -       | +       | +       | 2.05    | 0.01    | 1.06    | 0.09    | 1.8      |
| 18 | +       | -       | -       | -       | -       | -       | +       | 1.83    | 0.07    | 0.07    | 5.05    | 2.16     |
| 19 | +       | -       | -       | -       | -       | +       | +       | 7.47    | 1.61    | 0.38    | 0.35    | 0.33     |
| 20 | +       | -       | -       | -       | -       | -       | +       | 0.37    | 0.12    | 0.03    | 0.13    | 5.63     |
| 21 | +       | -       | +       | -       | -       | +       | +       | 0.01    | 0.005   | 0.13    | 0.004   | 10.3     |
| 22 | +       | -       | -       | -       | -       | +       | +       | 8.47    | 2.2     | 0.01    | 0.003   | 4.45     |
| 23 | +       | -       | -       | -       | -       | +       | +       | 1.04    | 0.04    | 0.03    | 0.8     | 0.18     |
| 24 | +       | -       | -       | +       | -       | +       | +       | 29.3    | 0.01    | 0.2     | 13.3    | 16.8     |
| 25 | +       | -       | -       | +       | -       | +       | +       | 1.7     | 15.9    | 31.2    | 4.85    | 101      |
| 26 | +       | -       | +       | +       | -       | +       | +       | 1.85    | 0.004   | 0.18    | 0.03    | 2.35     |
| 27 | +       | -       | -       | -       | -       | +       | +       | 0.09    | 0.05    | 0.07    | 0.6     | 0.06     |
| 28 | +       | -       | -       | -       | -       | -       | +       | 1       | 0.06    | 0.003   | 0.08    | 0.03     |
| 29 | +       | -       | -       | -       | -       | +       | -       | 1.68    | 0.15    | 0.02    | 1.32    | 6.34     |
| 30 | +       | -       | +       | -       | -       | +       | +       | 2.59    | 0.01    | 0.45    | 0.4     | 1.12     |
| 31 | +       | -       | -       | -       | -       | +       | -       | 24      | 0.01    | 0.55    | 2.17    | 2.45     |
| 32 | +       | -       | -       | -       | -       | -       | +       | 4.03    | 0.004   | 1.39    | 0.51    | 15.9     |
| 33 | ND      | ND      | ND      | ND      | ND      | ND      | ND      | 5.39    | 5.1     | 0.67    | 0.04    | 19.3     |
| 34 | ND      | ND      | ND      | ND      | ND      | ND      | ND      | 3.68    | 0.003   | 0.03    | 0.32    | 0.64     |
| 35 | +       | +       | +       | +       | +       | +       | +       | 5.64    | 53      | 2.3     | 0.82    | 36.2     |
| 36 | ND      | ND      | ND      | ND      | ND      | ND      | ND      | 0.42    | 2.75    | 0.15    | 0.96    | 1.15     |
| 37 | +       | -       | -       | +       | -       | +       | -       | 0.4     | 1.23    | 0.04    | 0.67    | 0.7      |
| 38 | +       | -       | -       | -       | -       | +       | -       | 0.06    | 0.01    | 0.01    | 0.21    | 0.06     |
| 39 | -       | -       | -       | -       | -       | -       | -       | 0.07    | 0.83    | 0.03    | 0.02    | 1.7      |
| 40 | +       | -       | -       | -       | -       | -       | -       | 0.64    | 0.03    | 0.24    | 7.46    | 13.4     |

(continued)
Raw venom of *A. mellifera* was obtained from ecological hives by electrostimulation. Its composition was analyzed using SDS-PAGE (Figure 1A and B), in which the presence of 10 bands can be seen, the most abundant having molecular weights compatible with Api m 4 and Api m 1. Bands were identified by mass spectrometry (Figure 1A and C). The 10 bands belonged to 9 of the 12 allergens described for *A. mellifera* (International Union of Immunological Societies). Shotgun identification of proteins in venom was performed to complete the analysis. The presence of 11 of the 12 allergens of *A. mellifera* venom was confirmed by limiting the 2 techniques applied to the high molecular weight of the protein (200 kDa), with only Api m 12 (vitellogenin) remaining unidentified. LC-MS data enabled relative quantification of each protein in the total extract to be obtained (Table S1 in Appendix). The SDS-PAGE stained with high-sensitivity silver (Figure 1B) demonstrated the presence of Api m 10 in the venom of *A. mellifera*.

The results of the sIgE determination in the patients’ sera are summarized in Table 2 and Figure 1. rApi m 1 and rApi m 10 were the major allergens in our population, sensitizing 88.2% and 74.5% of the patients tested, respectively (Table 3). Two patients had no sIgE to any of the recombinant allergens analyzed. At a cut-off point of >0.1 kU/L, they were positive to rApi m 1 and rApi m 5, respectively. Based on both rApi m 1 and rApi m 10, 96.1% of honeybee venom–allergic patients were diagnosed using ImmunoCAP with a cut-off point >0.35 kU/L. The sensitivity increased to 98% with the addition of rApi m 5. Six patients showed specific IgE (>0.35 kU/L) against CCDs.

**Discussion**

In this study, Api m 1, Api m 6, and Api m 10 were shown to be major allergens. Api m 6 has previously been described as a new *A. mellifera* venom allergen with detectable IgE in 26% to 42% of bee venom–hypersensitive patients [13,14]. Despite representing only 1% to 2% of the total amount of honeybee venom, it was capable of sensitizing 85.4% of the patients we studied, thus revealing itself to be a major allergen in our population. These variances concerning prevalence of sensitization in our study and in previous publications may be due to geographical differences in sensitization profile. Similar findings have been reported with vespid antigens [15]. More studies are needed to determine the possible role of sensitization to Api m 6 in the clinical picture and the response to immunotherapy of patients sensitized to this allergen.

It is essential to have an allergenic extract that contains all the allergens present in raw honeybee venom, since all the relevant allergens are not always present in commercial extracts [4]. The proteomic identification techniques applied in this study allow not only for the presence of relevant allergens to be checked, but also for their relative abundance within the extract to be determined. The selection and improvement of proteomic techniques will make it possible to improve the proteomic depth in venom extracts, especially for proteins that are present in very low amounts, such as Api m 10. The future application of these techniques could be a breakthrough in terms of standardizing bee venom in terms of all its allergenic components and not only for Api m 1 and Api m 2. It is important that a honeybee venom extract represents as

### Table 2. Presence of Allergens in Immunoblotting and Specific IgE Values for Each Patient (continuation)

| ID | Api m 1 | Api m 2 | Api m 3 | Api m 4 | Api m 5 | Api m 6 | Api m 10 | Specific IgE values, kU/L |
|----|--------|--------|--------|--------|--------|--------|---------|--------------------------|
| 41 | +      | -      | +      | -      | -      | -      | +       | 0.83 1.99 0.01 0.25 0.04|
| 42 | +      | -      | -      | -      | -      | -      | +       | 1.1 0.98 0.21 0.04 2.74|
| 43 | +      | -      | -      | -      | -      | -      | +       | 12.3 0.01 0.05 0.69 2.32|
| 44 | +      | -      | -      | -      | -      | -      | +       | 1.61 0.69 0.59 2.18 9.7 |
| 45 | +      | -      | -      | -      | -      | -      | +       | 0.45 0.88 0.16 0.06 1.02|
| 46 | +      | -      | +      | -      | -      | +      | +       | 40.9 0.05 16.8 26.7 30.3|
| 47 | +      | -      | -      | -      | -      | -      | +       | 1.52 0 0 0 7.1 |
| 48 | +      | -      | -      | -      | -      | -      | +       | 0.83 2.46 0.03 0.16 1.06|
| 49 | +      | -      | -      | -      | -      | +      | +       | 0.59 5.53 0.01 0.1 0.33|
| 50 | +      | -      | -      | -      | -      | +      | +       | 1.11 4.92 0.27 0.21 0.23|
| 51 | +      | -      | -      | -      | -      | -      | +       | 0.63 0.63 0.07 2.42 0.66|

Abbreviation: ND, not determined.
faithfully as possible the allergens of the raw venom, thus minimizing the presence of excipients and avoiding multidose compositions that could see their allergenic composition compromised through degradation [4,6].

The immunoblotting assay enables the identification of sIgE against relevant allergens that are not available in commercial systems, as is the case of Api m 6. It also provides greater sensitivity for some allergens, such as Api m 1, identifying 5 patients more than with the detection of sIgE. However, a lower percentage of patient recognition was obtained for Api m 2 and Api m 5. There is no clear explanation for the discrepancy in the results obtained with immunoblotting and ImmunoCAP. It may be that differential recognition of IgE depending on the technique employed (ImmunoCAP, Immulite, Advia Centaur) and the use of recombinant and native allergens give rise to different recognition rates. This has been confirmed for Api m 1 [2,16], but not for other allergens such as Api m 5. In view of the results, the correlation between both diagnostic techniques would not be good. However, the objective of the study was not to investigate the correlation between the 2 techniques, but to use them in a complementary way to obtain more in-depth information on patient sensitization. Immunoblot enables us to work with the natural allergens to which the patient is exposed and to detect allergens that are not available on commercial diagnostic platforms. While specific IgE detection using the diagnostic platforms enables us to use recombinant allergens, the results do not depend on the concentration at which the allergen is found in the natural allergen extract.

### Table 3. ImmunoCAP and Immunoblotting Sensitization

| Allergen | ImmunoCAP | Immunoblotting | P Value |
|----------|------------|----------------|---------|
| Api m 1  | 45 (88.2%) | 47 (97.9%)     | .0625   |
| Api m 2  | 21 (41.2%) | 4 (8.3%)       | <.001   |
| Api m 3  | 12 (23.5%) | 6 (12.5%)      | .227    |
| Api m 4  | NA         | 7 (14.6%)      | NA      |
| Api m 5  | 24 (47.1%) | 3 (6.3%)       | <.001   |
| Api m 6  | NA         | 41 (85.4%)     | NA      |
| Api m 10 | 38 (74.5%) | 26 (54.2%)     | .1078   |

Abbreviation: NA, not applicable.

*Number and percentage of patients sensitized to the different allergens of *Apis mellifera* according to ImmunoCAP with recombinant allergens and immunoblotting with raw venom.
One factor that diminishes sensitivity in detecting bee venom–allergic patients is the use of a single antigen to perform the diagnosis. Detection of Api m 1 sIgE varies depending on the technique and form used, the highest prevalence of sensitization being that observed using native Api m 1 [16,17]. Nevertheless, we must be aware that native Api m 1 contains CCDs and, hence, detects not only protein-specific IgE antibodies, but also anti-CCD IgE [18]. Several authors have reported that recombinant Api m 1 has limited clinical usefulness for the detection of HVA based on ImmunoCAP because of its low diagnostic sensitivity, which was about 58%-80% [1,19,20]. Therefore, the authors recommend the addition of more allergens to improve diagnosis: the combination of 2 allergens (Api m 1 and 10) enables diagnosis in 86.8% of cases [19]. We can diagnose 82.6% of patients with HVA using rApi m 1; this percentage rises to 96.1% when combined with Api m 10.

In conclusion, despite the possible limitations of this study, such as the nondetection of Api m 12 due to the limitations of the techniques used, the low number of patients, and the difficulty assigning the identity of the proteins in the immunoblot, the study demonstrates the need to use techniques other than those that are commercially available in order to identify all the sensitizations of patients who attend the allergy clinic. On the other hand, the concept of the major allergen is a local one, and each population will be different, as in the case of Api m 6 in the present study.

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Conflicts of Interest

AV reports having received fees for lectures from ALK, Allergy Therapeutics, Laboratorios Dieter, and Leti outside the submitted work. The remaining authors declare that they have no conflicts of interest.

Previous Presentation

Some of the data included in this manuscript were presented in poster format during the Spanish Congress of Allergy and Clinical Immunology in Valencia, 2018.

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