Clinical Commentary Review

Venom Component Allergen IgE Measurement in the Diagnosis and Management of Insect Sting Allergy

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Accurate identification of allergy-eliciting stinging insect(s) is essential to ensuring effective management of Hymenoptera venom-allergic individuals with venom-specific immunotherapy. Diagnostic testing using whole-venom extracts with skin tests and serologic-based analyses remains the first level of discrimination for honeybee versus vespid venom sensitization in patients with a positive clinical history. As a second-level evaluation, serologic testing using molecular venom allergens can further discriminate genuine sensitization (honeybee venom: Api m 1, 3, 4, and 10 vs yellow jacket venom/*Polistes dominula* venom Ves v 1/Pol d 1 and Ves v 5/Pol d 5) from interspecies cross-reactivity (hyaluronidases [Api m 2, Ves v 2, and Pol d 2] and dipeptidyl peptidases IV [Api m 5, Ves v 3, and Pol d 3]). Clinical laboratories use a number of singleplex, oligoplex, and multiplex immunoassays that employ both extracted whole-venom and molecular venom allergens (highlighted earlier) for confirmation of allergic venom sensitization. Established quantitative singleplex autoanalyzers have general governmental regulatory clearance worldwide for venom-allergic patient testing with maximally achievable analytical sensitivity (0.1 kU_A/L) and confirmed reproducibility (interassay coefficient of variation <10%). Emerging oligoplex and multiplex (fixed-panel) assays conserve on serum and are more cost-effective, but they need regulatory clearance in some countries and are prone to higher rates of detecting asymptomatic sensitization. Ultimately, the patient's clinical history, combined with proof of sensitization, is the final arbiter in the diagnosis of Hymenoptera venom allergy. **© 2024 The Authors. Published by Elsevier Inc. on behalf of the American Academy of Allergy, Asthma & Immunology. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).** **(J Allergy Clin Immunol Pract 2024;■:■-■)**

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INTRODUCTION

Insect stings represent the leading cause of large local reactions and severe anaphylaxis in adults,[1] and they have a significant role in work-related morbidity and mortality.[2-4] Between 56.6% and 94.5% of the general population describes having been stung by members of the Hymenoptera order at least once.[5] Inadvertent Hymenoptera stings reportedly elicit large local reactions in up to 17% of the general population.[3,4] Whereas systemic reactions to Hymenoptera stings occur in only 0.3% to 8.9% of adults,[6] a study of a representative cohort found that 41.6% of the general adult population had venom-specific IgE antibody (sIgE) levels above 0.35 kU_A/L to honeybee venom (HBV) and/or yellow jacket venom (YJV). Yet only 2.8% of these sensitized individuals reported a history of systemic reactions to stings.[7] In another study, Golden et al[8] reported that 3% of adults provided a history of a systemic sting reaction in an adult population with a 26% sensitization rate, but 40% reported a sting in the previous 3 months. Sturm et al[9] reported a 5% risk of anaphylaxis, and Golden et al[10] reported a 17% risk of systemic reaction in subjects with asymptomatic sensitization. For most people with positive sIgE levels, the sensitization may be asymptomatic and not clinically relevant. The disparity between reports of sensitization and

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Abbreviations used
Ag5- Antigen 5
CCD- Cross-reactive carbohydrate determinant
CRD- Component-resolved diagnostics
HBV- Honeybee venom
HVA- Hymenoptera venom allergy
PLA1- Phospholipase A1
PLA2- Phospholipase A2
PDV- *Polistes dominula* venom
sIgE- Specific IgE antibody
VIT- Venom-specific immunotherapy
VV- Vespid venom
YJV- Yellow jacket venom

clinically relevant allergy could be partly explained by the IgE sialylation status,[11] variable circulating levels of non-IgE blocking antibodies,[12] intrinsic status of intracellular signaling pathways, inhibitory or augmentation factors, and compelling observations suggesting that venom-specific IgE has a role in a natural defense mechanism, enhancing mast cell responsiveness and facilitating the neutralization of venom components through the action of released enzymes.[13,14]

Venom-specific immunotherapy (VIT) is the only treatment for Hymenoptera venom allergy (HVA) that modifies the course of disease and offers a high likelihood of cure, effectively safeguarding against occupationally induced large local and severe sting reactions in the future. Venom-specific immunotherapy is recommended for adults and children who show detectable sensitization accompanied by systemic reactions beyond generalized skin reactions.[15] Additionally, it is recommended for occupationally exposed workers (honeybee keepers and pest management experts) experiencing generalized skin symptoms and large local reactions if their quality of life is impaired.[4]

Given the potential of life-threatening anaphylaxis in HVA,[16] a thorough diagnostic process is imperative to ensure effective allergy management with VIT. Diagnosis begins with a thorough clinical history and physical examination that involves identifying the allergy-eliciting insect and evaluating risk factors that may predispose an individual to severe reactions or affect the treatment outcome. This is followed in a patient with a positive clinical by the identification of sensitization (IgE antivenom positivity) by *in vivo* (skin test) and *in vitro* (serology) testing methods, first with whole purified venom extracts and subsequently with *in vitro* assay reflex testing using molecular venom components. Molecular allergens are currently not cleared by governmental regulatory agencies for application to the skin. Quantitative singleplex serum IgE antibody tests and intradermal skin tests with venom extracts are considered complementary to each other. There remains approximately 10% of insect-allergic patients who are negative in any one test but positive in another.[17]

Molecular techniques have revolutionized our diagnostic approach to Hymenoptera venom-related sensitization by expanding our focus from whole-venom extracts to individual molecular components.[18] This shift has facilitated the development of molecular allergen-based sIgE diagnostics (component-resolved diagnostics [CRD]), which are valuable tools for dissecting venom allergies by enabling the analysis of patients' sensitization profiles to specific venom allergens.[19] In recent years, particular allergens from HBV (*Apis mellifera*), YJV (*Vespula vulgaris*), and European paper wasp venom (*Polistes dominula*) (PDV) have become available for analysis in clinical immunology laboratories worldwide (Tables I and II). Moreover, allergens can be produced so that they are free from cross-reactive carbohydrate determinants (CCDs),[20] or CCD-binding can be blocked,[21] circumventing a significant source of interference in sIgE assays.[22,23] Component-resolved diagnostics has introduced sophisticated strategies to discern cross-reactivity from primary sensitization and confirm multiple versus single venom sensitization, streamlining therapeutic decisions. This is particularly beneficial for patients who cannot pinpoint the offending insect, or when there is a mismatch between clinical history and traditional venom extract-based diagnostic results.[24]

VESPID VENOM MOLECULAR ALLERGENS IN CLINICAL APPLICATION

Various vespid species across different regions of the world can cause venom allergy. Yellow jackets (*Vespula* spp.) are prominent as allergy-eliciting species, particularly in the Northern Hemisphere. In contrast, paper wasps (*Polistes* spp.) hold greater significance in the United States and the Mediterranean regions of Europe. In South America, other Polistinae, such as *Polybia* spp., are of particular importance. Additionally, allergies to hornet stings (*Vespa* spp. and *Dolichovespula* spp.) are common and may be increasing owing to the spread of invasive species such as the Asian hornet, *Vespa velutina nigrithorax*.[25]

One common feature of most vespid venoms (VVs) is that they contain two allergens of particularly high allergologic relevance: phospholipase A1 (PLA1) (Ves v 1 and Pol d 1) and antigen 5 (Ag5) (Ves v 5 and Pol d 5) (Figure 1 and Table I). To date, molecular allergens exclusively from the venoms of the common yellow jacket (*V vulgaris*) and the European paper wasp (*P dominula*) are available commercially for routine laboratory diagnosis (Table II).

Vespid PLA1s exhibit no cross-reactivity with HBV phospholipase A2 (PLA2) (Api m 1) but share a high degree of structural similarity among themselves,[26] and cross-reactivity can be observed between PLA1s of most Vespoidea species.[27,28] Because the PLA1s of different *Vespula* species share approximately 95% sequence identity (around 70% with the American species *Vespula squamosa* and *Vespula vidua*, which belong to a different subgenus) and are thought to be almost entirely cross-reactive,[29] it can be assumed that the commercially available Ves v 1 is a valuable diagnostic tool for generally detecting YJV sensitization. In sting-allergic patients, IgE sensitization to Ves v 1 and Pol d 1 ranges between 39% to 66%[30-34] and 87% to 100%,[28,35] respectively.

Although Ag5 allergens are highly abundant in most VVs, their biological function remains unknown. IgE sensitization to Ves v 5 can be found in 82% to 98% of YJV-allergic patients.[31-34,36-41] Sensitization to Pol d 5 is more challenging to assess because substantial numbers of patients in the relevant populations are double-sensitized to PDV and YJV, with the primary sensitizer unknown. However, available studies suggest sensitization rates between 20% and 72%.[28,35] Antigen 5 allergens of different vespid species show a high degree of cross-reactivity among each other in *in vitro* sIgE measurements and basophil activation assays.[41] Interestingly, an Ag5-like protein was also identified at the transcriptomic level in the venom glands of winter bees. However, the resulting protein product shows no cross-reactivity with YJV Ves v 5.[42]

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TABLE I. Characteristics and significance of Hymenoptera venom allergens available for routine component allergen IgE measurement as of June 2024

Allergen	Biochemical name	Specific IgE antibody sensitization*	Cross-reactivity	Significance
		Honeybee venom (*Apis mellifera*)		
Api m 1	Phospholipase A2	57% to 97%	—	Marker for HBV sensitization. Allows discrimination between HBV and VV sensitization.
Api m 2	Hyaluronidase	28% to 60%	Ves v 2, Pol d 2	Potential marker for HBV sensitization (limited cross-reactivity with Ves v 2/Pol d 2 in absence of CCDs). Results should be interpreted with care in the context of clinical history.
Api m 3	Acid phosphatase	28% to 63%	—	Marker for HBV sensitization. Allows discrimination between HBV and VV sensitization. Valuable marker to diagnose HBV allergy in Api m 1-negative patients.
Api m 4	Melittin	17% to 54%	—	Marker for HBV sensitization. Allows discrimination between HBV and VV sensitization. Putative marker for severe VIT side-effects.
Api m 5	Dipeptidyl peptidase IV	16% to 70%	Ves v 3, Pol d 3	Cross-reactivity with Ves v 3 and Pol d 3 prevents use as marker for HBV sensitization. Api m 5-sIgE does not exclude primary VV sensitization. In cases where HBV allergy is highly likely but other tests have turned out negative, the use of Api m 5 still remains a diagnostic option to be considered.
Api m 10	Icarapin	35% to 73%	—	Marker for HBV sensitization. Allows discrimination between HBV and VV sensitization. Valuable marker to diagnose HBV allergy in Api m 1-negative patients. Dominant sensitization as putative marker for risk of venom-specific immunotherapy failure.
		Yellow jacket venom (*Vespula vulgaris*)		
Ves v 1	Phospholipase A1	39% to 66%	Pol d 1	Marker for VV sensitization. Allows discrimination between YJV and HBV sensitization. Cross-reactivity with Pol d 1 prevents use as marker to discriminate between YJV and PDV sensitization.
Ves v 5	Antigen 5	82% to 98%	Pol d 5	Marker for VV sensitization. Allows discrimination between YJV and HBV sensitization. Cross-reactivity with Pol d 5 prevents use as marker to discriminate between YJV and PDV sensitization.

(continued)

TABLE I. *(Continued)*

Allergen	Biochemical name	Specific IgE antibody sensitization*	Cross-reactivity	Significance
European paper wasp venom (*Polistes dominula*)				
Pol d 1	Phospholipase A1	87% to 100%	Ves v 1	Marker for VV sensitization. Allows discrimination between PDV and HBV sensitization. Cross-reactivity with Ves v 1 prevents use as marker to discriminate between PDV and YJV sensitization.
Pol d 5	Antigen 5	20% to 72%	Ves v 5	Marker for VV sensitization. Allows discrimination between PDV and HBV sensitization. Cross-reactivity with Ves v 5 prevents use as marker to discriminate between PDV and YJV sensitization.

CCDs, cross-reactive carbohydrate determinants; *HBV*, honeybee venom; *PDV*, *Polistes dominula* venom; *YJV*, yellow jacket venom.
*Measured by different assay systems and in patient populations with different inclusion criteria. References can be found in the main text.

As outlined in the section on HBV molecular allergens, PLA1 (Ves v 1 and Pol d 1) and Ag5 (Ves v 5 and Pol d 5) allergens are valuable markers for discriminating between HBV and VV allergy (Figures 1, *B* and 3 and Table I). However, the pronounced cross-reactivity of VV allergens among each other limits their reliability as marker allergens for detecting primary sensitization to different vespid species (Figure 1, *B* and Table I). Although an algorithm for using available vespid allergens to diagnose YJV and PDV allergy is proposed in Figure 2, a definite resolution of cross-reactivity and primary sensitization is rarely possible. Nevertheless, Monsalve et al[28] demonstrated that comparing the levels of sIgE with the homologous allergen pairs Ves v 5 and Pol d 5, and Ves v 1 and Pol d 1 enables a reliable identification of the allergy-eliciting venom in 67% of double-sensitized patients, an observation confirmed in a subsequent study.[43] However, the current reference standard for resolving double sensitization in YJV and PDV allergy is sIgE-venom-inhibition assays with YJV and PDV.[44-47]

Notably, the VV allergens available on routine laboratory tests achieve a high diagnostic sensitivity; for instance, the combination of Ves v 1 and Ves v 5 can detect sensitization in 92% to 100% of patients with YJV allergy.[31,33,34,37-40,48] The YJV and PDV hyaluronidases (Ves v 2, Pol d 2) and dipeptidyl peptidases IV (Ves v 3, Pol d 3) are further discussed in the subsequent section on HBV molecular allergens and are currently unavailable for routine diagnosis. Their availability, in addition to the currently available HBV homologs (Table II), would likely further improve diagnostic accuracy by enabling comparative measurements and facilitating the diagnostic profiling of patients with multiple positive IgE test results.

HONEYBEE VENOM MOLECULAR ALLERGENS IN CLINICAL APPLICATION

The most prominent honeybee species known to elicit venom allergy worldwide is *A mellifera* (European, common, or Western honeybee).[18,19] Therefore, allergenic proteins in its venom have been thoroughly investigated.[49] Currently, the most relevant HBV allergens are commercially available for CRD (Tables I and II). However, their availability varies depending on the particular sIgE assay platform used and the country of operation.

Detailed data on sIgE sensitization rates in HBV-allergic individuals are available for Api m 1 (57% to 97%),[24,32,36,37,40,48,50-57] Api m 2 (28% to 60%),[32,37,40,50-52,56,57] Api m 3 (28% to 63%),[32,50,51,57] Api m 4 (17% to 54%),[32,52,56,58] Api m 5 (16% to 70%),[32,50,51,57] and Api m 10 (35% to 73%)[32,50,51,57,59] (Figure, 1*A*). However, these rates can vary significantly based on the patient inclusion criteria.[60]

Api m 1, a PLA2, stands out as the most prominent allergen in HBV,[61] constituting up to 12% of its dry weight.[62] Although PLA1 allergens in VVs catalyze a related enzymatic reaction to PLA2, they do not share sequence identity and structural similarity with Api m 1.[63] The resulting lack of cross-reactivity and high sensitization rates make Api m 1 an ideal marker allergen for differentiating between sensitization to HBV and VV.[32,48]

Api m 2 belongs to the hyaluronidase protein family,[64] a group from which allergens have been identified across various Hymenoptera species.[65,66] Importantly, evidence suggests that Api m 2 may serve as a helpful marker allergen for detecting primary sensitization to HBV. Although Api m 2 is a major allergen in HBV,[32,37,40,50-52,56,57] the corresponding hyaluronidases in YJV (Ves v 2) and PDV (Pol d 2) are considered to have lesser relevance in VV allergies. Moreover, cross-reactivity between Api m 2 and its VV homologs, apart from CCD reactivity, is limited[20,67] (unpublished data). However, because cross-reactivity cannot be entirely ruled out, sIgE to Api m 2 must be interpreted cautiously and always considered within the broader context of the patient's clinical history.

To date, acid phosphatase (Api m 3) has been uniquely annotated as an allergen in HBV.[68] Hence, according to current knowledge, it represents a marker allergen for primary HBV sensitization. Api m 4 (melittin), a 3-kD cytotoxic peptide, also lacks a homolog in VVs, and constituting at least 50% of the dry weight, it is the predominant component of HBV.[69] IgE antibody to Api m 4 has been detected in 53% of Japanese honeybee keepers and 23% of German HBV-allergic patients.[32,70] Dipeptidyl peptidase IV allergens (Api m 5 in HBV) are found in HBV and VVs (Ves v 3 and Pol d 3) and exhibit pronounced cross-reactivity among each other.[71,72] Consequently, Api m 5 cannot be reliably used as a marker allergen to identify primary sensitization to HBV, especially because its homologs from VVs are unavailable for comparative sIgE testing in routine diagnostic practices.

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TABLE II. Commonly used assays for routine *in vitro* diagnostics that employ molecular Hymenoptera venom allergens

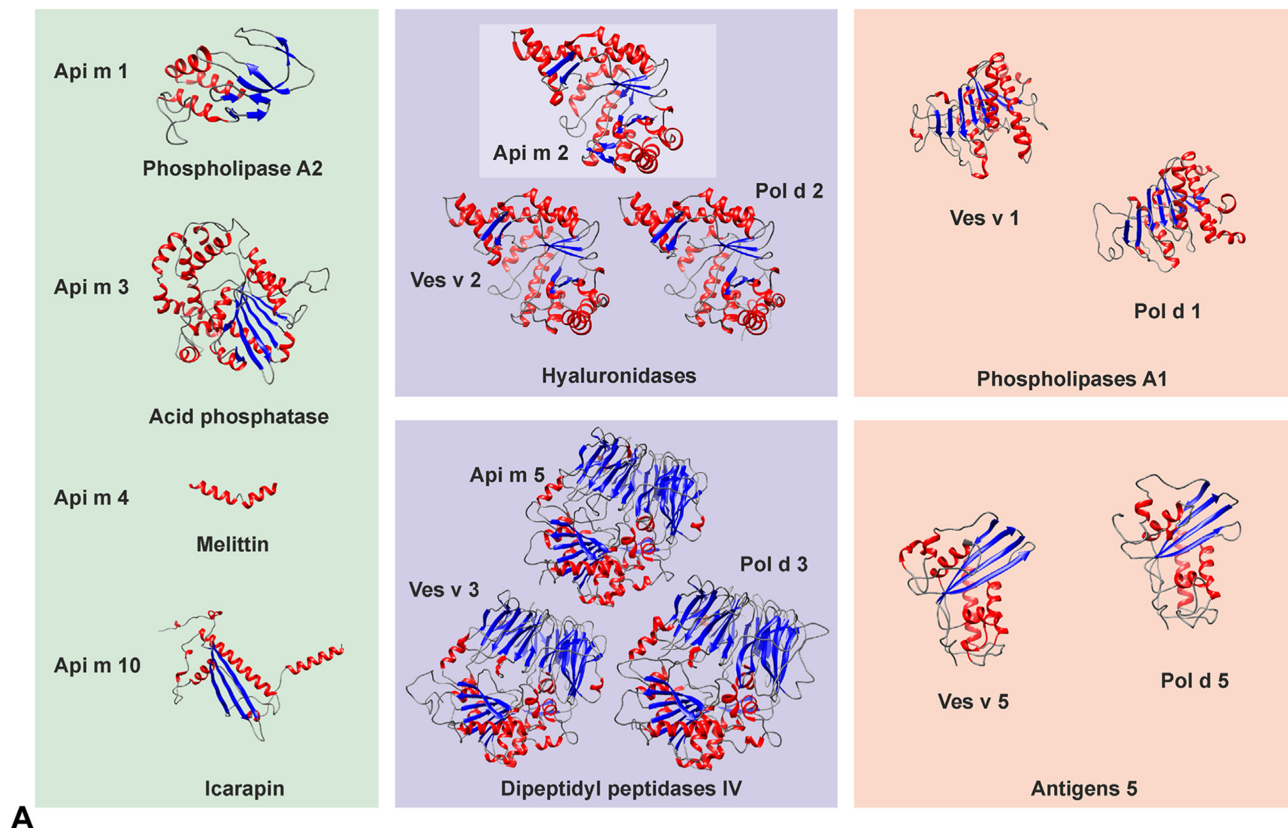
Assay	Manufacturer	Available allergens			Comments
		HBV	YJV	PDV	
Singleplex assays					
ALFA, REAST	Dr Fooke-Achterrath Laboratorien GmbH	Api m 1 Api m 2 Api m 10	Ves v 1 Ves v 5		CCD-free allergens
ImmunoCAP	Thermo Fisher Scientific	Api m 1 Api m 2 Api m 3 Api m 4 Api m 5 Api m 10	Ves v 1 Ves v 5	Pol d 5	CCD-free allergens
NOVEOS	Hycor Biomedical		Ves v 1 Ves v 5		CCD-free allergens
3gAllergy	Siemens Healthineers	Api m 1 Api m 2	Ves v 5		CCD-free allergens
Oligoplex assays					
EUROLINE	Euroimmun Medizinische Labordiagnostika	Api m 1 Api m 2 Api m 10	Ves v 1 Ves v 5	Pol d 1 Pol d 5	One assay carries HBV and YJV allergens One assay carries HBV, YJV, and PDV allergens CCD-free allergens The assays additionally carry venom extracts and a CCD marker
EUROASSAY	Euroimmun Medizinische Labordiagnostika	Api m 1 Api m 2 Api m 10	Ves v 1 Ves v 5		CCD-free allergens The assay additionally carries venom extracts and a CCD marker Available only in Germany
Multiplex assays					
ALEX[2]	Macro Array Diagnostics	Api m 1 Api m 10	Ves v 1 Ves v 5	Pol d 5	The assay uses a CCD inhibitor, which blocks CCD-specific IgE binding The assay additionally carries venom extracts and a CCD marker
FABER	ADL Srl	Api m 1 Api m 4			Api m 1 contains CCDs The assay additionally carries venom extracts

CCDs, cross-reactive carbohydrate determinants; *HBV*, honeybee venom; *PDV*, *Polistes dominula* venom; *YJV*, yellow jacket venom.
There are additional IgE antibody immunoassays reported in the peer-reviewed literature (eg, MARIA, Indoor Biotechnologies, Charlottesville, VA) that do not offer molecular Hymenoptera venom allergens; thus they are not listed here.

Api m 10 (icarapin), a labile protein with unknown function, stands out as a significant allergen in HBV despite its low concentration.[59] Although a homologous protein has been identified in PDV,[73] Api m 10 is a marker for primary sensitization to HBV.[74] This is because patients sensitized to VV lack sIgE reactivity to this allergen,[32,59] and preliminary, unpublished data suggest an absence of cross-reactivity between Api m 10 and its PDV homolog. Notably, Api m 10 contains one major IgE epitope recognized by all patients reactive to Api m 10,[75] which is not present in the PDV homolog.

The HBV allergens Api m 1, Api m 3, Api m 4, and Api m 10 are pivotal as marker allergens for detecting primary sensitization to HBV (Figure 1, *B*).[32] When used alongside vespid phospholipases A1 (Ves v 1/Pol d 1) and antigens 5 (Ves v 5/Pol d 5), these markers facilitate accurate differentiation between allergies to HBV and VVs (Figure 3).[19,60,76-78] With these marker allergens, CRD is being used to provide a diagnosis more accurately to patients who are double-sensitized to HBV and VV and those unable to identify the insect species that caused the allergic reaction.

The complexity of diagnosing HBV allergy with CRD is greater than that for VV allergy when using the patient's clinical history as the comparator because of the larger panel of relevant allergens and the diverse sensitization profiles observed in HBV-allergic patients. The first HBV allergen that became available for sIgE testing in the clinical laboratory, Api m 1, demonstrated a wide range of diagnostic sensitivity in HBV-allergic patients, from 58% to 97%.[32,36,53-56] This variability is influenced by factors such as the patient population's inclusion criteria, geographic variations, and the sensitivity of the immunoassay platform. Consequently, an absence of sensitization to Api m 1 does not rule out an HBV allergy. A pioneering study that used



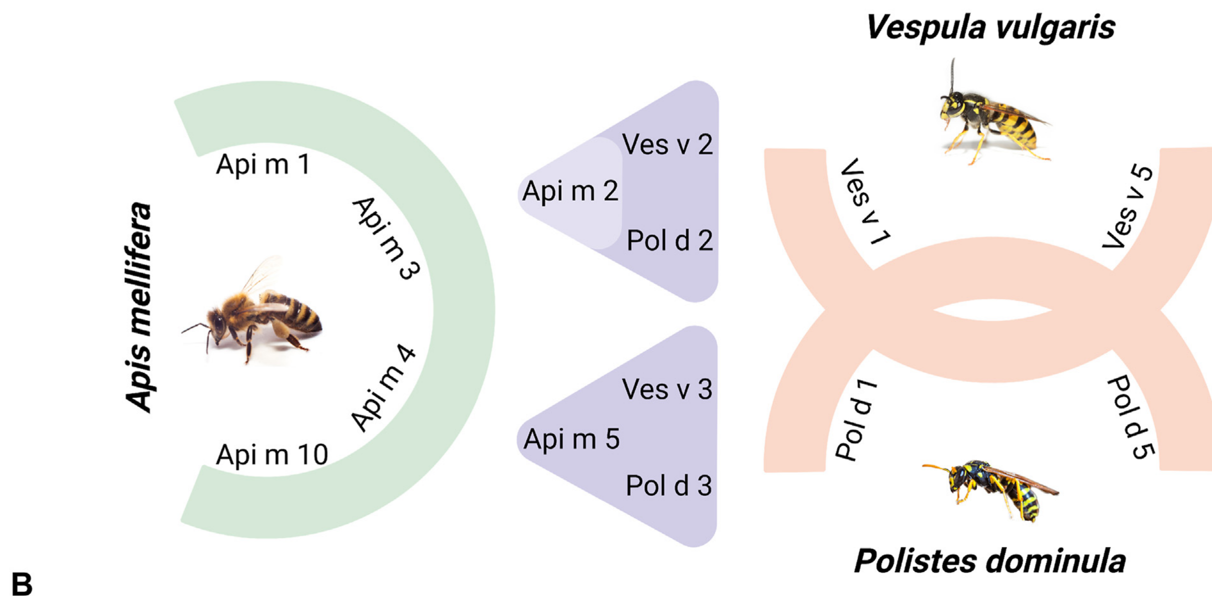


FIGURE 1. Overview of structures and cross-reactivity of Hymenoptera venom allergens with particular relevance for diagnostic purposes. (**A**) Comparison of three-dimensional structures. Illustrated are the structures of key allergens from honeybee venom (HBV), yellow jacket venom (YJV), and *Polistes dominula* venom (PDV). The structures highlight α-helices (shown in *red*), β-strands (*blue*), and coiled regions (*gray*). Included are the structures for Api m 1 (Protein Data Bank [PDB]: 1POC) and Api m 2 (PDB: 1FCU) from HBV and Ves v 2 (PDB: 2ATM) and Ves v 5 (PDB: 1QNX) from YJV, determined through x-ray diffraction. Structures not solved experimentally were generated by computational modeling. This visualization underscores the structural basis for antigenicity and potential cross-reactivity. (**B**) Diagnostic relevance of allergen cross-reactivity. Differentiation between primary sensitization and cross-reactivity to HBV and vespid venom can be achieved using the HBV allergens Api m 1, Api m 3, Api m 4, and Api m 10 and the YJV/PDV allergens Ves v 1/Pol d 1 and

the full spectrum of relevant HBV allergens (Api m 1-5 and 10) for diagnosing HBV allergy achieved a diagnostic sensitivity of 94.4%.[32] In contrast, another study employing the same assay platform and the Api m 1-3 and 10 allergen panel reported a lower diagnostic sensitivity of 79%.[50] This discrepancy likely stems from the varied composition of the patient cohort, especially considering the proportion of patients solely sensitized to HBV versus those double-sensitized to HBV and YJV. Yet another study found the diagnostic sensitivity of the latter panel to be 92% (90% and 94% for mono- and double-sensitized patients, respectively).[57]

Given the importance of CRD in clarifying cases of double sensitization, the available allergen panel can be deemed highly beneficial. Expanding the commercially available panel of HBV allergens beyond Api m 1 has added clinical value, for example, enabling the diagnosis of two thirds of patients who test negative for sIgE to Api m 1 by adding Api m 3 and Api m 10. For double-sensitized patients unable to identify the insect causing the allergy, this combined panel improved the sensitivity of HBV allergy verification to 78.6%, a significant increase from the previous 54% achieved with Api m 1 alone.[24]

Because of the close taxonomic relationship between the two genera, there are currently no marker allergens able to distinguish between primary HBV and bumblebee venom allergies.[19,79] On the other hand, despite the lack of detailed studies, this close relationship among different bee species suggests that the existing allergen panel could also effectively detect allergies to other bee (sub)species, including those commonly used in beekeeping. In line with this, a recent study has shown that sIgE measurements for Api m 1 and Api m 10 are effective in diagnosing allergy to *Apis dorsata* (Giant Asian Honeybee), highlighting the potential broader applicability of these markers.[80]

Furthermore, it has been demonstrated that allergen component-based testing in the basophil activation test is helpful for determining the appropriate VIT strategy in patients with inconclusive test outcomes.[81] However, standardized and validated venom allergens are not yet commercially available for basophil and mast cell-based diagnostic IgE antibody tests.

Besides shedding light on cross-reactivity and identifying primary sensitization, the significance of CRD and patients' sensitization profiles in the risk stratification for VIT has sparked considerable debate. A prospective study identified sensitization to Api m 4 (sIgE > 0.98 kU_A/L) as a potential risk factor for systemic reactions during the VIT initiation phase and more severe reactions after a sting.[82] Moreover, a retrospective multicenter study suggested that predominant sensitization to Api m 10 ($>$50% of sIgE relative to sIgE to whole HBV) may significantly increase the risk of VIT failure.[51] However, the current body of research does not yet provide a definitive conclusion regarding whether specific sensitization profiles can predict clinical severity and/or the likelihood of VIT success.[83] Despite this, in the future, understanding these profiles may enhance risk stratification and enable more personalized VIT approaches.[84]

VENOM ALLERGEN EPITOPES

A molecular understanding of recognition of venom allergens by monoclonal IgE antibodies is minimal, and peptide assays based on plasma or sera for identifying IgE epitopes, which were recently clinically evaluated for Ara h 2 epitopes in peanut-allergic patients,[85] are lacking in the field of venom allergy. Some nonlinear Api m 1 IgE epitopes have been characterized, but they were not further clinically evaluated.[86] Additionally, Api m 10 contains only one major IgE epitope, recognized by all Api m 10-reactive HBV-allergic patients.[75] Potentially, monoclonal IgE antibody convergence to immunodominant and pro-anaphylactic venom epitopes (such as those recently described for Ara h 2 epitopes in peanut allergy[87]) might be an essential mechanism behind clinically relevant versus nonrelevant (asymptomatic) Hymenoptera venom sensitization and double-sensitization to HBV and VV in sting reactors to single insect species.

DIAGNOSTIC AND MANAGEMENT STRATEGIES FOR THE USE OF MOLECULAR VENOM DIAGNOSTICS ACROSS REGIONS OF THE WORLD

Insects causing venom allergic reactions vary by region worldwide. In some areas, Formicidae (ants) stings are the most common cause of Hymenoptera venom anaphylaxis.[88,89] Further, insect migration and changes in venom allergy owing to climate change will intensify in the following decades, and many species are already expanding their range toward the poles.[90] Thus, in addition to allergens from HBV (*Apis*) and YJV (*Vespula*, which is highly cross-reactive with *Dolichovespula* and *Vespa*), allergens from paper wasp venom (*Polistes*) and distinct Hymenoptera allergens from stinging ant venoms (*Solenopsis*, *Myrmecia*, *Pachycondyla*, and *Pogonomyrmex*) are becoming more important for both diagnosis and immunotherapy.[88]

Allergy to paper wasp venom is particularly relevant in Southern Europe and parts of the United States, with recent evidence for the spread of paper wasps from south to north.[35,91] Pol d 1 helps to distinguish primary sensitization to *Polistes* and can corroborate sIgE-inhibition testing.[28,35,47] However, Pol d 1 does not distinguish primary *Polistes* sensitization from primary *Vespula* sensitization owing to cross-reactivity. Further, most therapeutic preparations for VIT of *Polistes* venom allergy seem to have a comparable amount of major *Polistes* allergens, including the products of *P dominula* venom and a venom mixture of American *Polistes* species.[92]

The fire ants (*Solenopsis*) native to South America spread or were imported to Central America, Southeastern United States, Australia, New Zealand, and several European, Asian, and Caribbean countries, and in heavily infested areas, the incidence of anaphylaxis resulting from fire ant stings exceeds that of other species of Hymenoptera.[89,93,94] The high frequency of imported fire ant anaphylaxis in infested areas is due to the almost 50% attack rate compared with the about 10% attack rate for *Vespula* in the United States, and not necessarily the venom itself. Fire ant venom has four major allergens: Sol i 1, 2, 3, and 4.[95] Sol i 1

Ves v 5/Pol d 5. Conversely, relevant allergens from YJV and PDV show significant cross-reactivity with each other, complicating the identification of the relevant allergy. The hyaluronidases (Api m 2, Ves v 2, and Pol d 2) and dipeptidyl peptidases IV (Api m 5, Ves v 3, and Pol d 3) present in all three venoms display variable degrees of cross-reactivity. Api m 2 shows limited cross-reactivity with its vespid homologs, whereas dipeptidyl peptidases IV across all species are highly cross-reactive.

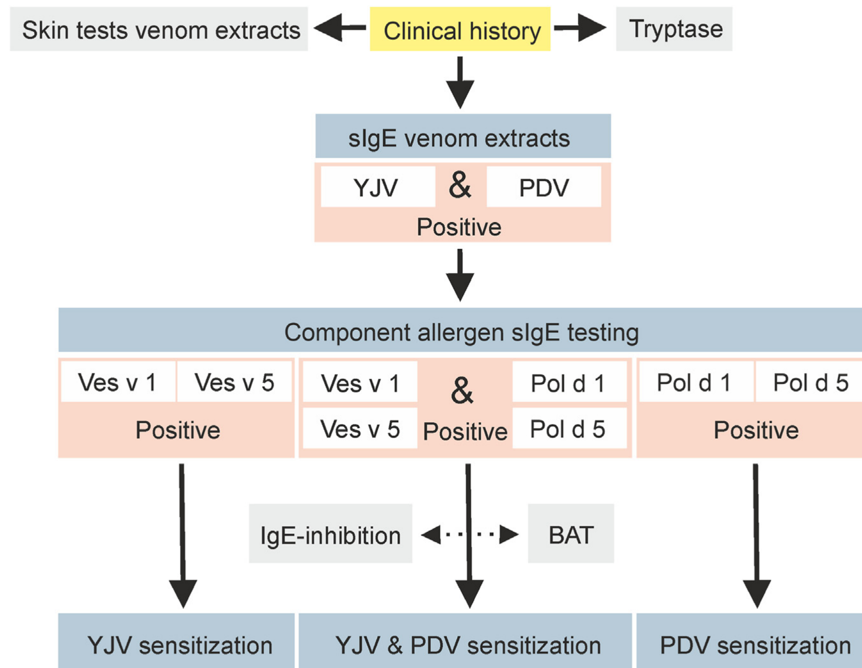


FIGURE 2. Diagnostic algorithm for component allergen IgE testing of yellow jacket venom (YJV) and *Polistes dominula* venom (PDV) allergy. In allergen component testing, not all allergens in a group must test positive to indicate sensitization; individual reactivities are sufficient to demonstrate sensitization. Pol d 1 is currently available for only a selected multiplex specific IgE platform. Despite the promise of component allergen testing, clinical history, skin tests, and the measurement of venom-specific IgE and serum tryptase form an essential basis for precise diagnosis in Hymenoptera venom allergy. Furthermore, cellular tests such as the basophil activation test (BAT) and IgE-inhibition assays can be valuable in unraveling primary sensitization. This algorithm is a simplification with considerable limitations. It cannot reflect all individual parameters, circumstances, and possible decision pathways that need to be considered when making a definitive diagnosis of Hymenoptera venom allergy. *sIgE*, specific IgE antibody.

has a PLA1 property and some cross-reactivity with PLA1 in VVs.[96] Sol i 3 is an Ag5-related protein and shows limited cross-reactivity with VV Ag5.[41,96] Fire ant whole-body extract immunotherapy seems effective and safe and is currently recommended for treatment.[97]

Jack jumper ant (*Myrmeciapilosula* species) is a predominant cause of ant sting anaphylaxis in Australia and Tasmania.[98,99] Major allergens in jack jumper venom are peptide Myr p 1, Myr p 2, and Myr p 3 (80% of patients are sensitized to Myr p 2), and venom preparations used in the diagnosis of jack jumper allergy and immunotherapy are standardized to those three allergens.[100-102] Sensitization to Myr p 1 seems to correlate with an increased risk for adverse reactions during jack jumper VIT and sting challenge failure.[103] Specific IgE testing (ImmunoCAP, Thermo Fisher Scientific, Uppsala, Sweden) is available for fire ant whole-body extract and jack jumper ant venom. However, none of the ants' venom allergens are currently available for CRD.

ANALYTICAL CELL AND IMMUNOASSAY METHODS EMPLOYING MOLECULAR VENOMS IN ASSESSMENT OF ALLERGIC SENSITIZATION

The diagnosis of allergic disease begins with a thorough clinical history and physical examination and transitions from decisions based on symptoms to IgE antibody testing for sensitization with complex venom extracts and newly available allergenic venom components.[19] For patients suspected of an HVA, the clinical history provides information on the type and severity of objective reactions, and it aids in identifying the culprit insect, which is helpful in planning VIT. Sting autotomy is almost universal among honeybees, but it is not unique to honeybees; it also commonly occurs with *Vespula maculifrons* stings.[104] Identification of large local and/or systemic allergic symptoms that are temporally associated with one or several stinging events is routinely followed by confirmation of sensitization (presence of venom-sIgE antibodies) to one or multiple Hymenoptera venoms using one of several diagnostic tests. Both direct *in vivo* (skin test and intentional insect sting challenge, in which more recent guidelines recommend sting challenges only for VIT monitoring[15]) and indirect *in vitro* cell-based (basophil or mast cell activation test) or humoral (serum/plasma-based immunoassays) methods have been used to detect IgE antibodies to Hymenoptera allergens.[19,105] Initial selection of a sensitization test varies as a result of the clinician's experience and bias, the age of the patient, national practice parameters, and the type of reimbursement provided by the medical program in the patient's country. For instance, allergists in the United States have historically preferred an intradermal skin test with purified venom extracts over *in vitro* methods, whereas in other countries serologic methods may be initially favored.[106] Moreover, an intentional venom sting challenge test[105] with live insects is not performed in routine patient care owing to logistic and safety concerns. Rather, it is reserved for research programs that investigate mechanistic and therapeutic efficacy hypotheses. Venom extracts are initially used in the diagnostic evaluation of a patient with a

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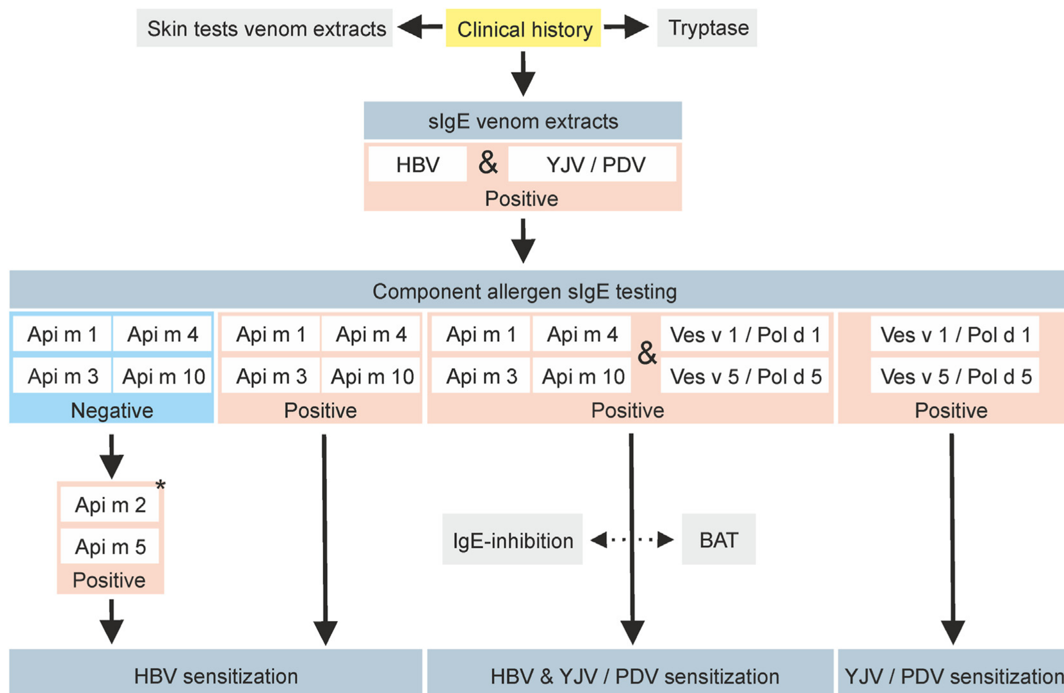

FIGURE 3. Diagnostic algorithm for component allergen IgE testing of honeybee venom (HBV) vespid venom (yellow jacket venom [YJV]) and *Polistes dominula* venom [PDV]) allergy. In allergen component testing, not all allergens in a group must test positive to indicate sensitization; individual reactivities are sufficient to demonstrate sensitization. Despite the promise of component allergen testing, clinical history, skin tests, and the measurement of venom-specific IgE and serum tryptase form an essential basis for precise diagnosis in Hymenoptera venom allergy. Furthermore, cellular tests such as the basophil activation test and IgE-inhibition assays can be valuable in unraveling primary sensitization. The HBV allergen Api m 2 might show limited cross-reactivity to homologous allergens of YJV and PDV that are not commercially available, so a positive test result does not necessarily exclude YJV or PDV allergy. Api m 5 is highly cross-reactive with homologs from vespid venom, which are currently are unavailable for comparative IgE testing. However, in cases where HBV allergy is highly likely, but other tests have turned out negative, the use of Api m 5 still remains a diagnostic option to be considered. This algorithm is a simplification with considerable limitations. It cannot reflect all individual parameters, circumstances, and possible decision pathways that need to be considered when making a definitive diagnosis of Hymenoptera venom allergy.

suspected venom allergy, followed by the use of molecular venom allergens when they can provide diagnostic clarity, especially with regard to suspected bee—vespid and hornet—yellow jacket dual sensitizations. Because regulatory agencies have not cleared venom components for *in vivo* skin testing, venom component-sIgE testing is thus restricted to the use of serologic assay methods. Molecular venom allergens provide a more definitive assessment of both genuine Hymenoptera venom specificity (honeybee: Api m 1, 2, 4, and 10; yellow jacket: Ves v 1, 5) and venom cross-reactivity—dependent sensitization (hyaluronidases: Api m 2 and Ves v 2; and dipeptidyl peptidases IV: Api m 5 and Ves v 3) (Table I).

Table II lists commonly used *in vitro* immunoassays that are employed in routine clinical testing worldwide and also offer molecular Hymenoptera venom allergen-sIgE testing. Missing from this list of *in vitro* assays is the basophil activation test, which is considered an excellent research tool for studying mechanisms of effector cell activation and confirming sensitization as a secondary test.[19]

The singleplex, oligoplex, and multiplex assays (Figure 4) in Table II share a common basic assay design, and they have the ability to detect IgE antibodies using both whole-venom extracts and Hymenoptera venom molecular allergens.[107] As a general design, allergen-specific antibodies are bound from human serum onto immobilized allergens on a solid-phase allergosorbent. After a buffer wash, bound IgE antibody is detected with a labeled anti-human IgE reagent. Response data from test sera are interpolated into allergy units of IgE antibody per unit volume (kU_A/L) from a total IgE reference curve. These assays, however, differ greatly in both analytical and diagnostic sensitivity and specificity. They use various solid-phase matrices and differ in the number of individual allergen specificities they can test at one time (range, 1 to about 300). Their source and quality of allergens differ along with their allergosorbent antibody binding capacity and many other technical variables (incubation conditions, buffers, labels, substrates, interpolation algorithms, reported units, degree of quantitation, level of automation, instrumentation requirements, degree of CCD blocking, and level of regulatory clearance and geographic availability).

Highly allergen-dense allergosorbents used in singleplex assays enhance their analytical sensitivity, which allows them to detect smaller amounts of venom-sIgE antibodies than multiplex assays. Their higher total venom-specific antibody binding capacity is less interfered with by non-IgE venom-specific antibodies that are commonly found in patients' sera. Multiplex allergosorbents tend to immobilize orders of magnitude smaller amounts of allergen. The density of allergens on the solid phase, the affinity of the patient's IgE antibodies, and the amount of allergen-specific non-IgE

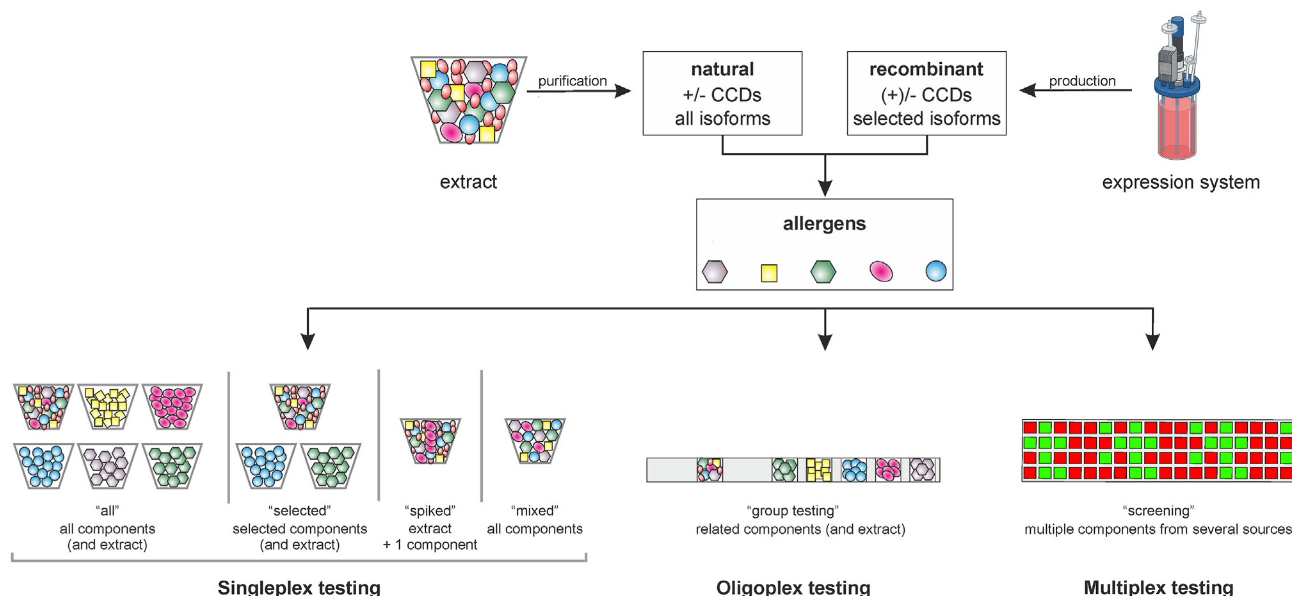

FIGURE 4. Concepts of singleplex, oligoplex, and multiplex testing in component allergen IgE measurement. Natural allergens, purified from their sources, may contain cross-reactive carbohydrate determinants (CCDs) based on their glycosylation status and the nature of the source, and may represent a mixture of allergen isoforms. Recombinant allergens, produced biotechnologically in various expression systems, can be specifically engineered to be free of CCDs. Selected isoforms are cloned and produced. In singleplex testing, a single type of target is tested in one assay. Specific IgE antibody can be tested against extracts and all available allergen components ("all"), selected analytes for specific questions ("selected"), or extracts supplemented with single components (eg, those underrepresented) to increase sensitivity ("spiked"). Using all available allergenic molecules from an allergen source as a mixture ("mixed") has not been considered so far and implies a potential loss of diagnostically helpful information about component-resolved sensitization profiles. Oligoplex testing simultaneously measures a small number of (related) analytes relevant to a given clinical context. Multiplex testing in a microarray format involves simultaneously measuring IgE antibodies against multiple allergens from various sources in a single assay ("screening"). Despite all its strengths (specificity completeness, serum conservation, and low cost), as a fixed panel, multiplex assays have a predisposition to identify asymptomatic but sensitized patients, which makes interpretation to the patient complex. Parts of the figure were created with BioRender.com.

antibodies in the patient's serum all affect what minimum detectable dose the assay can achieve. Until proven otherwise with definitive peer-reviewed and cross-validated data, singleplex assays, whether with venom extracts or components, are currently considered more analytically quantitative and analytically sensitive than multiplex assays. However, because the presence of IgE indicates sensitization (a risk factor for allergic disease) and not the absolute kU_A/L quantity of IgE antibodies, the analytical sensitivity (minimum detectable dose) of the venom-sIgE assay becomes the most important diagnostic performance parameter for selecting a venom extract and component (CRD)-sIgE assay.

By definition, multiplex assays[108] use a fixed allergen panel that cannot be tailored to sensitivities indicated by the clinical history of a given patient. Thus, one criticism leveled at the multiplex assay is its tendency to encourage abusive testing with the measurement of unwanted or unneeded IgE antibody specificities and, as a result, an increase in the chance of detecting asymptomatic sensitizations.[109] Its allergosorbent contains allergens from many different classes (food, inhalant, and venom) such that a patient who is being tested for allergic rhinitis may show positivity for venom-specific IgE (because of 20% to 40% asymptomatic sensitization in the population), leading to unnecessary fear, epinephrine prescriptions, and even venom immunotherapy.

Despite these limitations, chip-based multiplex assays such as the Allergy Explorer ($ALEX^2$) (MacroArray Diagnostics, Vienna, Austria) have a number of strengths to recommend their use compared with established singleplex autoanalyzers. Multiplexing allows simultaneous extract and component-sIgE measurements across all major allergen groups, which appears to be particularly useful in the context of the diagnostic evaluation of the allergic disease burden on a population level.[110] This permits a more accurate identification of both genuine and cross-reactive venom allergen-related sensitizations. Multiplex assays conserve sample volume (eg, 40 μL for one singleplex analysis vs 100 μL for about 300 multiplex analyses), and they require fewer reagents, which lowers the overall cost. They have a more rapid turnaround time (on a per-allergen basis), especially when assessing polysensitized venom-allergic patients.

CONCLUSIONS

Molecular bee and VV allergens provide new tools for precisely identifying the sensitization patterns of venom-allergic patients with the goal of optimizing VIT. In the short term, venom extract-based singleplex autoanalyzers will remain the dominant quantitative assay method together with intradermal skin testing to confirm sensitization in the diagnostic workup of a venom-allergic patient. Increasingly, venom components will be incorporated in this assessment to confirm genuine sensitization to one or several venoms and to clarify dual bee and

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vespid cross-sensitizations. Future use of a chip-based multiplex assay to assess venom sensitization will require the physician to compromise between a targeted venom singleplex IgE antibody assay strategy in which individual venom specificities are selected based on the patient's history and the use of a rigid comprehensive venom extract and molecular allergen array panel, which also contains prescribed numbers of non-venom allergen specificities, many of which will not be relevant to evaluating the patient for HVA.

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S. Blank, P. Korošec, M. Ollert, and R.G. Hamilton wrote the manuscript. B.O. Slusarenko designed the figures and revised the manuscript. All authors have agreed to the final version of the manuscript that has been submitted.

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