

Is epitope recognition of shrimp allergens useful to predict clinical reactivity?

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Summary

Background Shrimp is a frequent cause of severe allergic reactions world-wide. Due to issues such as cross-reactivity, diagnosis of shrimp allergy is still inaccurate, requiring the need for double-blind, placebo-controlled food challenges (DBPCFC). A better understanding of the relationship between laboratory findings and clinical reactivity is needed.

Objective To determine whether sensitization to certain shrimp allergens or recognition of particular IgE epitopes of those allergens are good biomarkers of clinical reactivity to shrimp. **Methods** Thirty-seven consecutive patients were selected with clinical histories of shrimp allergy. Skin prick test, specific IgE determinations, DBPCFC and immunoblot assays to shrimp extract were performed. IgE binding to synthetic overlapping peptides representing the sequence of the four allergens from the Pacific white shrimp (*Litopenaeus vannamei*) identified to date (Lit v1, Lit v2, Lit v3 and Lit v4) was analysed.

Results Of 37 (46%) patients, 17 had a positive challenge to shrimp (11 children and 6 adults). By microarray, patients with positive challenges showed more intense binding to shrimp peptides than those with negative challenges. Statistically significant differences in terms of the frequency and intensity of IgE binding to some epitopes were observed between the two groups. Diagnostic efficiency was higher for individual epitopes than for proteins. Particularly, efficiency was highest for certain Lit v 1 and Lit v 2 epitopes, followed by Lit v 3 and Lit v 4 epitopes.

Conclusion and Clinical Relevance Patients with positive shrimp challenges present in general more intense and diverse epitope recognition to all four shrimp allergens. IgE antibodies to these shrimp epitopes could be used as biomarkers for prediction of clinical reactivity in subjects with sensitization to shrimp. Patients with positive shrimp challenges show more intense sensitization and more diverse epitope recognition. Several IgE-binding shrimp epitopes could be used as biomarkers for predicting clinical reactivity in subjects with sensitization to shrimp.

Keywords allergen, arginine kinase, DBPCFC, food allergy, IgE epitope, microarray immunoassay, myosin light chain, peptide, sarcoplasmic calcium-binding protein, shellfish, shrimp, tropomyosin

Abbreviations Lit v 1, tropomyosin from *Litopenaeus vannamei*; Lit v 2, arginine kinase (AK) from *L. vannamei*; Lit v 3, myosin light chain (MLC) from *L. vannamei*; Lit v 4, sarcoplasmic calcium-binding protein (SCP) from *L. vannamei*; DBPCFC, double-blind, placebo-controlled food challenge; FDR, false discovery rate; HSA, human serum albumin; PBS-T, PBS containing 0.05% Tween 20; SPT, skin prick test; rPen a 1, recombinant tropomyosin from *Penaeus aztecus*.

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Introduction

Shellfish allergy is a long-lasting, life threatening disorder, which is common in both children and adults [1]. The actual prevalence of shellfish allergy is difficult to determine due to a lack of accurate controlled population-based studies incorporating double-blind, placebo-controlled food challenges (DBPCFCs). Reported rates of shellfish allergy vary among countries: Canada, 1.42%; United States, 2% and Singapore, 5.2% (14–16 years olds) [2]. In a prospective Spanish study, shellfish was the offending food in 22% of the subjects diagnosed with food allergy [3]. In addition, recent studies estimated the frequency of US emergency department (ED) visits/year at 203 000 for food-related acute allergic reactions and 90 000 for those with anaphylaxis [4]. According to the National Electronic Injury Surveillance System in the United States, shellfish may account for up to 24% of the ED visits for food-allergic events [5, 6]. Despite the high prevalence and severity of these food-allergic reactions, there is still no cure and accurate diagnosis and avoidance are the only recommended options.

Shellfish includes crustaceans and mollusks. Most species provoking allergic reactions belong to the class Crustaceans, which includes shrimp, by far the most frequently involved. The muscle protein tropomyosin (MW 34 kDa) is considered to be responsible for most of the allergenic activity of shrimp [7]. More recently, other allergens have been identified in crustaceans including arginine kinase (AK) with a MW 40 kDa [8], myosin light chain (MLC) [9] and a sarcoplasmic calcium-binding protein (SCP) [10] both with MW of 20 kDa. The latter two may be of particular importance in children [9, 10].

Diagnosis of shellfish allergy is aided by clinical history, skin prick test (SPT) and serum-specific IgE levels. Since extensive *in vitro* cross-reactivity is observed among crustaceans and other arthropods, the DBPCFC remains the gold standard for diagnosis and to identify causative species [11]. However, DBPCFCs are not entirely practical considering time requirements and the risk of potential severe reactions. Therefore, research is focusing on the development of diagnostic methodology that may eliminate the need for oral food challenges. Recently, the relevance of sensitization to tropomyosin has been studied in dust mite-sensitized subjects, also sensitized to shrimp. Tropomyosin-specific IgE was found to have greater diagnostic efficiency compared with whole shrimp-specific IgE or SPT (88.5%, 74.2% and 65.7%, respectively) to predict a positive oral challenge [12]. However, no study to date has evaluated the relevance of sensitization to all four main shrimp allergens or their epitopes with respect to clinical reactivity.

IgE epitope mapping with peptide micro arrays may provide an additional tool for allergy diagnosis and prognosis [13]. Informative IgE-binding epitopes have been identified as biomarkers of clinical severity, persistence or both in patients' with some food allergies. For peanut allergy, clinical sensitivity determined by means of DBPCFC has been positively related to broader epitope recognition [14]. Also, patients with persistent milk allergy showed increased epitope diversity and stronger IgE affinity binding compared with patients with transient milk allergy or tolerance to extensively heated milk [15]. Regarding shellfish allergy, the IgE-binding epitopes of the four shrimp allergens have been recently identified. Statistically significant differences in epitope recognition, in terms of frequency, diversity and intensity were observed between children and adults, suggesting that shrimp sensitization may decrease with age [16]. However, no attempt was made to correlate epitope or allergen recognition with clinical reactivity. The aim of this study is to determine whether sensitization to certain shrimp allergens or recognition of particular IgE shrimp protein epitopes is a good predictive marker of clinical reactivity to shrimp.

Methods

Patient selection

Patients were recruited between September 2007 and September 2008 from the Allergy Clinic of Fundación Jimenez Diaz and Hospital Infantil Universitario Niño Jesús, Madrid, Spain. Thirty-seven consecutive subjects with reported immediate allergic reactions following shrimp ingestion were selected. Some of the patients were previously diagnosed with shellfish allergy based on a suggestive history and positive SPT and/or shrimp-specific serum IgE and regularly followed-up in the clinic, and some were seen for an initial evaluation of shrimp allergy. The local ethical committee approved the study, and informed consent was obtained from all patients.

SPTs and specific IgE

All subjects underwent prick-by-prick tests with raw shrimp (*Solanocera melantho*) obtained from a local market. Tests were performed according to European guidelines [17]. Specific IgE to shrimp (f24 Phadia, Sweden), recombinant shrimp tropomyosin from gulf shrimp *Penaeus aztecus* (rPen a 1) and house dust mites (HDM, *Dermatophagoides pteronyssinus*/Dermatophagoides *farinae*) were measured by ImmunoCAP (Phadia, Uppsala, Sweden). Specific IgE antibodies > 0.35 kU_A/L was considered positive. Sera were stored at –80°C.

Oral food challenges

Double-blind, placebo-controlled food challenge were performed in all patients, except those with a moderate and severe anaphylactic reaction following shrimp ingestion within the previous 3 months. A meal was prepared with chocolate and vanilla ice cream, cocoa powder and vanilla extract. The active meal also contained ground boiled shrimp. Each subject received up to 24 g of shrimp in a blinded challenge. Five investigators confirmed that preparation was adequately blinded. None of the subjects were lactose intolerant or allergic to any compound in the meal. In case of a negative DBPCFC, the subject received an open shrimp challenge.

SDS-page and immunoblotting analysis

Boiled and raw extracts from the Pacific white shrimp (*Litopenaeus vannamei*) were prepared; proteins were separated by SDS-PAGE (Nupage 4-12% Zoom Gels; Invitrogen, Carlsbad, CA, USA) and transferred onto Immobilon-P (Millipore, Bedford, MA, USA) for detection of IgE binding as previously described [9]. Iodine 125-labelled goat anti-human IgE (DiaMed, Windham, ME, USA) was used as secondary antibody. Membranes were exposed to Kodak Imaging Film (Carestream Health Inc, Rochester, NY, USA) for 10 days. One non-atopic subject was used as negative control (NC).

Synthetic peptides, printing conditions and immunolabelling

Overlapping peptides (15-mers, overlapping by 12 amino acids) corresponding to the primary sequence of the four allergens identified in *L. vannamei*, Lit v 1 (tropomyosin), Lit v 2 (AK), Lit v 3 (MLC) and Lit v 4 (SCP) were commercially synthesized (JPT Peptide Technologies, Berlin, Germany; peptide sequences were previously reported [16]). Peptides were diluted, printed and immunolabelling was performed with individual sera as previously described [16, 18]. A mix of three monoclonal biotinylated anti-human IgE antibodies were used as secondary antibodies [16, 18], incubated overnight at 4°C. Afterwards, slides were incubated for 3 h at 31°C with Anti-Biotin-Dendrimer Oyster 550 (350; Genisphere, Hatfield, PA, USA) in Dendrimer Buffer (Genisphere). After successive washes, slides were centrifuge dried and scanned with a ScanArray Gx (PerkinElmer, Waltham, MA, USA). Images were saved in TIFF format.

For inhibition experiments, raw and boiled shrimp extracts were used as inhibitors. A serum pool of five patients with a positive oral challenge was used. The serum pool diluted 1/5 was pre-incubated for 1 h at

room temperature with raw or boiled shrimp extract, or chickpea extract as control, all of them at a final concentration of 0.5 mg/mL of inhibitor. Then, the serum pool-inhibitor mix was incubated with the peptide-containing slide and processed as described before.

Microarray data analysis

Fluorescence signal of each spot was digitized with the program Scan Array Express (Perkin Elmer), and data were exported as comma-delimited text files and transformed to z scores, as previously described [16, 18]. As peptides overlap by 12 amino acids, they might share the same epitope. Therefore, IgE-binding epitopes are defined as including two or more overlapping peptides. Subsequent analysis was done adjusting each Z score [calculated for each array element (spot) using protein printing buffer values within the same array as background signal] to the median of itself (i.e. of the six replicate spots in the array for the same peptide) and the two flanking peptides (weighted average Z score), by using the following formula $Z = 0.25 * Z_{-1} + 0.5 * Z_0 + 0.25 * Z_{+1}$. An IgE-binding epitope contained at least two contiguous peptides with a weighted average z score greater than 3. TileMap, a tool for tiling array analysis even when the sample size is small [19], was applied to identify IgE epitopes with statistical differences between patients with positive or negative challenge and also between children and adults. The false discovery rate (FDR) was used for adjustment of multiple comparisons in the peptide microarray study. A P-value of less than 0.01 and FDR of less than 0.05 were selected as significant thresholds.

Statistical analysis to describe the diagnostic value of each test

Statistical analysis was performed with Prism 4 for Macintosh version 4.0c (GraphPad Software, Inc, San Diego, CA, USA). Two-by-two tables were used to calculate sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and efficiency of sensitization to the different epitopes or proteins. Only individual epitopes identified as statistically significant in this study were considered for the analysis. An allergen was considered positive when at least one epitope of those showing statistically significant differences was recognized.

Specificity was defined as the proportion of true-negative results detected among negative challenge subjects and sensitivity as the proportion of true-positive results among positive challenge subjects. PPV describes the proportion of positive challenge subjects among those having a positive test result (recognition of an epitope or allergen), whereas NPV describes the proportion of

the negative challenge subjects among those having a negative test result. Efficiency was defined as the proportion of true-positive (TP) and true-negative (TN) results detected among the total number of tests, and it was calculated dividing the sum of TP and TN by the total number of tests.

Results

Patient characteristics

A total of 37 patients were recruited, 17 women (46%) and 20 men (54%). Overall, 23 were adults (13 women/

10 men; age range 18–70 years, median 34.3 years) and 14 were children (4 women/10 men; age range 3–16 years, median 7.5 years). Immediate allergic reactions after contact with shrimp ranged from mild (oral symptoms) to severe (anaphylaxis). Twenty were new and 17 had been previously diagnosed with shell-fish allergy (time after diagnosis, median: 46 months; range: 10–270 months) (Table 1).

Oral food challenges

Double-blind, placebo-controlled food challenges were performed in 31 of the 37 patients. In six subjects, a

Table 1. Patients' clinical characteristics, challenge outcome, SPT and specific IgE measurement

Group	Subject	Age (years)	Sex	DBPCFC	SPT	Symptoms	Time Dx	Shrimp IgE	rPen a 1 IgE	DP IgE
A <i>n</i> = 17	1	33	F	NI	+	Ax, inh-A	0	100.0	100	46.0
	2	8	M	NI	+	Ax	36	100.0	100	14.1
	3	7	F	NI	+	Ax	24	100.0	61.8	11.1
	4	15	F	+	+	U, RC	20	96.7	95.5	18.7
	5	5	M	+	+	U	30	50.4	40.3	10.3
	6	14	M	+	+	U, A	24	24.2	16.6	10.5
	7	3	M	+	+	A	0	13.7	7.85	0.86
	8	9	M	+	+	U, AE	96	11.7	9.48	5.75
	9	28	F	NI	+	Ax, inh-A	270	7.03	2.03	ND
	10	33	M	NI	+	Ax	0	6.15	2.83	2.27
	11	26	F	NI	+	Ax	0	5.60	2.60	<0.35
	12	13	M	+	+	U, A, GI	75	5.5	0.79	2.15
	13	16	F	+	+	GI	0	5.24	4.34	26.70
	14	33	M	+	+	OAS, GI	250	2.72	0.46	13.90
	15	48	M	+	+	OAS, GI	80	2.47	<0.35	0.82
	16	12	F	+	+	U, inh-A	46	5.98	<0.35	1.56
	17	8	M	+	+	AE	40	15.4	8.62	1.94
B <i>n</i> = 11	18	29	F	—	+	U	10	0.44	<0.35	0.75
	19	31	M	—	+	U, AE, A	0	0.62	<0.35	0.46
	20	18	F	—	+	OAS	0	0.55	0.38	1.26
	21	41	F	—	+	RC	0	0.48	<0.35	9.75
	22	22	M	—	+	OAS	60	2.32	<0.35	4.13
	23	24	F	—	+	U	0	2.15	0.71	0.35
	24	31	M	—	+	U	0	5.58	4.43	12.20
	25	29	F	—	+	U	0	1.45	<0.35	60.20
	26	7	M	—	+	GI	24	0.6	0.41	<0.35
	27	7	M	—	+	RC	0	16.5	10.1	2.0
C <i>n</i> = 9	28	5	M	—	—	U, GI	0	0.81	0.48	0.68
	29	36	F	—	—*	U	120	<0.35	<0.35	1.19
	30	36	M	—	—*	GI	160	<0.35	<0.35	2.03
	31	34	M	—	—	U	0	<0.35	<0.35	<0.35
	32	47	M	—	—	U	0	<0.35	<0.35	<0.35
	33	39	F	—	—	U	0	<0.35	<0.35	0.66
	34	24	F	—	—	U	0	<0.35	<0.35	<0.35
	35	26	M	—	—	GI, RC	0	<0.35	<0.35	<0.35
	36	70	F	—	—	U, AE	0	<0.35	<0.35	<0.35
	37	52	F	—	—	U	0	<0.35	<0.35	<0.35

*SPT-positive in past.

Results of food challenge: (+), positive; (—), negative; NI, not indicated; ND, not done. Results of SPT: (+), positive; (—), negative; Ax, anaphylaxis; A, asthma; inh-A, asthma by inhalation; U, urticaria; AE, angioedema; OAS, oral allergy syndrome; GI, gastrointestinal symptoms; RC, rhinoconjunctivitis; time Dx, time since diagnosis of shrimp allergy (months); DP, *Dermatophagoides pteronyssinus*.

challenge was not indicated due to a recent history of anaphylaxis following shrimp ingestion. DBPCFC confirmed the diagnosis of shrimp allergy in 10 patients (32%), 8 children and 2 adults. The 21 patients (57%) with negative DBPCFCs, including 4 children (19%) and 17 adults (81%), received an open challenge, which was positive in one child only. Symptoms after challenge were immediate, except for a child who developed urticaria after 6 h. Interestingly, the levels of shrimp and rPen a 1-specific IgE in this child were 96 and 94 kU_A/L, respectively.

Based on our results, we differentiated three groups of patients. Seventeen patients had positive oral challenges, SPTs and shrimp-specific IgE levels (group A). Eleven patients had negative challenges but positive SPTs and/or shrimp-specific IgE levels (group B), and nine patients, who despite reporting symptoms following shrimp ingestion, had negative challenges, SPTs and shrimp-specific IgE levels (group C). All patients in this latter group were adults (Table 1). Unfortunately, data about the last reported reaction were not available.

Skin prick testing and shrimp-specific IgE levels

Prick-by-prick skin tests with raw shrimp were performed in all patients and were positive in 27/37 (73%), (13/14 children, 14/23 adults) (Table 1). Ten had a negative test (27%), but two had previously documented positive shrimp SPTs. Serum shrimp-specific IgE was positive in 28/37 patients (76%), with 14 children (100%) and 14 adults (61%). Of these 28 subjects, 21 (57%) were positive for IgE to rPen a 1 (13 children and 8 adults). Subjects in group A had higher values of specific IgE to shrimp (range: 2.47 to >100 kU_A/L, median: 11.70 kU_A/L) and r

Pen a 1 (range: <0.35 to >100 kU_A/L, median: 7.85 kU_A/L) than those in group B (range: 0.44–16.5 kU_A/L, median: 0.81 kU_A/L) and < 0.35–10.1 kU_A/L, median: 0.38 kU_A/L, for shrimp and rPen a 1 specific IgE, respectively. Of the 37 (76%) patients, 28 were sensitized to HDM (13/14 children and 16/23 adults).

IgE immunoblotting

In general, patients in group A had IgE antibodies recognizing more shrimp proteins and with greater intensity as shown by IgE immunoblotting than those in group B. In addition, both groups appeared to recognize different proteins. Most subjects of group A showed IgE binding to proteins ranging from 6 to 90 kDa, in raw and boiled shrimp extract with strong intensity (Fig. 1a). Similar proteins were recognized in both extracts. Two IgE-binding proteins of approximately 20 kDa and 34 kDa were recognized by most subjects. In group B, a different pattern of protein recognition was seen (Fig. 1b). In general, IgE recognition was weaker than in group A, and several subjects recognized proteins of high molecular weight in the boiled extract. Patients from group C showed no protein recognition of any of the extracts (data not shown).

IgE reactivity to shrimp peptides by peptide microarray analysis

Sera from 15 of the 17 subjects of group A and all the subjects of group B (11) were used for IgE epitope mapping. Seven atopic and non-atopic subjects were included as NC. Since patients from group C did not recognize any protein by immunolabelling and had

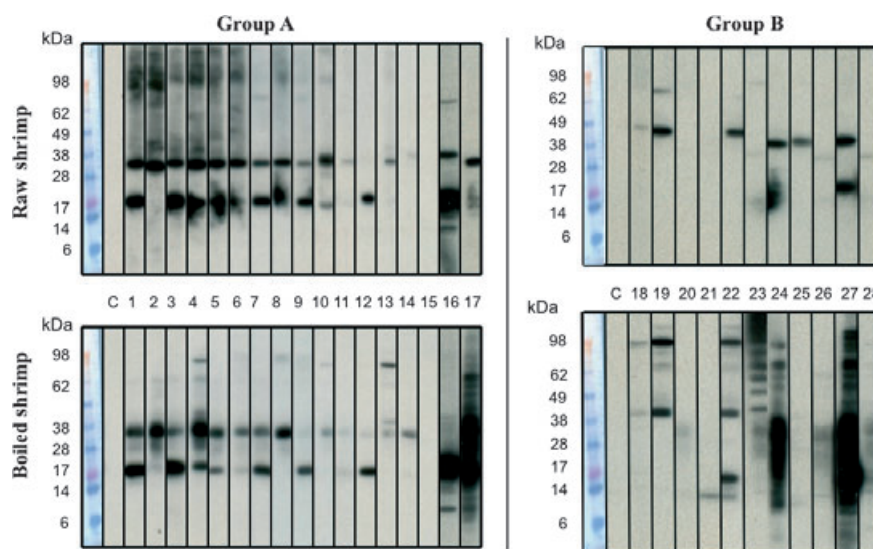


Fig. 1. Immunoblotting with raw and boiled shrimp extracts. (a) Patients with positive DBPCFC (group A). (b) Patients with negative DBPCFC (group B). Top: raw shrimp. Bottom: boiled shrimp.

negative SPTs and shrimp-specific IgE levels, this group was not included in the microarray analyses.

The same major IgE epitopes previously described by our group for Lit v 1, Lit v 2, Lit v 3 and Lit v 4 [16] were identified in our current study including a different population. Recognized epitopes and their frequencies are shown in Table 2 and Fig. 2 (group A vs. NC), with their intensity of recognition (Fig. 3). Frequency of recognition of each epitope for each protein represents the number of patients recognizing each epitope divided by the total number of patients in each group recognizing that protein.

To determine the frequency of recognition of each allergen, all epitopes were considered. In general, frequency and intensity of recognition for all four allergens was higher in group A than in group B, as represented by fold change in Z scores (Fig. 2). For group A, the frequency of allergen recognition was as follows: Lit v 1, 15/15 (100%); Lit v 3, 14/15 (93%); Lit v 2, 13/15 (87%) and Lit v 4, 10/15 (67%); for group B, Lit v 1, 6/11 (54%); Lit v 3, 7/11 (64%); Lit v 2, 7/11 (64%) and Lit v 4 3/11 (27%). For both groups, the intensity of IgE binding was higher for Lit v 1 than for the other allergens (Fig. 2).

Patients of group A showed more intense binding than those of group B. Statistically significant differences in frequency and intensity of recognition as determined in the Tile map analysis ($P < 0.01$) with FDR < 0.05 were observed among groups A and B only for some epitopes in each allergen. These epitopes were Lit v 1 epitopes 1, 2, 5a/5b/5c, 7; Lit v 2 epitopes 6 and 7, Lit v 3 epitopes 1, 2, 4a/4b and 5, and Lit v 4 epitope 1 (Fig. 2). Sequences of these epitopes have been previously published [16]. Among subjects of group A, no differences in epitope recognition were observed between children and adults.

Diagnostic efficiency of sensitization to shrimp allergens and their epitopes

The results of sensitivity, specificity, PPV, NPV and diagnostic efficiency of specific IgE measurements to shrimp allergens and to individual epitopes are shown on Table 3A and B. For the analysis of individual epitopes, only the epitopes with statistically significant differences between groups were considered. Also, an allergen was considered positive when at least one epitope of those showing statistically significant

Table 2. Frequency of recognition of IgE epitopes identified in tropomyosin, MLC, SCP and AK for each group (A = positive challenge; B = negative challenge)

	Epitope 1	Epitope 2	Epitope 3	Epitope 4	Epitope 5a	Epitope 5b	Epitope 5c	Epitope 6	Epitope 7
Frequency									
Tropomyosin									
A (Ch+), %	73.3	93.3	46.6	40	73.3	80	86.6	53.3	80
B (Ch-), %	50	66.6	33.3	33.3	16.6	16.6	66.6	33.3	50
AA	1–36	37–63	61–81	82–105	115–150	142–162	157–183	190–210	246–284
	Epitope 1	Epitope 2	Epitope 3	Epitope 4a	Epitope 4b	Epitope 5			
Frequency									
Myosin light chain									
A (Ch+), %	50	57.1	35.7	92.9	50			64.3	
B (Ch-), %	14.3	14.3	14.3	86.7	14.3			28.6	
AA	13–30	22–48	49–66	58–90	79–99			118–141	
	Epitope 1	Epitope 2	Epitope 3	Epitope 4	Epitope 5	Epitope 6	Epitope 7		
Frequency									
Sarcoplasmic calcium-binding protein									
A (Ch+), %		90		70				0	
B (Ch-), %		100		66.6				0	
AA		10–36		49–72				130–147	
	Epitope 1	Epitope 2	Epitope 3	Epitope 4a	Epitope 4b	Epitope 5	Epitope 6	Epitope 7	
Frequency									
Arginine kinase									
A (Ch+), %	38.5	7.7	69.2	92.3	15.4	53.8	84.6	84.6	
B (Ch-), %	14.3	14.3	14.3	86.7	14.3	28.6	0	28.6	
AA	1–18	25–42	64–96	121–141	142–159	160–192	232–255	319–342	

AA, amino acid number.

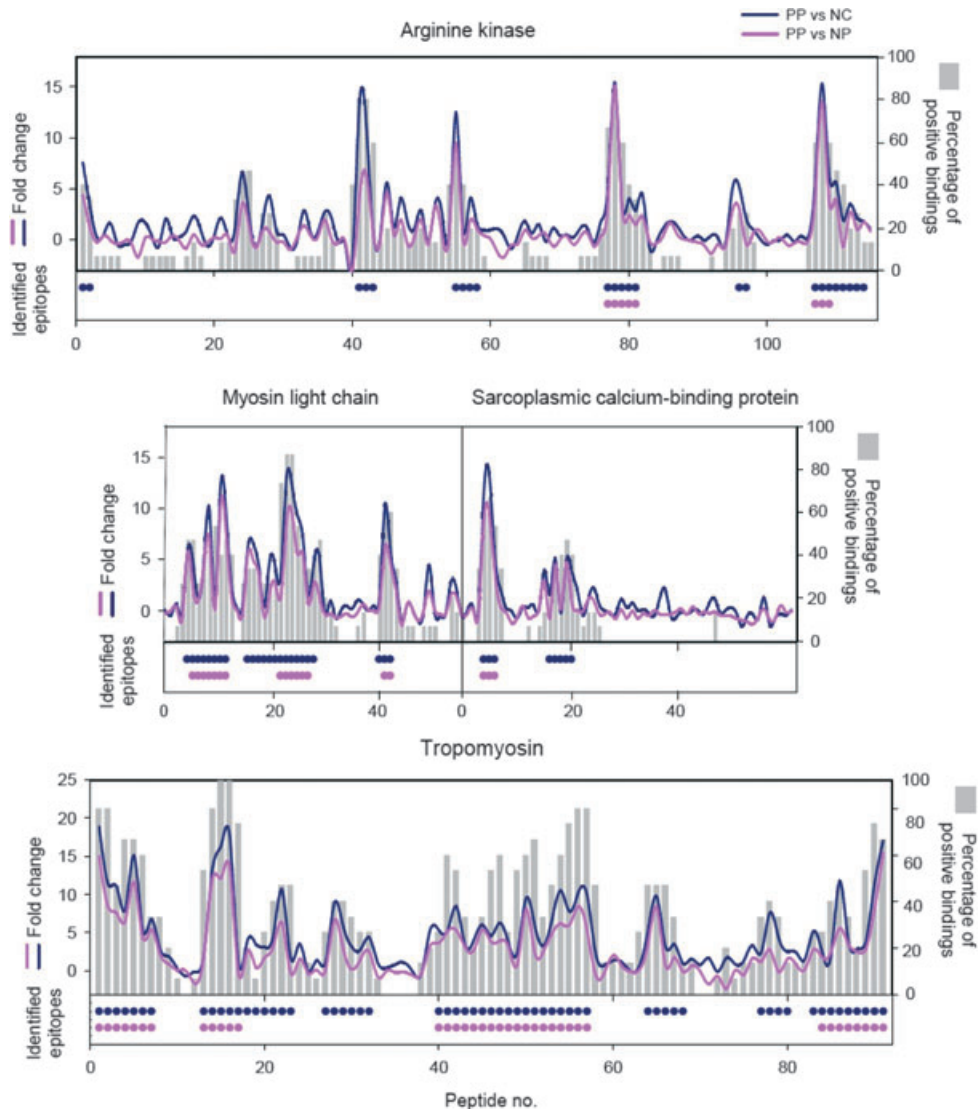


Fig. 2. TileMap analysis. Blue line: fold change intensity, positive challenge patients (group A) vs. negative controls (NC). Purple line: positive challenge (group A) vs. negative challenge (group B). Blue dots: epitopes with statistically significant differences between group A and NCs. Purple dots: epitopes with statistically significant differences between group A and group B. All significant epitopes as determined by TileMap analysis have $P < 0.01$, FDR < 0.05 .

differences was recognized. Also combinations of different allergens were analysed. In general, diagnostic efficiency of sensitization to certain individual epitopes is superior to IgE determination to the whole allergen. Regarding individual epitopes, the highest diagnostic efficiency was seen for Lit v 2 epitope 6 (85%) and Lit v 1 epitopes 2, 5a and 5b (81%). The highest NPV was for Lit v 1 epitopes 2 and 5c (87.5% and 85.71%, respectively). The greatest PPV was found for Lit v 2 epitope 6 (100%), Lit v 1 epitope 5a and 5b (90%), and Lit v 3 epitopes 1, 2 and 4b (88%). All these epitopes had greater than 90% specificity. As for sensitivity, these epitopes showed lower values: Lit v 2 epitope 6 and Lit v 1 epitopes 5a and 5b (73%), Lit v 3 epitopes 1, 2 and 4b (50%).

Regarding efficiency of sensitization to the different allergens, Lit v 2 and Lit v 1 showed equal diagnostic efficiency (77%), followed by Lit v 3 (70%) and Lit v 4 (65%). Sensitization to Lit v 2 (epitopes 6 and/or 7) had the highest PPV (85%), followed by Lit v 4 (epitope 1) (75%), Lit v 1 (epitopes 1, 2, 5a, 5b, 5c, 7) (71%) and Lit v 3 (epitopes 1, 2, 4a, 4b, 5) (67%). Lit v 1 and Lit v 3 showed the highest NPV (100% and 80%, respectively), and Lit v 1 showed the highest sensitivity (100%) of all four proteins but the lowest specificity (45%) while Lit v 3 sensitization had a sensitivity of 93% and a specificity of 73%. Serum-specific IgE to recombinant Pen a 1 showed slightly lower diagnostic efficiency than Lit v 1 measured by microarray (71% vs. 77%).

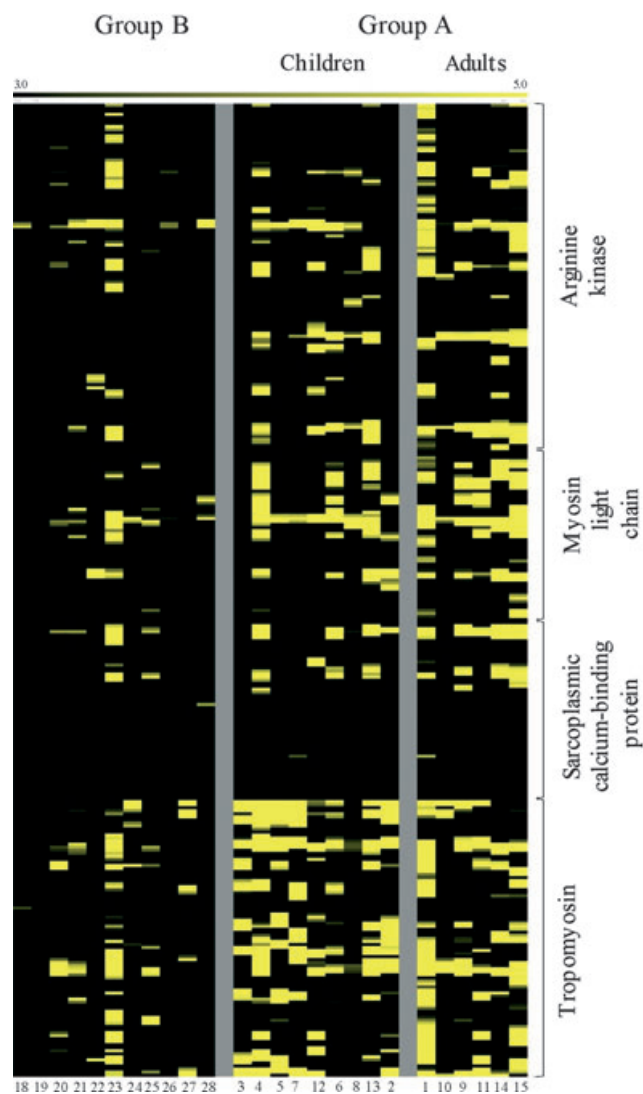


Fig. 3. Comparison of shrimp epitope recognition by positive challenge (group A) (adults and children) and negative challenge (group B) patients. All the peptides from the four shrimp allergens are represented in rows. The recognition of peptides by each patient is shown in columns. Intensity of IgE binding is represented by weighted average z scores, in grading scale ranging from less than 3 (black) to 5 or greater (yellow).

Patients of group A recognized more allergens than those of group B (Fig. 3). Of 15 patients in group A, 12 recognized at least three different allergens, but only 3 of 11 did in group B. Therefore, combinations of sensitization to two, three or four different proteins were analysed to see whether certain associations would improve the diagnostic value of the test (data not shown). Sensitization to an increasing number of allergens by microarray did not significantly increase the diagnostic efficiency. However, it was noted that the intensity of binding was in general stronger of group A for all four proteins. Simultaneous sensitization to all four shrimp allergens had a

diagnostic efficiency of 65%, which was similar to the diagnostic value of certain combinations of two allergens (Lit v1 and Lit v4, Lit v3 and Lit v4, Lit v2 and Lit v4).

Inhibition experiments

Due to the length of results and to present with more clarity, we are showing only results for tropomyosin and MLC peptides (Fig. 4). However, we found similar results with peptides of all four proteins. Experiments performed on two different occasions showed similar results, IgE binding to peptides of all four proteins was partially inhibited with the raw shrimp extract. Boiled shrimp extract almost completely inhibited IgE binding to the shrimp peptides, while chickpea extract (NC) did not inhibit binding.

Discussion

Shellfish allergy is a common disorder, with shrimp species being among the most frequent offending foods in both children and adults. In the United States, it is among the four foods most commonly provoking severe food anaphylaxis [20, 21]. Shellfish are also one of the most frequent foods causing ED visits in adults for food allergy and a significant number in children 6 years of age and older [5, 6]. Despite its prevalence and the potential severity of the reactions there is still no treatment available [22].

Unlike other food allergies, there is limited information about the relevance of sensitization to particular shrimp allergens. For instance, specific IgE to Ara h 2 is more accurate in predicting clinical peanut allergy than other peanut allergens [23]. In addition, sensitization to particular epitopes of milk [24], egg [25] or peanut [14] has been used as biomarkers for severity and disease persistence. However, regarding shellfish allergy, the role of particular allergens or their epitopes in clinical reactivity still needs to be elucidated. In this study, we aimed to determine whether sensitization to sequential epitopes of the four shrimp allergens identified to date would be good predictive markers of clinical reactivity to shrimp.

The outcome of oral challenges in 37 successive patients reporting shrimp allergy was analysed. Noteworthy, only 17 (46%) resulted in a positive challenge. This is in agreement with the result of DBPCFCs with other foods [11] and emphasizes the importance of oral food challenges in daily clinical practice, given that more than 50% of subjects tolerated shrimp. This is important, particularly when analysing self-reported, questionnaire-based studies of shellfish allergy, as our study shows that clinical reactivity is actually much lower than that self-reported.

Table 3. Sensitivity, specificity, PPV, NPV and efficiency of specific IgE measurements: (A) individual shrimp proteins and rPen a 1 and (B) individual epitopes showing statistically significant differences between groups

Protein	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Efficiency, %	P-value
Lit v 2	73.3% (44.9–92.2)	81.8% (48.2–97.7)	84.6% (54.6–98.1)	69.2% (38.6–90.0)	76.9	0.015
Lit v 3	93.3% (68.1–99.8)	72.7% (39.0–94.0)	66.7% (43.0–85.4)	80% (28.4–99.5)	69.2	0.127
Lit v 4	60% (32.3–83.7)	72.7% (39.0–94.0)	75% (42.8–94.5)	57.1% (28.9–82.3)	65.4	0.130
Lit v 1	100% (78.2–100)	45.5% (16.8–76.6)	71.4% (47.8–88.7)	100% (47.8–100)	76.9	0.007
rPen a 1	88.2% (63.6–98.5)	45.5% (16.8–76.6)	71.4% (47.8–88.7)	71.4% (29.0–96.3)	71.4	0.076

Protein	Epitope	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Efficiency, %	P-value
Lit v 2	6	73.3% (44.9–92.2)	100% (71.5–100)	100% (71.5–100)	73.3% (44.9–92.2%)	84.6	0.0002
	7	73.3% (44.9–92.2)	81.8% (48.2–97.7)	84.6% (54.6–98.1)	69.2% (38.6–90.9)	76.9	0.015
Lit v 3	1	46.7% (21.3–73.4)	90.9% (58.7–99.8)	87.5% (47.4–99.7)	55.6% (30.8–78.5)	65.4	0.083
	2	53.3% (26.6–78.7)	90.9% (58.7–99.8)	88.9% (51.8–99.7)	58.8% (32.9–81.6)	69.2	0.036
	4a	86.7% (59.5–98.3)	45.5% (16.8–76.6)	68.4% (43.5–87.4)	71.4% (29.0–96.3)	69.2	0.094
	4b	46.7% (21.3–73.4)	90.9% (58.7–99.8)	87.5% (47.4–99.7)	55.6% (30.8–78.5)	65.4	0.083
	5	60% (32.3–83.7)	81.8% (48.2–97.7)	81.8% (48.2–97.7)	60% (32.3–83.7)	69.2	0.050
Lit v 4	1	60% (32.3–83.7)	72.7% (39.0–94.0)	75% (42.8–94.5)	57.1% (28.9–82.3)	65.4	0.130
Lit v 1	1	73.3% (44.9–92.2)	72.7% (39.0–94.0)	78.6% (49.2–95.3)	66.7% (34.9–90.1)	73.1	0.044
	2	93.3% (68.1–99.8)	63.6% (30.8–89.1)	77.8% (52.4–93.6)	87.5% (47.4–99.7)	80.8	0.003
	5a	73.3% (44.9–92.2)	90.9% (58.7–99.8)	91.7% (61.5–99.8)	71.4% (41.9–91.6)	80.8	0.001
	5b	73.3% (44.9–92.2)	90.9% (58.7–99.8)	91.7% (61.5–99.8)	71.4% (41.9–91.6)	80.8	0.001
	5c	92.9% (66.1–99.8)	54.6% (23.4–83.3)	72.2% (46.5–90.3)	85.7% (42.1–99.6)	73.1	0.021
	7	80% (51.9–95.7)	72.7% (39.0–94.0)	80% (51.9–95.7)	72.7% (39.0–94.0)	76.9	0.014

CI, confidence interval, in %; PPV, positive predictive value; NPV, negative predictive value.

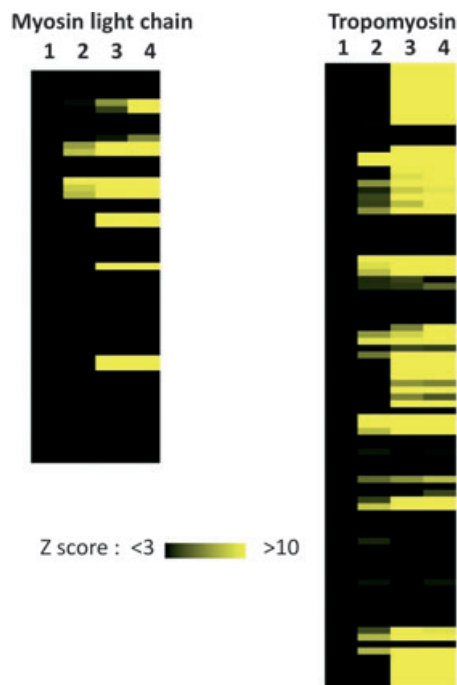


Fig. 4. Inhibition of IgE binding to Lit v 1 (tropomyosin) and Lit v 3 (MLC) peptides with raw and boiled shrimp extracts. Tropomyosin peptides (1–91) and MLC peptides (1–55) are shown in rows. 4: peptides recognized by the serum pool. Reactivity to the same peptides after pre-inhibition with boiled shrimp extract (1), raw shrimp extract (2) or chickpea extract (3). The intensity of recognition is graded from white (Z score < 3) to yellow (10 or higher).

Out of the 20 patients with negative DBPCFC, 11 (55%) had positive shrimp-specific IgE levels and/or positive SPT. The remaining nine (45%) were negative for all *in vivo* and *in vitro* tests, although two of these nine subjects had had a previous diagnosis of shrimp allergy with positive SPTs. Even if the seven subjects with negative tests are not included, the percentage of negative challenges among our patients is 43.3% (13/30 patients). We decided to include initially all 37 subjects in the study although some had negative SPT/IgE to reflect the results of oral challenges in all subjects reporting shrimp allergy in our clinic population. Interestingly all subjects in group C were adults, who appeared to have more subjective symptoms than children.

Previous publications have shown similar results regarding the outcome of oral shrimp challenges. In a study by Daul et al. [26], 30% of their adult patients had positive DBPCFCs to shrimp, as determined by objective signs during the challenge. However, recently a positive challenge rate of 88% was found in a Thai shrimp-allergic cohort [27]. Interestingly, in this study, all subjects were children. Regarding results of DBPCFCs by age, in our group of children 78% (11/14) had a positive challenge in contrast to 26% (6/23) of the adults, also suggesting that the history in children may be more reliable than in adults. These findings also raise the possibility that shellfish allergy may wane overtime [16]. The natural history of shellfish allergy has been analysed in only one short-term study [28].

Eleven subjects with shrimp allergy were followed for 24 months. Seven of them had positive DBPCFCs and four only subjective symptoms during challenge (pruritus). Shrimp-specific IgE levels remained stable in all patients overtime. Larger prospective studies using DBPCFCs are required to address whether sensitization actually decreases with age.

Patients with positive food challenges had higher frequencies of allergen and epitope recognition than those previously observed in 53 non-challenged patients [16] and also higher than in patients with negative challenges. However, identified IgE epitopes in all four allergens by our Spanish population are similar to those described before with a mixed population from Spain and United States [16]. Interestingly, among those patients with positive shrimp challenges, no differences were detected in terms of allergen or epitope recognition between adults and children. It appears that although subjects with positive challenges (either children or adults) recognize the same shrimp allergens and allergenic epitopes with high intensity, the frequency of positive challenges and intensity of epitope recognition in adults are generally lower than in children. This is consistent with our previous publication, which showed that the degree of sensitization to shrimp proteins seems to be less in adults [16]. Our study did not find allergens recognized uniquely by children compared with adults. However, prospective studies are needed to determine whether sensitization and clinical reactivity to shrimp decrease overtime.

In this manuscript, we couple diagnostic food challenges with testing of IgE binding to synthetic peptides to define which allergens or IgE epitopes are most closely related to clinical food allergy. Diagnostic efficiency was highest for Lit v 2 epitope 6 (85%) and Lit v 1 epitopes 2, 5a and 5b (81%); and for allergens Lit v 2 and Lit v 1 (77% both), Lit v 3 (70%) and Lit v 4 (65%). Diagnostic efficiency of sensitization to rPen a 1 (71%) is in agreement with diagnostic efficiency of Lit v 1 by microarray (77%) supporting the validity of microarray testing.

Sensitization to Lit v 2, especially to epitope 6 appears associated with a positive oral food challenge (PPV 100%). Also epitopes 5a and 5b for Lit v 1 (PPV 91%) as well as epitopes 1, 2 and 4b of Lit v 3 (PPV 88%) appear to be relevant in predicting the clinical reactivity of a patient. As the microarray technique is more sensitive than standard *in vitro* assays, it has been argued that microarray results need to be interpreted cautiously [29]. Therefore, the analysis of the results of this study focused only on the epitopes with statistically significant differences between positive and negative challenge patients.

Sensitization to Lit v 2 epitopes 6 and 7 seems to be a good predictor of clinical reactivity in this study. PPV

is the highest whether the patient is sensitized only to Lit v 2 (85%) or in combination with Lit v 1 and/or Lit v 3 (85%). However, the clinical relevance of sensitization to Lit v 2 needs to be confirmed with a larger cohort. The existing cross-reactivity between Lit v 2 from shrimp and other arthropods [8] might play a role in the population of this study, which is highly sensitized to dust mites. Although tropomyosin-specific IgE levels are usually used in the routine diagnosis of shellfish allergy, we only find a specificity of 45.5%. Therefore, it may not be the most informative test in terms of clinical relevance and accurate diagnosis. In this regard, Yang et al. [12] recently reported that measurement of IgE to shrimp tropomyosin improves specificity, PPV and diagnostic efficiency (88%) to predict a positive oral food challenge in comparison to shrimp-SPT and shrimp-specific IgE. However, these were patients sensitized to house dust mites and some to shrimp, and tropomyosin sensitization was used as indicator of possible shrimp allergy. In contrast, our study provides information on allergen and epitope recognition to determine clinical reactivity in subjects with a history of shrimp allergy. In our study, recognition of particular epitopes seems to offer the best diagnostic efficiency. Probably due to cross-reactivity issues, recognition of particular Lit v 1 epitopes might have better diagnostic efficiency than IgE determination to the whole rPen a 1 commercially available. However, diagnostic efficiency might change if a lower threshold (0.1 kU_A/L) had been considered. Further studies need to be done comparing peptides and recombinant and purified shrimp allergens and larger populations in a component-based diagnosis to determine the global relevance of each allergen and epitopes in shellfish allergy.

Although we have no doubt that conformational epitopes present in native or recombinant allergens are important for environmental allergens and food allergens, the importance of sequential epitopes in food allergens has been demonstrated in multiple studies [14, 24, 30, 31]. In addition, previous studies have shown that when hypoallergenic tropomyosin variants were designed on the basis of results of linear epitope disruption experiments using several amino acid substitutions, very important reduction of allergenicity was obtained [32]. However, the relevance of IgE binding to short peptides has been challenged in a recent publication by Albrecht et al. [29] who found that IgE binding to short peptides, contribute little to the IgE binding of the two food allergens studied, particularly in fluid phase antibody/target interaction. Although Pen a 1 peptides displayed low but detectable IgE binding in fluid phase, significant binding occurred when the epitope regions were grafted into the molecular framework of a non-allergenic mammalian tropomyosin suggesting that conformational epitopes are more important.

In contrast to aeroallergens, many foods and their allergens undergo heat processing before consumption and enzymatic digestion in the stomach and the gut. Because these modified allergens are not in their native conformation, the humoral immune response to linear epitopes may be favoured. To demonstrate the relevance of binding to IgE our shrimp peptides, an additional experiment was designed based on the same principle as Albrecht et al. [29]. If the IgE-binding sequences recognized by individual patients represented true epitopes, these structures would be able to inhibit IgE binding to the parent allergen significantly, or *vice versa*, the full-length allergen would be able to inhibit binding to the peptides, demonstrating that they are not only important, but that they may be exposed on the surface of the allergen. Inhibition experiments were done using raw and boiled shrimp extract as inhibitors, which theoretically contain all four shrimp allergens. Due to the length of results, we are showing only results for tropomyosin (Lit v 1) and MLC (Lit v 3) peptides in Fig. 4. However, we found similar results with peptides of all four proteins. IgE binding to peptides of all four proteins was partially inhibited with the raw shrimp extract, while the boiled extract almost completely inhibited IgE binding to the shrimp peptides. This clearly demonstrates that more linear epitopes become exposed and available for IgE binding in the boiled extract than in the raw form, but they are accessible in both extracts. Since allergens in the raw extract are present more in their conformational form, they do not accomplish as much inhibition as the boiled extract, probably just because the epitopes are partially hidden and not available for inhibition. Only when the molecules are denatured in the boiled extract, more sequential epitopes become exposed that can inhibit binding to the peptides on the slide.

In conclusion, patients with positive oral shrimp challenges (children or adults) have greater recognition (frequency and intensity) of certain IgE epitopes of all four shrimp allergens. IgE to these shrimp epitopes could be used as biomarkers for prediction of clinical reactivity in subjects with sensitization to shrimp. This is a pilot study intended to determine whether certain epitope recognition may be most closely related to clinical shrimp allergy. Candidate epitopes were identified with statistical significance, but clear clinical evidence still has to be found. Since sample size is small, validation of these findings in a blinded analysis of larger groups of sera of subjects with a clinically well-characterized status of sensitization vs. clinical reactivity is needed. These larger studies, possibly including patients from different ethnicities, will be needed before such tests can be considered for use in the clinical setting.

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