



Defining specific allergens for improved component-resolved diagnosis of shrimp allergy in adults



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ABSTRACT

Shrimp is one of the predominant causes of food allergy among adults, often presenting with severe reactions. Current *in vitro* diagnostics are based on quantification of patient specific-IgE (sIgE) to shrimp extract. Tropomyosin is the known major shrimp allergen, but IgE sensitisation to other allergens is poorly characterised.

In this study, the binding of IgE to various shrimp allergens, additional to tropomyosin, was investigated using sera from 21 subjects who had clinical reactions to one or more shellfish species. Total shrimp-sIgE was quantified using ImmunoCAP, while allergen-sIgEs were quantified using immunoblotting and mass spectrometry, and immuno-PCR to recombinant shrimp tropomyosin.

Sixty-two percent of subjects (13/21) were positive to shrimp by ImmunoCAP. IgE from 43% of subjects (9/21) bound tropomyosin, while an additional 29% of subjects (6/21) demonstrated IgE-binding solely to other shrimp allergens, including sarcoplasmic calcium-binding protein, arginine kinase and hemocyanin. Furthermore, IgE sensitisation to other shrimp allergens was demonstrated in 50% of subjects (4/8) who were ImmunoCAP negative.

The lack of standardised shrimp allergens and inadequacy of current extracts for shrimp allergy diagnosis is highlighted by this study. Comprehensive knowledge of less studied allergens and their inclusion in component-resolved diagnostics will improve diagnostic accuracy, benefitting the wider population suffering from shellfish allergy.

1. Introduction

An increase in seafood consumption has seen a concomitant rise in associated seafood allergies. The prevalence of shellfish allergy in the general population ranges from 0.2% to over 5%, depending on the geographic region and study design (Pedrosa et al., 2015; Moonesinghe et al., 2016; Le et al., 2018; Ruethers et al., 2018). Shrimp is the predominant species causing over 80% of reactions to shellfish that result in severe clinical outcomes (Lopata et al., 2010). The diagnostic process

for shrimp allergy includes assessment of shrimp-specific IgE antibodies (shrimp-sIgE) by *in vitro* measurement in blood or skin prick testing (SPT), combined with a detailed dietary and clinical history, and, if possible, an oral food challenge (OFC) (Lopata et al., 2016).

The ImmunoCAP assay (Pastorello et al., 1991; Paganelli et al., 1998) for shrimp-sIgE (f24, Thermo Scientific, MA, USA) is currently one of the most widely used allergen extract-based *in vitro* detection tests for clinical allergy. The whole shrimp extracts commonly used for diagnostics comprise of a mixture of raw and/or boiled shrimp species.

Abbreviations: sIgE, specific IgE; TM, tropomyosin; AK, arginine kinase; SCP, sarcoplasmic calcium-binding protein; HC, hemocyanin

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However, it is not known if these extracts contain an optimal mix of all the clinically relevant shrimp allergens. Furthermore, it is becoming increasingly evident that the detection of IgE antibodies specific to individual allergens improves the specificity of allergy diagnosis (Pascal et al., 2015; Lopata et al., 2016). Tropomyosin, Pen m 1 (TM) (Shanti et al., 1993; Daul et al., 1994; Leung et al., 1998) is the most frequently recognised allergen in shrimp allergy (Lopata et al., 2010; Kamath et al., 2014). In addition to total shrimp-sIgE, TM-sIgE (f351, ImmunoCAP, Thermo Scientific, MA, USA and Immulite, Siemens, Munich, Germany (Li et al., 2004)) can be measured in routine clinical practice for *in vitro* diagnosis of shrimp allergy. A study by Yang et al. showed that the measurement of TM-sIgE is more accurate in the prediction of clinically relevant allergic reactions to shrimp, compared to the use of whole shrimp extract by SPT and shrimp-sIgE blood tests (Yang et al., 2010). Similarly, a 2011 study by Gamez et al. demonstrated that a positive result from a TM-sIgE test adds value to the current diagnostic methods in confirming shrimp allergy, with a positive predictive value of 0.72 (Gámez et al., 2011).

We recently developed a sensitive immuno-PCR method to quantify TM-sIgE utilising a specialised recombinant TM (Johnston et al., 2014). This method captures TM-sIgE from serum that can then be measured by quantitative PCR using a protein-DNA conjugate. The detection device is the recombinant allergen (TM) produced in fusion with Tus, a DNA-binding protein originating from *Escherichia coli*, that binds the specific DNA sequence *Ter* (Tus-*Ter*-lock) (Morin et al., 2010, 2011). This allows the binding of IgE to TM in solution, and its subsequent detection by quantitative real-time PCR, without the need of any detection antibodies, making this method very sensitive and specific.

Previous studies have demonstrated the clinical relevance of IgE reactivity to additional shrimp allergens (Pedrosa et al., 2015) including myosin light chain – Pen m 3 (MLC) (Ayuso et al., 2008), sarcoplasmic calcium-binding protein – Pen m 4 (SCP) (Ayuso et al., 2009), arginine kinase – Pen m 2 (AK) (Garcia-Orozco et al., 2007) and hemocyanin (HC) (Piboonpocanun et al., 2011; Giuffrida et al., 2014). These less investigated shrimp allergens have also been demonstrated to play a role in clinical cross-reactivity to other invertebrate allergen sources, including dust-mites (Arlan et al., 2009; Kamath et al., 2017). Thus, for the accurate diagnosis of shrimp allergy, it would be beneficial to perform immunoassays using a complete set of clinically relevant allergens, in addition to a detailed clinical history.

In this study, we analysed the IgE binding patterns of sera from a cohort of shrimp-allergic subjects to all known shrimp allergens from raw (unheated) shrimp extract and compared these to the results of ImmunoCAP, the widely used *in vitro* IgE quantification platform. We demonstrated considerable frequency of IgE binding to minor allergens showing their diagnostic value, in addition to TM. Furthermore, we compared the detection of TM-sIgE by immunoblot and immuno-PCR to investigate the value of different component-resolved diagnostic tests.

2. Material and methods

2.1. Subjects

Adult subjects were recruited at The Alfred Hospital Allergy Clinic, Melbourne, Victoria, Australia with a convincing history of clinical symptoms to shrimp and/or other shellfish species ($n = 21$; Table 1). Oral food challenge with shrimp is not conducted routinely in this clinic for adult patients with frequent comorbidities, in keeping with the clinicians' preference for safer serum sIgE testing and clinical history of reactions on exposure. Shrimp-sIgE antibodies to whole shrimp extract were quantified using ImmunoCAP (shrimp CAP f24) (Thermo Scientific, MA, USA). This ImmunoCAP contains protein extracts from four different shrimp species: *Pandalus borealis*, *Penaeus monodon*, *Metapenaeus barbata*, *Metapenaeus joyneri*.

The study was approved by the Ethics Committees of James Cook University (Project number H4313), The Alfred Hospital (Project

number 192/07) and Monash University (MUHREC CF08/0225).

2.2. Detection of shrimp allergen-specific IgE by immunoblotting

Serum IgE binding to Black Tiger Shrimp (*Penaeus monodon*) allergens was analysed by SDS-PAGE and IgE immunoblotting. Raw shrimp allergen extract was prepared according to our previously published method (Kamath et al., 2013). Proteins separated by SDS-PAGE (12% polyacrylamide) were immobilised on a PVDF membrane (20 V for 16 min), blocked with 5% skim milk powder in phosphate buffered saline with 0.05% Tween-20 (PBS-T) and incubated overnight with 1:10 diluted sera, using a surf blot apparatus (Idea Scientific, MN, USA). Rabbit anti-human IgE polyclonal antibody (1:10,000; DAKO Corporation, USA) and horseradish peroxidase labelled goat anti-rabbit IgG polyclonal antibody were used to detect IgE binding, visualised using enhanced chemiluminescence (ECL) according to the manufacturer's instructions (BD Biosciences, Sydney, Australia). For the semi-quantitative analysis of IgE reactivity, densitometric analysis of the blots was performed using ImageJ software v1.50i (Schneider et al., 2012). Bands were visually inspected and a pixel area of ≥ 2000 under the peak was designated as the cut-off for a positive test.

2.3. Allergen identification by mass spectrometry

Protein bands demonstrating IgE antibody reactivity were excised from the gel, de-stained and digested with trypsin as described previously (Koeberl et al., 2014). Digested samples were analysed with a LTQ Orbitrap Elite coupled to an Ultimate 3000 RSLC nanosystem, Dionex (Thermo Scientific, MA, USA). Two microlitres of the peptide mix were loaded onto an Acclaim Pepmap nanotrap column of 3% acetonitrile containing 0.1% formic acid for 5 min before the enrichment column was switched in-line with the Acclaim Pepmap analytical column. Data analysis was performed using Mascot v2.4 (Matrix Science Inc, USA) against the Uniprot database. Search parameters included precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.6 Da (CID). The fixed modification was set as carbamidomethyl of cysteine and the variable modification was oxidised methionine. The cleavage enzyme was trypsin with a maximum of one missed cleavage, using a 1% false discovery rate threshold.

2.4. Detection of TM-specific IgE by Immuno-PCR

Recombinant TM containing a fusion to the Tus DNA-binding protein (Tus-TM) was produced and purified, as described previously (Johnston et al., 2014). TM-sIgE antibodies were recognised in patient serum by the Tus-*Ter*-lock immuno-PCR using the previously described mixture format (Johnston et al., 2014). 96-well plates were coated overnight with 50 μ L anti-IgE antibody (10 μ g/mL; rabbit anti-human IgE, A0094 (Agilent, Sydney, Australia) in phosphate buffer (50 mM NaH₂PO₄/Na₂HPO₄, pH 7.8), washed once with wash buffer (BW: 20 mM Tris-HCl (pH 8), 150 mM NaCl, 0.005% Tween 20) and blocked with block buffer (BW + 1% bovine serum albumin (w/v)). A detection device was created by incubating Tus-TM with its specific DNA (*Ter*-lock) to allow binding (1 μ M and 125 nM, respectively), followed by 50-fold dilution in block buffer. Serum samples were diluted 1/20, and 4 μ L of detection device was added and mixed gently. Fifty microlitres of the serum mixtures were applied to the anti-IgE coated wells and incubated for 90 min at room temperature. This allowed the detection device to bind to TM-sIgE in the serum, which was then captured on the anti-IgE surface. Each serum sample was tested in duplicate on three separate occasions ($n = 6$). Wells were washed five times with BW, followed by the addition of a primer mix (primers specific for the amplification of the *Ter*-lock DNA; 0.5 μ M in water) and incubation for 60 min to allow dissociation of the complexes. Ten microlitre aliquots were transferred to 96-well PCR plates, 10 μ L of real-time PCR mix (iQaq, Biorad) was added and quantitative real-time PCR performed

Table 1
Summary of clinical features of shellfish-allergic subjects.

No.	Age	Sex	Clinical presentation to shellfish		SPT Shrimp (mm)	Specific IgE to shrimp (kU/L) (ImmunoCAP)
			Symptoms	Known shellfish species		
1	36	F	AE, U	Shrimp, scallops	–	9.82
2	48	M	R, OAS	Calamari, squid	–	9.03
3	26	F	A, R, AE, U	Shrimp, lobster	–	6.65
4	32	F	AN, AE	Shrimp	–	5.93
5	52	M	AN, U	Shrimp	–	5.37
6	49	M	OAS	Shrimp, scallops	–	4.54
7	40	F	OAS, U, LE	Shrimp	–	3.33
8	30	F	OAS	Shrimp	17	1.82
9	30	M	OAS	Shrimp	–	1.65
10	41	M	R, G, AE	Shrimp, lobster, crayfish	10	1.41
11	23	F	A, R	Shrimp	–	1.32
12	58	F	OAS, U	Shrimp, crab	–	0.5
13	26	F	R, OAS	Shrimp, crab, oyster, lobster, mussels	–	0.36
14	39	M	R, AE, U	Shrimp, lobster	7	0.21
15	71	F	A, R, OAS	Shrimp	10	0.16
16	60	M	A, R, OAS, LE	Shrimp, crab	3	0.1
17	26	F	LE, U, AE	Shrimp, crab, oyster, mussel, marron	0	0.07
18	34	F	A, R, LE, U	Crab	–	0.05
19	57	M	R, H	Shrimp	4	0.04
20	38	F	OAS	Shrimp	0	0.04
21	35	F	R, U	Shrimp	0	0.03

Note: ‘–’ denotes test not performed.

Key: A: asthma, R: rhinitis, AN: anaphylaxis, U: urticaria, AE: angioedema, OAS: oral allergy syndrome, LE: laryngeal edema, H: hypotension.

(Bio-Rad iQ5 Thermocycler). PCR conditions were set to 10 min at 95 °C, followed by 40 cycles of: 10 s at 95 °C, 10 s at 55 °C, 10 s at 60 °C. The ‘no template controls’ contained 10 µL primer mix and 10 µL PCR mix only. PCR positive controls contained 1 µL of detection device. The cut-off for a positive test was set at two standard deviations below the average Ct value for negative control subjects ($n = 11$, Fig. S1A). Negative controls were atopic adults with no history of shrimp allergy, a negative shrimp-specific IgE by ImmunoCAP, and negative to recombinant shrimp TM by immunoblot; $n = 11$ (Fig. S1B). Results from quantitative PCRs were analysed using the iQ5 software (Bio-Rad, CA, USA) and graphed using GraphPad Prism (version 5.04 for Windows, GraphPad Software, CA, USA). Immuno-PCR results were compared to the densitometric analysis of the immunoblot TM bands using Spearman’s rank correlation and Cohen’s kappa (R statistical package).

3. Results

3.1. Patient characterisation

Twenty-one adult subjects with a convincing history of clinical symptoms to one or more shellfish species were recruited for this study (62% females and 38% males) with an average age of 41 years. For this study, subjects were numbered in descending order according to their ImmunoCAP values (Table 1). Nineteen subjects (91%) presented with clinical symptoms to shrimp. Therefore, we focused on analysing IgE binding to specific shrimp allergens. Out of the 21 subjects, 13 had positive shrimp-sIgE by ImmunoCAP (> 0.35 kU/L), as per the manufacturer’s manual.

Clinical presentation to shellfish ranged from rhinitis and urticaria to asthma and anaphylaxis. Fifty-two percent of the adult subjects had rhinitis, 48% had oral allergy syndrome, 24% had asthma and 2 subjects experienced anaphylactic reactions.

3.2. Investigation of IgE binding to shrimp allergens by immunoblot

Sixty-two percent of subjects (13 out of 21) were positive by shrimp ImmunoCAP testing (Fig. 1A). To determine which proteins were identified by serum IgE, traditional immunoblotting was performed. Immunoblot results demonstrated IgE binding to a number of shrimp protein bands corresponding to SCP – Pen m 4 (19 kDa), TM – Pen m 1

(35 kDa), AK – Pen m 2 (40 kDa) and HC (73 kDa) (Fig. 1B). The identity of these bands was confirmed by mass spectrometry (Table 2). Troponin C – Pen m 6, was also identified in the band corresponding to a molecular weight of 27 kDa. However, only very low intensity of IgE binding was observed (below the positive cut-off value of densitometry analysis) and for only a few subjects, and so this allergen was excluded from further analysis. Overall, 67% of subjects ($n = 14$) displayed IgE binding to one or more shrimp allergens (Fig. 1B, Table 3). IgE binding included eight subjects reactive to TM, eight subjects to HC, five subjects reactive to AK and two subjects to SCP. Twenty-nine percent of subjects ($n = 6$) were positive to only minor allergens with no reactivity to TM. Four subjects who were positive by ImmunoCAP did not display any IgE binding by immunoblot. Five subjects were negative by ImmunoCAP but displayed IgE binding to one or more allergens by immunoblot.

3.3. Immuno-PCR for the identification of TM-specific IgE

To explore the use of an alternative method for component-resolved diagnosis we used an immuno-PCR technique that we developed previously with high sensitivity for the detection of TM-sIgE from serum samples (Johnston et al., 2014; Kamath et al., 2017). This method is based on a recombinant TM and provides additional comparison to the natural shrimp extracts used in the immunoblot. Using immuno-PCR, eight patients demonstrated binding to TM, all of which were also positive by ImmunoCAP (Fig. 1D, Table 3). This is in comparison to eight patients who showed binding to TM by immunoblot (Fig. 1C). There was a strong correlation between the results of the immuno-PCR and the TM immunoblot (Spearman’s correlation, $\rho = -0.767$, $p = 5.01 \times 10^{-5}$; Fig. 2), with 19 of 21 observations in concordance (one subject positive to TM by immunoblot was negative by immuno-PCR and one subject negative to TM by immunoblot was positive by immuno-PCR; Cohen’s Kappa = 0.798, $p = 2.55 \times 10^{-4}$). Three subjects were negative in all three tests performed (Fig. 1, Table 3).

4. Discussion

In vitro allergy testing is used frequently to avoid the use of oral food challenges (OFC). Although OFC is considered the gold standard for diagnosing food allergy, it requires highly trained personnel, is time

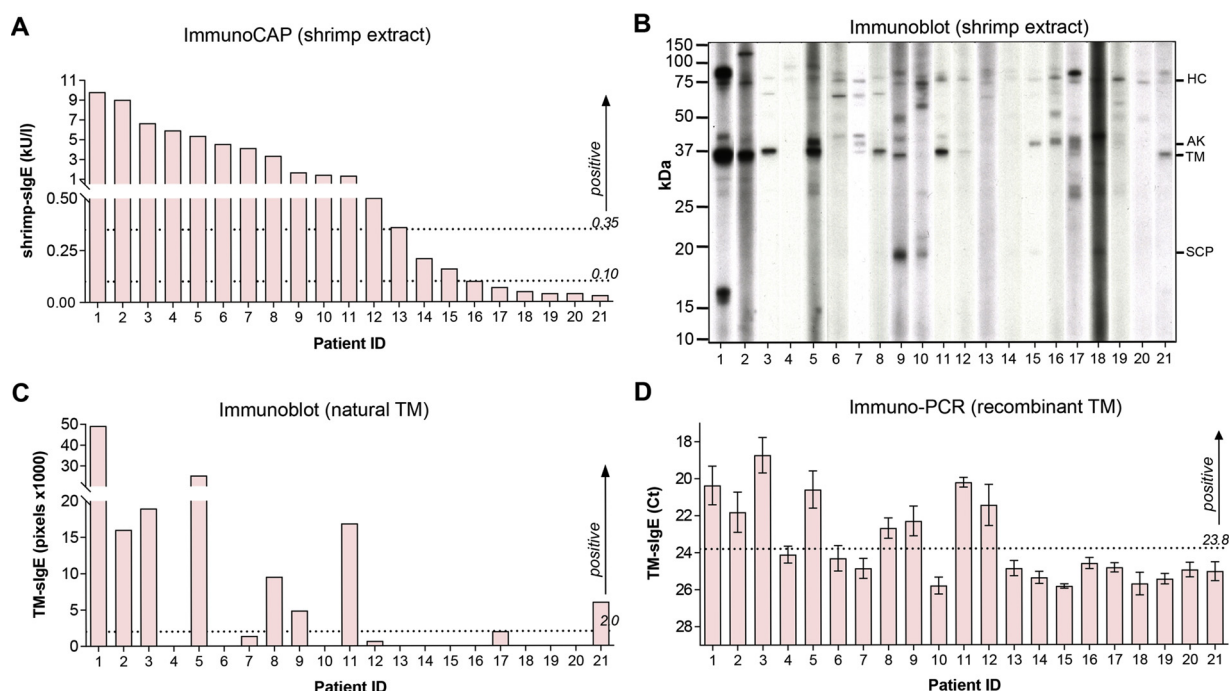


Fig. 1. Analysis of IgE binding to shrimp allergens using sera of twenty-one subjects with a convincing history of clinical symptoms on exposure to shellfish. (A) Results of shrimp ImmunoCAP test (f24). Subjects ordered by decreasing concentration of specific IgE in kU/L. Positive test indicated at ≥ 0.35 kU/L. (B) IgE immunoblotting analysis using Black Tiger Shrimp (*Penaeus monodon*) protein extract, IgE bands detected by enhanced chemiluminescence (ECL) and allergens identified using mass spectrometry, (C) Densitometry of TM-binding IgE bands on immunoblot. Pixel density measured as area under peak using ImageJ software. Positive test indicated at ≥ 2000 pixels. D. TM immuno-PCR results. Average and SD displayed, $n = 6$. Positive test indicated at ≤ 23.8 cycle threshold.

consuming, and poses a risk of severe clinical reactions, especially in adults who frequently have comorbidities. Skin prick testing, especially for food allergies, also presents a small but real risk of adverse reaction and its use in routine testing for allergy is debated (Kowalski et al., 2016; Jensen-Jarolim et al., 2017). In medical practice, a combination of clinical history and allergy test results are taken into consideration (Santos and Brough, 2017). Keeping this in mind, improvement in current *in vitro* quantitative allergen-sIgE tests is required to avoid potential misdiagnosis caused by the commonly used crude protein extract-based assays which may lack some important allergens.

Traditional *in vitro* IgE testing is performed using allergen extracts that contain an often unknown mixture of allergenic and non-allergenic proteins. Specific IgE to allergen extracts has been shown to have low specificity in diagnosing food allergy (Santos and Brough, 2017). On the other hand, single component testing involves the use of purified natural or recombinant allergens that can be used to better detect and quantify specific IgE due to an increased availability of appropriate allergens, thereby reducing the risk of misdiagnosis.

The most routinely used *in vitro* test for the diagnosis of shellfish allergy, ImmunoCAP Shrimp (f24), encompasses raw and boiled extracts of four shrimp species: *Pandalus borealis*, *Penaeus monodon*, *Metapenaeopsis barbata* and *Metapenaeus joyneri*. Allergens, especially heat-labile ones, may be present at below-detectable levels due to a dilution effect. Thus, a negative result towards whole protein extracts may not reliably confirm that the subject is not sensitised to a specific allergen in the shrimp extract. This is particularly important in subjects who are negative to TM, but sensitised to other shrimp allergens.

In this study, we investigated the IgE sensitisation profile to shrimp allergens in a cohort of subjects with a history of reactions to shrimp or other shellfish species. Of the 21 subjects in the cohort 62% tested positive for shrimp by ImmunoCAP. Since 91% of the study subjects had a convincing history of clinical reactions to shrimp we aimed to investigate the sensitisation patterns to shrimp allergens, in particular those that were testing negative by commercial test. IgE binding to shrimp allergens was investigated by immunoblotting against raw

shrimp extract. The IgE-binding allergens were subsequently identified using mass spectrometric techniques, an established tool used for identifying allergens in complex protein mixtures (Nugraha et al., 2018). Using immunoblotting, 38% of subjects displayed IgE binding to TM, 38% to HC, 24% to AK and 10% to SCP. TM is well known as the major allergen in shrimp, therefore the 38% IgE binding from subjects in this study seems to be low compared to previous studies which demonstrated a higher frequency of tropomyosin binding of up to 81% (Yang et al., 2010; Bauermeister et al., 2011; Gámez et al., 2011). In our study subjects were recruited based on clinical history rather than ImmunoCAP test scores. This enabled us to investigate IgE binding patterns of the 38% of clinically allergic individuals who tested negative using the current mainstream diagnostic test, an occurrence which is not uncommon (Rolland et al., 2013; Farioli et al., 2017). We demonstrated additional IgE reactivity to shrimp allergens by immunoblotting and using this component-resolved technique, showed that in addition to TM, other allergens are clearly important in sensitisation to shrimp in adults.

Twenty nine percent of subjects demonstrated IgE binding to shrimp allergens AK, SCP and HC, but not to the major allergen TM. Two of these allergens, AK and HC are known to have variable stability and solubility profiles (Piboonpocanun et al., 2011; Chen et al., 2013; Si et al., 2014; Zanjani et al., 2014; Dolashki et al., 2015; Khalil et al., 2016) so there is the potential for them to be missing from some extracts, depending on processing and storage conditions. Nineteen percent of subjects were shrimp ImmunoCAP negative but positive by immunoblot to AK or HC. This may suggest that ImmunoCAP reactivity occurs predominantly to the highly stable allergens. Although raw extracts are included in the ImmunoCAP for some of the shrimp species, it is possible that some allergens are also sensitive to the treatments used in the preparation of this test, or are not stable for long periods of time while in storage. Furthermore, it is possible that individuals may be sensitised to allergens of Australian shrimp species which are not present in the commercial mixture of four species (only two are found in Australia), thereby causing the negative test results in this patient

Table 2

Identification of IgE antibody-reactive proteins in raw shrimp extract using immunoblotting and mass spectrometric techniques. The sequence coverage is denoted as a percentage. IgE binding proteins have been matched to known allergens using Uniprot accession IDs.

Band No.	Estimated MW of Band (kDa)	Peptide sequences	Identified allergens	MASCOT Score	Coverage (%)	UniProtKB Accession I.D.	MW of native protein (kDa)	Species (common name)
1	73 kDa	DPSFFR DSLPPYTK YMDNIFK EHKDSLPPYTK	Hemocyanin B chain	159.64	4.24	P83180	65.3 kDa	<i>Astacus leptodactylus</i> (Turkish narrow-clawed crayfish)
		DPSFFR YMDNIFK	Hemocyanin A chain	80.78	1.98	P04254	75.7 kDa	<i>Panulirus interruptus</i> (California spiny lobster)
2	64 kDa	DPSFFR DSLPPYTK YMDNIFK TILQSDNGGK	Hemocyanin B chain	141.22	3.71	P83180	65.3 kDa	<i>Astacus leptodactylus</i> (Turkish narrow-clawed crayfish)
			Peptidase 1	35.02	3.11	Q1EIQ3	36.3 kDa	<i>Psoroptes ovis</i> (Sheep scab mite)
3	46 kDa	YNQILR DMYMEFCK AGAAELGIPLYR AAVPSGASTGVHEALEMR	Enolase	256.55	10.16	P56252	47.0 kDa	<i>Homarus gammarus</i> (European lobster)
4	40 kDa	YNLQVR AVFDQLK FLQAANACR LTSAVNEIEK MGLTEFQAVK LIDDHFLFK GTYYPLTGMSK EMQDGILELIK VSSTLSSLEGELK IISMQMGGDLGQVFR GEHTEAEGGIYDISNK LGFLTFCTNLGTTVR ALQASVLR	Arginine kinase	840.42	37.36	C7E3T4	40.1 kDa	<i>Penaeus monodon</i> (Black tiger prawn)
			Fructose-bisphosphate aldolase	62.37	2.22	P07764	39.0 kDa	<i>Drosophila melanogaster</i> (Fruit fly)
		AGAAELGIPLYR	Enolase	55.56	2.77	P56252	47.0 kDa	<i>Homarus gammarus</i> (European lobster)
5	35 kDa	ANIQLVEK SLEVSEK FLAEEADR MDALENQLK IVELEEELR LAMVEADLER IQLLEEDLER LAEASQAADSESR ALSNAEGEVAALNR YNLQVR AVFDQLK FLQAANACR LTSAVNEIEK MGLTEFQAVK LIDDHFLFK GTYYPLTGMSK VSSTLSSLEGELK GEHTEAEGGIYDISNK YNQILR AGAAELGIPLYR ALQASVLR	Tropomyosin	651.91	31.34	A1KYZ2	32.8 kDa	<i>Penaeus monodon</i> (Black tiger prawn)
			Arginine kinase	592.51	25.56	C7E3T4	40.1 kDa	<i>Penaeus monodon</i> (Black tiger prawn)
			Enolase	118.58	4.16	P56252	47.0 kDa	<i>Homarus gammarus</i> (European lobster)
			Fructose-bisphosphate aldolase	46.36	2.22	P07764	39.0 kDa	<i>Drosophila melanogaster</i> (Fruit fly)
6	27 kDa	YNLQVR FLQAANACR LTSAVNEIEK MGLTEFQAVK LIDDHFLFK GTYYPLTGMSK EMQDGILELIK VSSTLSSLEGELK IISMQMGGDLGQVFR GEHTEAEGGIYDISNK VISSIEQK NLLSVAYK YDDMAAAMK DSTLIMQLLR EVTETGVLSNEER QAFDDAIAELDTLNEDSYK GFMTTPER AAEFNFR DYEINELNIQVNDLR	Arginine kinase	739.77	30.90	C7E3T4	40.1 kDa	<i>Penaeus monodon</i> (Black tiger prawn)
			14-3-3 protein zeta	393.59	27.53	Q2F637	28.1 kDa	<i>Bombyx mori</i> (Silk moth)
			Troponin I	188.06	14.43	P05547	23.5 kDa	<i>Astacus leptodactylus</i> (Turkish narrow-clawed crayfish)

(continued on next page)

Table 2 (continued)

Band No.	Estimated MW of Band (kDa)	Peptide sequences	Identified allergens	MASCOT Score	Coverage (%)	UniProtKB Accession I.D.	MW of native protein (kDa)	Species (common name)
7	19 kDa	AGGINLAR	Sarcoplasmic calcium-binding protein, beta chain	354.99	29.69	P02635	22.0 kDa	<i>Penaeus</i> sp. (Penaeid shrimp)
		LDCITR						
		VTLEGR						
		VFIGNQFK						
		EIDDAYDK						
		AFANFPNAFK	Sarcoplasmic calcium-binding protein, alpha-B and alpha-A chains	330.43	23.96	P02636	22.0 kDa	<i>Penaeus</i> sp. (Penaeid shrimp)
		DGEVTVDEFK						
		LDCITR						
		NTLEGR						
		AGGLTLER						
		VGLDEYR	Troponin I	278.96	14.93	P05547	23.5 kDa	<i>Astacus leptodactylus</i> (Turkish narrow-clawed crayfish)
		EIDDAYDK						
		DGEVTVDEFK						
		GFMTPER						
		AAEFNFR						
		DYEINELNIQVNDLR	Arginine kinase	124.98	5.90	C7E3T4	40.1 kDa	<i>Penaeus monodon</i> (Black tiger prawn)
		KDYEINELNIQVNDLR						
		LTSAVNEIEK						
		EMQDGILELIK						

cohort. The high percentage of patients displaying reactivity to HC in this study indicates that it is an allergenic protein that requires careful consideration in clinical testing, aligning with previous studies which have demonstrated IgE binding to HC (Khanaruksombat et al., 2014; Kamath et al., 2017). The same situation applies for AK. Both HC and AK have also been implicated previously in seafood-mite cross-reactivity, and may be important molecular markers for consideration (Ayuso et al., 2009). It should be noted that a low coverage of mass spectrometric identification was observed for shrimp hemocyanin (maximum of 4% sequence coverage). A follow-up analysis of purified Black Tiger Prawn hemocyanin confirmed the identity of this IgE binding protein demonstrating up to 70% of amino acid sequence

coverage (data not shown). Our study as well as previous studies have shown the diagnostic relevance of IgE binding to minor shellfish allergens (Ayuso et al., 2012; Abramovitch et al., 2013; Pascal et al., 2015; Rolland et al., 2018), with 50% of our ImmunoCAP negative patients displaying reactivity to minor allergens. In this study, IgE binding was not observed to myosin light chain (Pen m 3) or triose-phosphate isomerase (Cra c 8) and there was very low IgE binding to Troponin C (Pen m 6). This may be due to the patient panel selected. Alternatively, it may be due to the low abundance of these allergens in the tested shrimp extract, or in case of Pen m 3 or Cra c 8, due to their resistance to tryptic digestion in the mass spectrometric analysis used to identify these allergens. It is therefore important to validate and

Table 3

Summary of results of different *in vitro* diagnostic methods for 21 adult subjects with convincing history of clinical symptoms to shellfish. Tests include ImmunoCAP (whole shrimp extract), immunoblot (component-resolved whole shrimp extract) and immuno-PCR (recombinant TM). Dark shading indicates a positive test result. Cut-offs for a positive test: ImmunoCAP ≥ 0.35 kU/L, immuno-PCR ≤ 23.8 Ct, immunoblot ≥ 2000 pixels. TM: tropomyosin, SCP: sarcoplasmic calcium binding protein, AK: arginine kinase, HC: hemocyanin.

Patient No.	ImmunoCAP Shrimp-sIgE (kU/L)	Immuno-PCR TM-sIgE (Ct)	Immunoblot TM-sIgE (pixels x10 ³)	Immunoblot SCP-sIgE (pixels x10 ³)	Immunoblot AK-sIgE (pixels x10 ³)	Immunoblot HC-sIgE (pixels x10 ³)
1	9.82	20.37	49.22	0	0	9.43
2	9.03	21.81	16.02	0	0	6.48
3	6.65	18.74	18.95	0	0	1.08
4	5.93	24.11	0	0	0	0.74
5	5.37	20.60	25.22	0	17.03	0
6	4.54	24.32	0	0	0	1.67
7	3.33	22.69	9.52	0	0	0.77
8	1.82	24.86	1.40	0	3.15	2.03
9	1.65	22.30	4.87	13.32	0	0.56
10	1.41	25.80	0	3.45	0	4.75
11	1.32	20.22	16.85	0	0	7.09
12	0.5	21.45	0.67	0	0	1.79
13	0.36	24.86	0	0	0	0
14	0.21	25.36	0	0	0	0
15	0.16	25.84	0	0	5.41	1.35
16	0.1	24.60	0	0	8.33	3.19
17	0.07	24.83	1.99	0	4.75	3.27
18	0.05	25.71	0	1.37	0	0.82
19	0.04	25.45	0	0	1.27	5.80
20	0.04	24.96	0	0	0	1.18
21	0.03	25.05	6.01	0	0	0
N positive	13 (62%)	8 (38%)	8 (38%)	2 (10%)	5 (24%)	8 (38%)

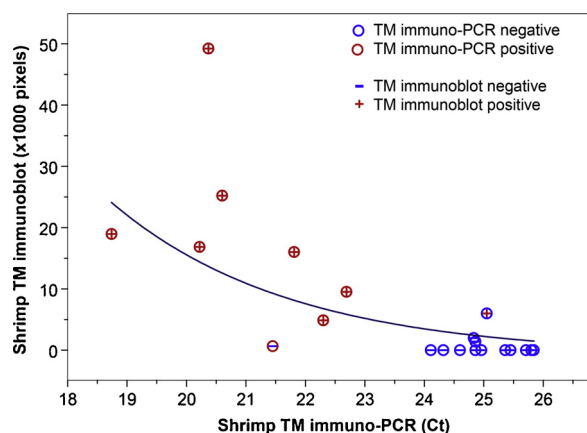


Fig. 2. Correlation of TM immuno-PCR versus TM immunoblot. Spearman rank correlation, $\rho = -0.767$, $p = 5.01 \times 10^{-5}$. Blue circle = negative immuno-PCR observation, red circle = positive immuno-PCR observation, blue ‘-’ = negative immunoblot observation, red ‘+’ = positive immunoblot observation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

monitor the presence of these less common allergens in diagnostic tests, especially for subjects who are mono-sensitised to allergens other than TM (Guillen et al., 2014; Kamath et al., 2017).

Using two different immunoassays, it was demonstrated that 43% of subjects displayed IgE binding to TM, as analysed by immunoblot and immuno-PCR, with a significant correlation between the two assays. Most diagnostic assays, including the ImmunoCAP, follow the direct ELISA principle where the allergen extract or purified molecule is directly immobilised onto a surface to allow IgE binding, which is subsequently detected using colorimetric or immunofluorescent detection. However, such covalent immobilisation may alter the allergen structure, leading to lower IgE binding. In addition, a caveat of the immunoblotting technique, which follows a similar principle, is the occurrence of non-specific binding of the secondary/tertiary antibodies to the allergenic proteins used for visualisation as seen in Figure S1.B. Due consideration should be given to such non-specific bands while confirming IgE binding to allergens immobilized on the membrane. We employed the Tus-TM TT-lock immuno-PCR system for the detection of TM-sIgE antibodies as a novel diagnostic assay that can quantify IgE binding to allergens that are in the liquid phase (Johnston et al., 2014). Our study indicated that IgE binding to TM was similar in its immobilised and free moving liquid state. Furthermore, the specialised recombinant TM resulted in comparable detection rates to the natural TM used in the immunoblot. While we have utilised a TM-sIgE immuno-PCR with high efficiency, this analytical approach could be implemented for other allergens including AK and SCP. When performed simultaneously, this method would reveal not only that the patient is sensitised to shrimp allergens, but also identify the specific responsible allergens.

5. Conclusions

In a cohort of 21 clinically confirmed shellfish-allergic individuals 62% tested positive by ImmunoCAP, a widely used commercial *in vitro* diagnostic test. Using component-resolved techniques IgE sensitisation to the major shrimp allergen TM was demonstrated in 43% of subjects analysed, while 52% of the cohort displayed sensitisation to other shrimp allergens. Out of the subjects sensitised to alternate allergens, 55% demonstrated specific IgE exclusively to an alternate allergen including AK, SCP or HC (but not TM). This highlights the need to assess IgE binding to other allergens in a larger cohort of clinically confirmed shrimp-allergic adults. Furthermore, in the subjects who tested negative by ImmunoCAP 50% displayed IgE binding to one or more of these

other allergens. This suggests the need for better standardisation of allergen content in commercial tests and the inclusion of more component-resolved techniques to improve the diagnosis of shrimp allergy. Diagnostic approaches including immunoblot and immuno-PCR appear advantageous especially when investigating cases where subjects are not IgE-reactive to major allergens. Efficient standardisation and validation of tests based on crude protein extracts are required, particularly for less investigated allergens such as AK, SCP and HC. The inclusion of these allergens in component-resolved diagnosis will lead to improved diagnostic outcomes in a wider population suffering from shrimp allergy.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.molimm.2019.05.006>.

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Supplementary Figures

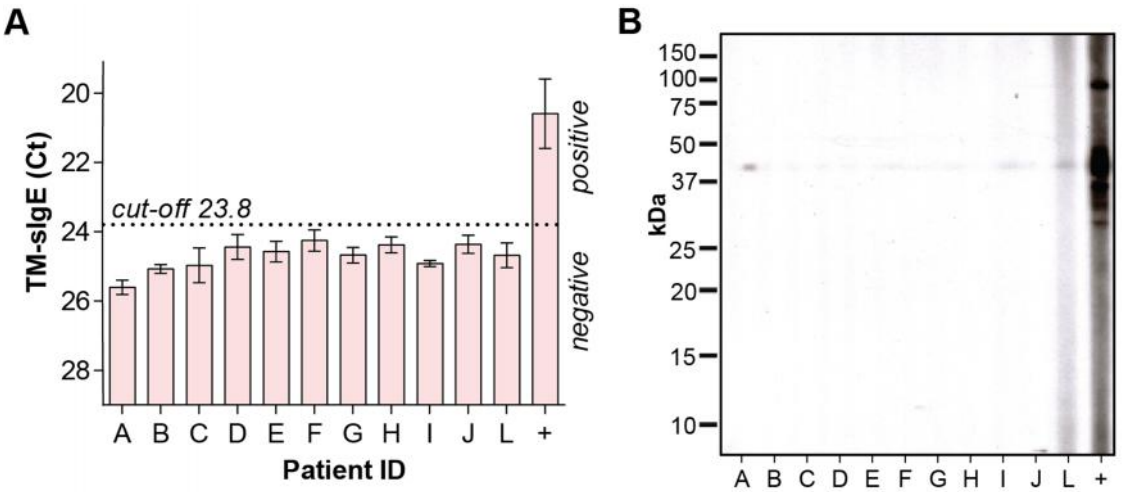


Figure S1: Analysis of eleven negative control subjects for the TM immuno-PCR. (A) Average and SD (n = 6 repeats) displayed for control subjects A-L and one positive control (+). A positive test was set at two standard deviations below the average cycle threshold (Ct) of the negative subjects, ≤ 23.8 . (B) IgE immunoblot of control subjects against recombinant shrimp TM, detected by ECL.