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Original Article

Cell-Based Functional IgE Assays Are Superior to Conventional Allergy Tests for Shrimp Allergy Diagnosis

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What is already known about this topic? Shrimp allergy is prevalent, but conventional diagnostic methods including skin prick test (SPT) and specific IgE (sIgE) measurements have low specificity. Testing for tropomyosin improves diagnostic accuracy for shrimp allergy in Caucasians, but this has not been replicated among Asians.

What does this article add to our knowledge? BAT has superior diagnostic performance for shrimp allergy than SPT and sIgE measurement. Tropomyosin may not be the most appropriate diagnostic marker in the Chinese population. The IgE crosslinking–induced luciferase expression (EXiLE) test can be a good alternative to BAT.

How does this study impact current management guidelines? A single step of BAT may replace SPT and sIgE in the diagnosis of patients with clinical history suggestive of shrimp allergy. The EXiLE test can be a suitable alternative with respect to cost and sampling constraint.

BACKGROUND: The diagnosis of shellfish allergy currently relies on patient history, skin prick test (SPT), and serum specific IgE (sIgE) quantification. These methods lack sufficient diagnostic accuracy, whereas the gold standard of oral food challenges is risky and burdensome. Markers of reactivity and severity of allergic reactions to shellfish will improve clinical care of these patients.

OBJECTIVES: This study compared the diagnostic performance of SPT, sIgE, basophil activation test (BAT), and IgE crosslinking–induced luciferase expression (EXiLE) test for shrimp allergy.

METHODS: Thirty-five subjects with documented history of shrimp allergic reactions were recruited and grouped according to results of double-blind, placebo-controlled food challenge (DBPCFC). In addition to routine diagnostics, BAT (Flow CAST) and EXiLE test with shrimp extract and tropomyosin were performed.

RESULTS: Of 35 subjects, 15 were shrimp allergic with pruritus, urticaria, and itchy mouth on DBPCFC, whereas 20 were tolerant to shrimp. Tropomyosin only accounted for 53.3% of sensitization among subjects with challenge-proven shrimp allergy. BAT using shrimp extract as stimulant showed the highest area under curve value (0.88), Youden Index (0.81), likelihood ratio (14.73), odds ratio (104), and variable importance (4.27) when compared with other assays and tropomyosin diagnosis. Results of BAT significantly correlated with those of EXiLE ($r = 0.664$, $P < .0001$).

CONCLUSIONS: BAT is a more accurate diagnostic marker for shrimp allergy than SPT and shrimp sIgE, whereas the EXiLE test based on an IgE crosslinking assay is a good alternative to

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Abbreviations used
AUC- Area under curve
BAT- Basophil activation test
DBPCFC- Double-blind, placebo-controlled food challenge
EDTA- Ethylenediaminetetraacetic acid
EXiLE- IgE crosslinking—induced luciferase expression
HR- Histamine release
LH- Likelihood ratio
NPV- Negative predictive value
OR- Odds ratio
PBS- Phosphate-buffered saline
PPV- Positive predictive value
PWH- Prince of Wales Hospital
ROC- Receiver operating characteristic
SCORAD- SCORing Atopic Dermatitis
sIgE- Specific IgE
SPT- Skin prick test

BAT. Tropomyosin may not be the most important shrimp allergen in Chinese, which warrants further investigation to search for other major allergens and diagnostic markers. © 2020 American Academy of Allergy, Asthma & Immunology (J Allergy Clin Immunol Pract 2021;9:236-44)

Key words: *Shrimp allergy; Basophil activation test; IgE crosslinking—induced luciferase expression test; DBPCFC; Tropomyosin; CD63; CCR3*

Shellfish is one of the most common food allergens, with shrimp being the most frequent offender causing anaphylaxis in both pediatric and adult populations.[1] In the United States, 2.9% of the adult population and 1.3% of children suffered from shellfish allergy.[2,3] In Europe, shrimp was one of the 3 commonest food allergens among adults with the IgE sensitization rate of 1.46%.[4] Shellfish was the dominant food allergen among Asian populations, with prevalence rates of shellfish allergy ranging from 0.9% to 6.9%.[5] In Hong Kong, Guangzhou, and Shaoguan, the prevalence of probable shellfish allergy as defined by reported symptoms together with positive skin prick test (SPT) or specific IgE (sIgE) was 1.05%, 0.18%, and 0.65%, respectively.[6] Shellfish allergy is a growing health care concern due to its increasing global prevalence and low resolution rate.[7,8]

Like other food allergies, double-blind, placebo-controlled food challenge (DBPCFC) is the gold standard for shrimp allergy diagnosis. However, it is risky, labor-intensive, and expensive. Although SPT and sIgE can aid in diagnosis, concerns about their poor diagnostic accuracy and correlation with clinical allergic reactions limited their application in routine patient care.[9-11] Therefore, there is an unmet need to identify objective biomarkers that can accurately diagnose shrimp allergy and reflect its severity.

Over the last decade, basophil activation test (BAT) has been advocated as "food challenge in test tube."[12] This functional assay quantifies fluorometrically the number of activated CD63-expressing basophils in response to IgE crosslinking with a given dose of the culprit allergen. BAT has been shown to closely reproduce patients' clinical phenotype, and basophil response can be a reliable biomarker for the severity and threshold of allergic reactions.[13-16] Incorporating BAT into the diagnostic workup was also shown to reduce the need for oral food challenges.[17] Nonetheless, the pitfalls of BAT include limited window of sample processing and presence of nonresponder basophils.[12] Mast cell activation test, histamine release assay (HR), passive HR,[18,19] and a rat basophilic leukemia cell line—based approach called the IgE crosslinking—induced luciferase expression (EXiLE) test were alternative methods adopting similar machinery.[20] In the EXiLE test, a rat basophil leukemia cell line that overexpresses the human FcεRI receptor and firefly luciferase reporter gene (RS-ATL8 cell line) is stimulated by the culprit allergen in human serum. The degree of allergen-IgE crosslinking was then quantified by the luciferase signals.[21] The area under curve (AUC) of the EXiLE test correlated well with the outcome of oral food challenges,[22] which was reported as a convenient and sensitive diagnostic biomarker for egg allergy.

To our knowledge, these cellular tests have not been evaluated for usefulness to diagnose shrimp allergy. This study aimed to evaluate and compare the diagnostic efficacy of SPT, sIgE measurement, BAT, and EXiLE test on shrimp-allergic and -tolerant subjects as defined by DBPCFC.

METHODS

Subjects

Patients aged 5 to 50 years who exhibited documented history of immediate allergic reactions within 2 hours of shrimp consumption on at least 2 occasions over the past 5 years were recruited from our 3 regional hospitals (Prince of Wales Hospital [PWH], Princess Margaret Hospital, and Yan Chai Hospital). All subjects underwent allergy evaluations and DBPCFCs according to the EuroPrevall protocol in PWH ("Methods" and Figure E1, available in this article's Online Repository at www.jaci-inpractice.org), who were classified into allergic and tolerant groups.[23] The study protocol was approved by the Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee (2018.484) and Kowloon West Research Ethics Committee (127.12).

Allergy tests

SPT was performed with commercial shrimp extract (ALK-Abelló, Madrid, Spain) together with 10 mg/mL histamine (positive control) and normal saline (negative control). The concentrations of total IgE and sIgE to shrimp (f24) and rPen a 1 (f351) were measured by ImmunoCAP (ThermoFisher Scientific, Uppsala, Sweden). *In vitro* BAT was performed on fresh venous blood using the Flow CAST kit (BÜHLMANN Laboratories, Schönenbuch, Switzerland) and predetermined concentrations of *Penaeus monodon* protein extract and recombinant Pen m 1 (rPen m 1) (Figure E2, available in this article's Online Repository at www.jaci-inpractice.org). The EXiLE test was performed by sensitizing the RS-ATL8 cells with 1:100 diluted serum, followed by stimulation with a predetermined concentration of *P. monodon* protein extract or rPen m 1.[21] Please refer to this article's Online Repository at www.jaci-inpractice.org for details of these assays.

Statistical analysis

GraphPad Prism (version 8.0, GraphPad Software, San Diego, Calif) and SPSS (version 26, IBM Corporation, Armonk, NY) were used for graphical presentation and statistical analyses. Differences in quantitative variables were analyzed by the Mann-Whitney *U* test, and categorical data were compared by the Fisher exact test. The diagnostic accuracy of allergy tests was evaluated by the AUC value on receiver operating characteristic (ROC). Optimal cutoff values to identify shrimp allergy were determined. Sensitivity, specificity, odds ratio (OR), likelihood ratios (LH), and positive (PPV) and negative

TABLE I. Characteristics of study participants

Demographic features	Allergic group	Tolerant group	*P* value
N	15	20	–
Gender: male, % (number)	66.7 (10)	45.0 (9)	.306*
Age (y): median [range]	25.0 [5-44]	30.5 [9-49]	.443†
Total IgE (kUA/L): median [range]	386 [25.9-2781]	464 [44.8-4081]	.705†
Other food allergies: % (number)	60.0 (9)	30.0 (6)	.097*
Eczema: % (number)	80.0 (12)	65.0 (13)	.458*
Asthma/allergic rhinitis: % (number)	66.7 (10)	40.0 (8)	.176*
Age of shrimp allergy onset (y): median [range]	5.5 [2-19]	7.0 [2-17]	.821†
History of shrimp allergy	+	+	–
DBPCFC	+	–	–
SPT			
Wheal size (mm): median [range]	5.5 [0-12]	3.3 [0-5]	**.0082**†
Sensitization rate: % (number)	92.8 (13/14)	72.2 (13/18)	.196*
sIgE to shrimp			
Level (kUA/L): median [range]	2.28 [0.24-33.2]	1.71 [0.01-11.1]	.122†
Sensitization rate: % (number)	93.3 (14)	65.0 (13)	.101*
sIgE to rPen a 1			
Level (kUA/L): median [range]	0.65 [0-24.5]	0.01 [0-7.04]	.097†
Sensitization rate: % (number)	53.3 (8)	20 (4)	.071*

DBPCFC, Double-blind, placebo-controlled food challenge; *SPT*, skin prick test; *sIgE*, specific IgE.
Statistical differences were assessed by the Fisher exact test for categorical data (*) and the Mann-Whitney *U* test for quantitative variables (†). Bold indicates the statistical significance ($P < .05$). "+", positive; "–", negative.

(NPV) predictive values were calculated using 2-by-2 contingency tables, whereas the Youden Index was calculated as follows: sensitivity + specificity − 1. The random forest method implemented in the SPSS add-on package for R system version 3.5 was used to investigate the ability of each allergy test to correctly discriminate between shrimp-allergic and intolerant subjects based on the calculated variable importance, in which the higher the variable importance, the more relevant will that variable be for disease discrimination. To obtain sufficiently stable variable importance estimates, each forest consisted of 10,000 trees and the unscaled variable importance was reported.[23] The correlation between allergy tests was assessed by the Pearson correlation. $P < .05$ was considered as statistical significance.

RESULTS

Subjects' allergy status

Table I summarizes the demographics, SPT, and sIgE results of 15 shrimp-allergic and 20 shrimp-tolerant subjects as defined by DBPCFC. The median (range) age of first shrimp-allergic reaction was 5.5 (2-19) years and 7.0 (2-17) years for the allergic and tolerant groups, respectively. These 2 groups were matched for age, gender, total IgE, other food allergies, and allergic diseases. The scores of allergic reactions that shrimp-allergic subjects developed during DBPCFC ranged from 3 (mild reactions) to 7 (severe reactions) (Table E1, available in this article's Online Repository at www.jaci-inpractice.org). The majority of these subjects reported skin reactions such as pruritus (80.0%) and urticaria (73.3%) as well as oral symptoms such as itchy mouth (66.7%). On the other hand, all shrimp-tolerant subjects did not report any objective or persistent and severe subjective reactions (Table E2, available in this article's Online Repository at www.jaci-inpractice.org).

SPT and sIgE results

The shrimp-allergic and -tolerant groups differed significantly with respect to SPT wheal ($P = .0082$; Figure 1, *A*) but not the rate for positive SPT ($P = .196$, Fisher exact test; Table I). Neither sIgE levels nor the sensitization rates to shrimp and rPen a 1 at 0.35 kUA/L cutoff differed significantly between allergic and tolerant groups (Table I and Figure 1, *B* and *C*). The "optimal decision points" (ie, highest Youden Index) for SPT and sIgE to shrimp and rPen a 1 were determined from ROC curves (Table II). At the cutoff wheal size of 4.75 mm, 9 of 14 (64.3%) allergic subjects and only 1 of 18 tolerant subjects were SPT positive ($P = .0006$, Fisher exact test). At a higher cutoff sIgE to shrimp of 0.89 kUA/L, 14 of 15 (93.3%) allergic and 11 of 20 (55.0%) tolerant subjects were positive ($P = .022$, Fisher exact test). Similarly, a higher cutoff sIgE to rPen a 1 of 0.56 kUA/L differentiated shrimp-allergic and -tolerant subjects ($P = .027$, Fisher exact test) in which 8 of 15 (53.3%) allergic subjects and only 3 of 20 (15.0%) tolerant subjects were sIgE positive.

Basophil activation test

Our group optimized BAT to shrimp and recombinant shrimp tropomyosin rPen m 1 before subject testing according to the methods and Figure E3 available in this article's Online Repository at www.jaci-inpractice.org.

Whole blood samples were collected from all shrimp-allergic subjects and all except 3 tolerant subjects. Of the 32 analyzed samples, none of the patients were nonresponders; all samples showed >10% CD63-expressing basophils on anti-FCεRI monoclonal antibody stimulation. Among allergic subjects, the median (range) %$CD63^+$basophil at 5000 and 10,000 ng/mL of shrimp extract were 67.1 (0.7-96.8) and 72.9 (0.9-94.7), respectively (Figure 1, *D* and *E*). In the tolerant group, the respective median (range) %$CD63^+$basophil were 2.4 (0.2-80.3)

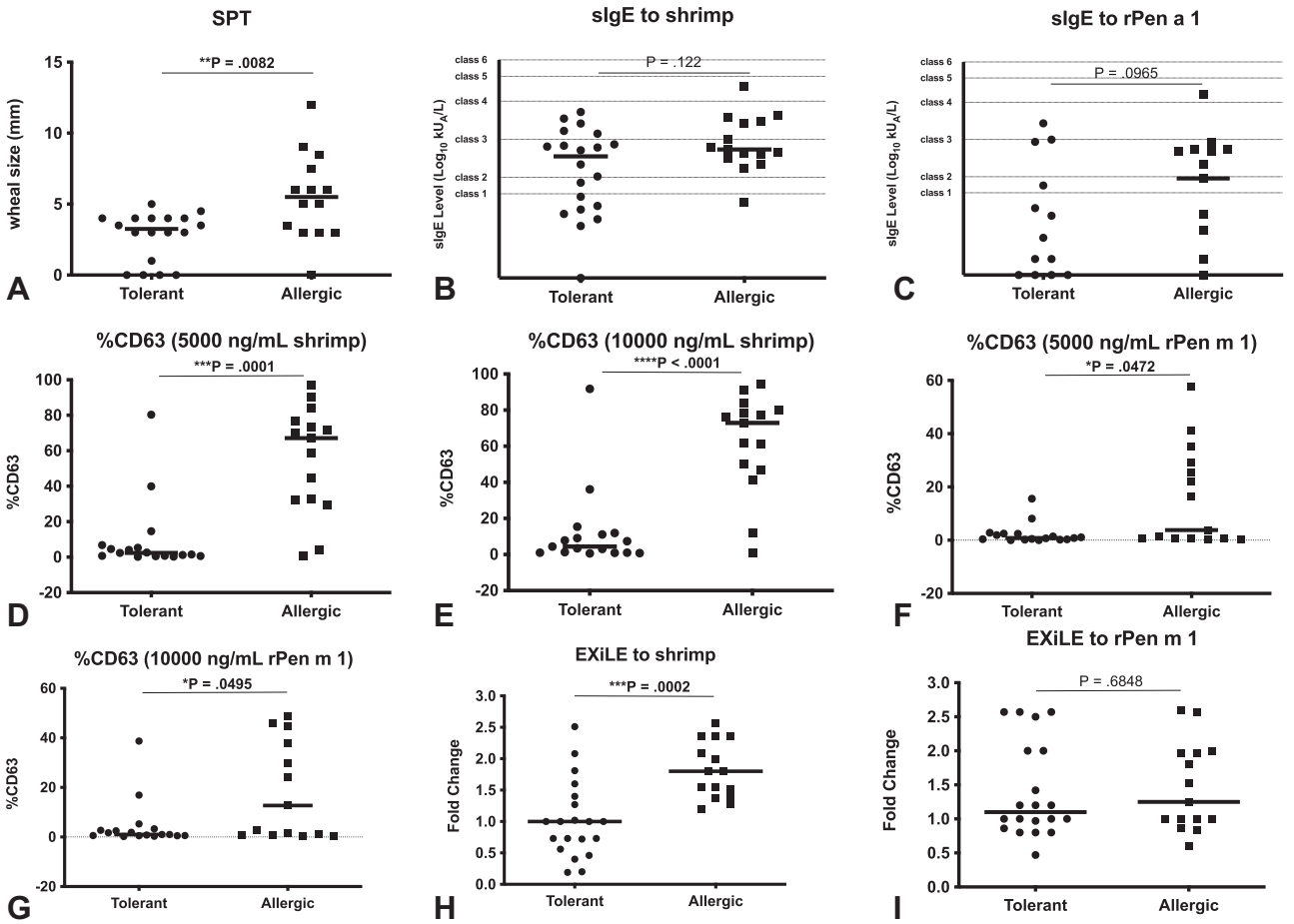

FIGURE 1. SPT (**A**), sIgE (**B** and **C**), BAT (**D-G**), and EXiLE (**H** and **I**) responses of shrimp-allergic and -tolerant subjects. Individual data points with median (line) are presented. Differences in the responses between 2 groups were assessed by the Mann-Whitney *U* test. Boldface indicates the statistically significant difference. *BAT*, Basophil activation test; *EXiLE*, IgE crosslinking–induced luciferase expression; *sIgE*, specific IgE; *SPT*, skin prick test.

and 4.4 (0.6-91.8). The intergroup differences in %CD63$^+$basophil were significant at both tested concentrations ($P = .0001$ and $P < .0001$, respectively). When stimulated with rPen m 1, the median (range) %CD63$^+$basophil were 3.8 (0.2-57.8) and 12.7 (0.2-61.0) in the allergic group (Figure 1, *F* and *G*), and 0.8 (0-15.6) and 1.0 (0.2-38.7) among tolerant subjects at the 2 tested concentrations. Such differences in %CD63$^+$basophil between the allergic and tolerant groups were also significant at both concentrations ($P = .0472$ and $P = .0495$, respectively).

Based on the Youden Index, the optimal cutoffs for % CD63$^+$basophil were 22.1% and 38.8% at the 2 shrimp extract concentrations, respectively (Table II). At these cutoffs, 13 of 15 (86.7%) shrimp-allergic subjects were BAT positive but only 1 of 17 tolerant subjects showed positive results ($P < .0001$, Fisher exact test; Table II). Using 5000 and 10,000 ng/mL rPen m 1 as stimulants for BAT, the respective optimal cutoffs were 16.1% and 9.0% (Table II). BAT positivity was detected in 7 of 15 (46.7%) allergic subjects, whereas negative BAT was found on all tolerant subjects at 5000 ng/mL rPen m 1 ($P = .0019$, Fisher exact test; Table II).

EXiLE test

Serum samples from all shrimp-allergic and -tolerant subjects were analyzed under optimized condition described in the methods and Figure E3 available in this article's Online Repository at www.jaci-inpractice.org. At 100 ng/mL of shrimp extract, the median (range) fold changes of luciferase expression were 1.8 (1.2-2.6) and 1.0 (0.2-2.5) in the allergic and tolerant groups, respectively ($P = .0002$; Figure 1, *H*). However, the fold changes induced by the same concentration of rPen m 1 did not differ between the 2 groups ($P = .6848$; Figure 1, *I*). BAT and EXiLE, both analyzed the degree of IgE cross-linking, showed significant correlation by the Pearson test ($r = 0.664$, $P < .0001$). From the ROC curve, the optimal cutoff for EXiLE with shrimp extract was a fold increase of 1.1 (Table II). All shrimp-allergic and 6 of 20 (30.0%) of tolerant subjects were positive ($P < .0001$, Fisher exact test). On the other hand, at the optimal cutoff for rPen m 1 at a fold increase of 1.2, 8 of 15 (53.3%) allergic subjects and 50% of tolerant subjects were positive ($P > .999$, Fisher exact test; Table II).

TABLE II. Properties of SPT, ImmunoCAP, BAT, and EXiLE test as diagnostic tests for challenge-confirmed shrimp allergy

Allergy test	*P* value by the Fisher exact test	AUC	Odds ratio	Cutoff	Sensitivity	Specificity	Youden Index (*J*)	PPV	NPV	Likelihood ratio
SPT	.196	0.77 [0.59-0.95]	5.00 [0.51-48.9]	3.0 mm	0.93 [0.66-1.00]	0.28 [0.10-0.53]	0.22	0.50 [0.30-0.70]	0.83 [0.36-1.00]	1.29
	.001		30.6 [3.09-303.4]	4.75 mm*	0.64 [0.35-0.87]	0.94 [0.73-1.00]	0.58	0.90 [0.56-1.00]	0.77 [0.55-0.92]	11.57
sIgE to shrimp	.101	0.66 [0.47-0.84]	7.53 [0.81-69.9]	0.35 kUA/L	0.93 [0.68-1.00]	0.35 [0.15-0.59]	0.28	0.52 [0.32-0.71]	0.88 [0.47-1.00]	1.44
	.022		11.5 [1.25-104.6]	0.89 kUA/L*		0.45 [0.23-0.68]	0.38	0.56 [0.35-75.6]	0.90 [0.56-1.00]	1.70
sIgE to rPen a 1	.071	0.67 [0.48-0.85]	4.57 [1.03-20.4]	0.35 kUA/L	0.53 [0.27-0.79]	0.80 [0.56-0.94]	0.33	0.67 [0.35-0.90]	0.70 [0.47-0.87]	2.67
	.027		6.49 [1.39-26.2]	0.56 kUA/L*	0.53 [0.30-0.75]	0.85 [0.64-0.95]	0.38	0.73 [0.43-0.90]	0.71 [0.51-0.85]	3.56
BAT to shrimp (5000 ng/mL)	**<.001**	0.87 [0.74-1.00]	104 [8.46-1279]	22.1%*	0.87 [0.60-0.98]	0.94 [0.71-1.00]	0.81	0.93 [0.66-1.00]	0.89 [0.65-0.99]	14.73
BAT to shrimp (10,000 ng/mL)		0.88 [0.74-1.00]		38.8%*						
BAT to rPen m 1 (5000 ng/mL)	**.002**	0.71 [0.52-0.89]	30.9 [1.57-606.8]	16.1%*	0.47 [0.21-0.73]	1.00 [0.80-1.00	0.47	1.00 [0.59-1.00]	0.68 [0.47-0.85]	NA†
BAT to rPen m 1 (10,000 ng/mL)	**.049**	0.70 [0.52-0.89]	6.56 [1.10-39.3]	9.0%*		0.88 [0.64-0.99]	0.35	0.78 [0.40-0.97]	0.65 [0.43-0.84]	3.97
EXiLE to shrimp	**<.001**	0.85 [0.72-0.98]	69.2 [3.57-1340]	1.1 FC*	1.00 [0.78-1.00]	0.70 [0.46-0.88]	0.70	0.71 [0.48-0.89]	1.00 [0.77-1.00]	3.33
EXiLE to rPen m 1	>.999	0.54 [0.35-0.74]	1.14 [0.27-4.04]	1.2 FC*	0.53 [0.30-0.75]	0.50 [0.30-0.70]	0.03	0.44 [0.25-0.66]	0.59 [0.36-0.78]	1.07

AUC, Area under curve; *BAT*, basophil activation test; *CI*, confidence interval; *EXiLE*, IgE crosslinking–induced luciferase expression; *ROC*, receiver operating characteristic; *sIgE*, specific IgE; *FC*, fold change; *NA*, data not available; *NPV*, negative predictive value; *PPV*, positive predictive value; *SPT*, skin prick test.

Values are shown as 95% CI. Odds ratio, sensitivity, specificity, Youden Index, PPV, NPV, and likelihood ratio were determined from the 2-by-2 contingency table. Bold indicates statistical significance ($P < .05$).

*Optimal cutoff point as determined by the ROC curve based on the highest Youden Index calculated as follows: sensitivity + specificity − 1.

†NA: likelihood ratio for the BAT assay using rPen m 1 (5000 ng/mL) could not be determined from the 2-by-2 contingency table.

Diagnostic performance of different allergy tests

We analyzed only allergy tests that showed significant differences in positivity between allergic and tolerant groups. Accordingly, we excluded conventional tests of SPT at 3 mm cutoff, sIgE to shrimp and rPen a 1 at 0.35 kUA/L cutoff points, and rPen m 1-EXiLE ($P > .05$, Fisher exact test; Table II).

Among remaining tests, shrimp-BAT had the highest Youden Index (0.81) with sensitivity and specificity of 0.87 and 0.94, respectively, regardless of stimulant concentrations (Table II). This test also showed the highest LH ratio (14.73), high PPV (0.93) and NPV (0.89). Despite having the highest sensitivity (1.00), shrimp-EXiLE had a lower Youden Index (0.7) and a much lower LH ratio (3.33). SPT at 4.75 mm wheal cutoff had a low Youden Index (0.58) but comparable specificity (0.94), PPV (0.90), and LH ratio (11.57) to shrimp-BAT.

The ability of allergy tests to discriminate between allergic and tolerant subjects was then compared by AUC values, OR, and variable importance from the random forest method (Table II, Figures 2 and 3). Shrimp-BAT at both concentrations had the highest AUC values (0.87 and 0.88, respectively), OR (104), and variable importance (3.20 and 4.27, respectively). These results indicated that BAT, particularly with 10,000 ng/mL shrimp extract as stimulant, was the best method to diagnose shrimp allergy. Despite the comparable AUC value (0.85), shrimp-EXiLE had a much lower OR (69.2) and variable importance (2.59). SPT at 4.75 mm cutoff had weak discriminative power (AUC value 0.77, OR 30.6, and variable importance 1.82), and the discriminative power was even weaker for conventional sIgE measurements at 0.89 kUA/L (shrimp extract) and 0.56 kUA/L (rPen m 1) cutoffs (Table II, Figures 2 and 3).

Diagnostic algorithm for shrimp allergy

Given the good diagnostic accuracy of shrimp-BAT and the comparable accuracy of shrimp-EXiLE, we analyzed if single-step tests with either SPT (at 3 mm wheal cutoff), shrimp sIgE (≥0.35 kUA/L), shrimp-BAT (≥38.1% $CD63^+$ basophil response), and shrimp-EXiLE (fold change ≥1.1) would predict DBPCFC outcomes (Figure 4, *A*). Only 56.6% of SPT-positive subjects and 61.9% of sIgE-positive subjects reacted during shrimp DBPCFC. Shrimp-EXiLE performed slightly better than 70% of shrimp-EXiLE−positive subjects reacted during shrimp DBPCFC. By contrast, 12 of 13 (92.3%) patients with positive BAT reacted in DBPCFC, whereas 14 of 16 (87.5%) patients with negative BAT results passed DBPCFC. We also compared algorithms with sequential use of different allergy tests (Figure 4, *B*). Because SPT was widely used in clinical practice, we chose this allergy test as the first step in both algorithms. For the algorithm with SPT followed by shrimp-sIgE, 12 of 17 (70.6%) patients with positive tests reacted during DBPCFC, whereas 5 (29.4%) subjects were tolerant. This workflow showed acceptable sensitivity but only 0.29 specificity. For the algorithm with SPT followed by shrimp-EXiLE, 13 of 16 (81.3%) patients with positive SPT and shrimp-EXiLE reacted in DBPCFC. The algorithm showed higher sensitivity and specificity (0.40) than that of sequential SPT and sIgE. By contrast, 12 of 13 (92.3%) patients with positive SPT and shrimp-BAT reacted in DBPCFC. This stepwise algorithm with SPT followed by BAT had the best sensitivity (0.92) and specificity (0.83). However, this stepwise approach did not improve the overall diagnostic accuracy when compared with the single shrimp-BAT approach.

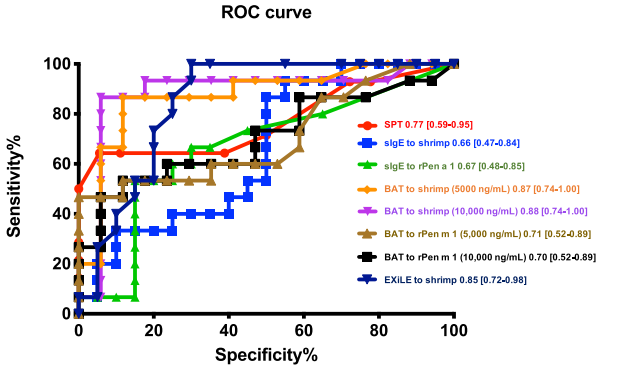

FIGURE 2. ROC curves plotting the proportion of the true allergic and tolerant subjects. The curve for each type of allergy test (SPT, BAT using 5000 and 10,000 ng/mL of shrimp extract or 5000 ng/mL of rPen m 1, as well as the EXiLE test using shrimp extract) is depicted by a different color. AUC values (95% CI) are shown. *BAT*, Basophil activation test; *CI*, confidence interval; *EXiLE*, IgE crosslinking–induced luciferase expression; *ROC*, receiver operating characteristic; *sIgE*, specific IgE; *SPT*, skin prick test.

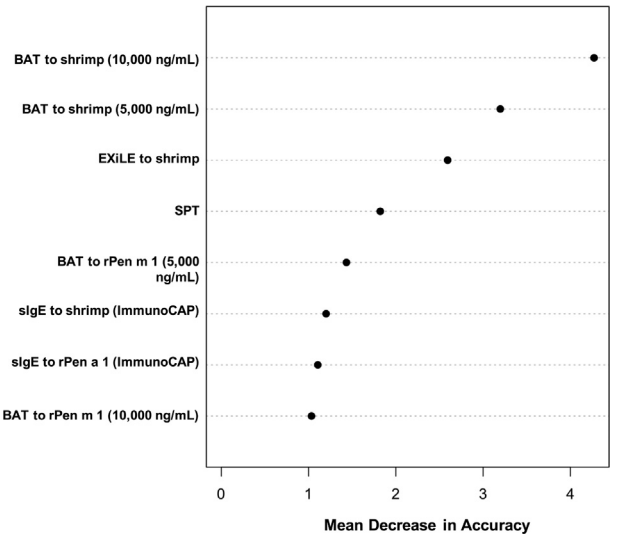

FIGURE 3. Variable importance plot. High positive values of the mean decrease in accuracy mean a higher variable importance, whereas the small positive values indicate a variable that is less relevant for discrimination. *BAT*, Basophil activation test; *EXiLE*, IgE crosslinking–induced luciferase expression; *sIgE*, specific IgE; *SPT*, skin prick test.

DISCUSSION

This study demonstrated that standard assessment methods for shrimp sensitization could not accurately identify shrimp allergy. Our data indicated that basophil parameters were possible biomarkers of the clinical outcome of patients with shrimp allergy.

Although DBPCFC remains the gold standard diagnosing food allergy, the clinical utility is limited by the cost and safety considerations. Accurate alternative diagnostic methods are therefore important to circumvent the need for oral food challenges. SPT and measurement of sIgE levels to shrimp extract remain the standard clinical procedures for helping to diagnose shrimp allergy. From our data, SPT is a more accurate predictor of shrimp allergy compared with shrimp sIgE that is also similarly reported for sesame allergy.[24] Our study also corroborated with published studies that SPT and sIgE levels had a high sensitivity of >90% but a low specificity of 28% (SPT) and 35% (shrimp sIgE) at the standard cutoff points, as well as low AUC values that indicate their low diagnostic power to shrimp allergy.[25,26]

IgE crosslinking assays were shown to closely resemble the clinical phenotype of food-allergic patients and reflect the severity and threshold of allergic reactions,[15,27,28] as these tests took into account the levels, specificity, diversity, and affinity of allergen-specific IgE as well as possible interference by other allergen-specific antibodies such as IgG4. This type of assays involved liquid phase rather than immobilized allergens as in the ImmunoCAP test, so the former would more closely reflect the physiological interaction between allergens and IgE.[29] Implementation of BAT in cow's milk allergy diagnosis was reported to reduce 30% to 40% of oral food challenges.[17] However, the BAT assay has intrinsic disadvantages of a small window period (4-24 hours) for sample processing after collection, presence of nonreleaser basophils, and high operational cost.[30] With the possibility to overcome these obstacles, our data showed that the EXiLE test significantly correlated with BAT results. EXiLE could also discriminate between shrimp-allergic and -tolerant subjects with comparable sensitivity and AUC value to BAT. A single-step shrimp-EXiLE analysis could already improve diagnostic accuracy compared with conventional allergy tests such as SPT and sIgE measurement. The EXiLE test may thus be a suitable alternative to BAT in terms of providing a cheaper option and when fresh blood samples and flow cytometric facilities are not available. This test also allows longer time from blood sampling to testing because EXiLE is done on archived serum instead of viable cells as required by BAT. However, it is noteworthy that BAT remained superior as a diagnostic test with the highest Youden Index (ie, high sensitivity and specificity) and a much higher LH ratio, OR, and variable importance measure compared with EXiLE and other allergy tests. This discrepancy is attributed to the functional heterogeneity of basophils and existence of different allergic manifestations among different subjects that cannot be reflected from an engineered cell line used in the EXiLE test.

We demonstrated herein, for the first time, that BAT to shrimp was the most accurate in diagnosing shrimp allergy in challenge-confirmed patients. Our data demonstrated that in contrast to ImmunoCAP tests that only consider sIgE concentrations, BAT using shrimp extract as stimulant was able to discriminate true allergic patients from a pool of individuals positive for shrimp-sIgE. This reflects the functional nature of BAT that takes into account both the affinity of sIgE and presence and level of sIgE. The discriminative performance of BAT for shrimp allergy diagnosis was equivalent to those for sesame and peanut allergies, with AUC values being 0.88 in our analysis and 0.86 and 0.97 for the latter foods, respectively.[13,28] Our study and other publications also similarly showed higher sensitivity and specificity of BAT over conventional allergy tests. On the other hand, our findings demonstrated that even a single diagnostic step involving shrimp-BAT could significantly improve the diagnostic accuracy for shrimp allergy. However, the

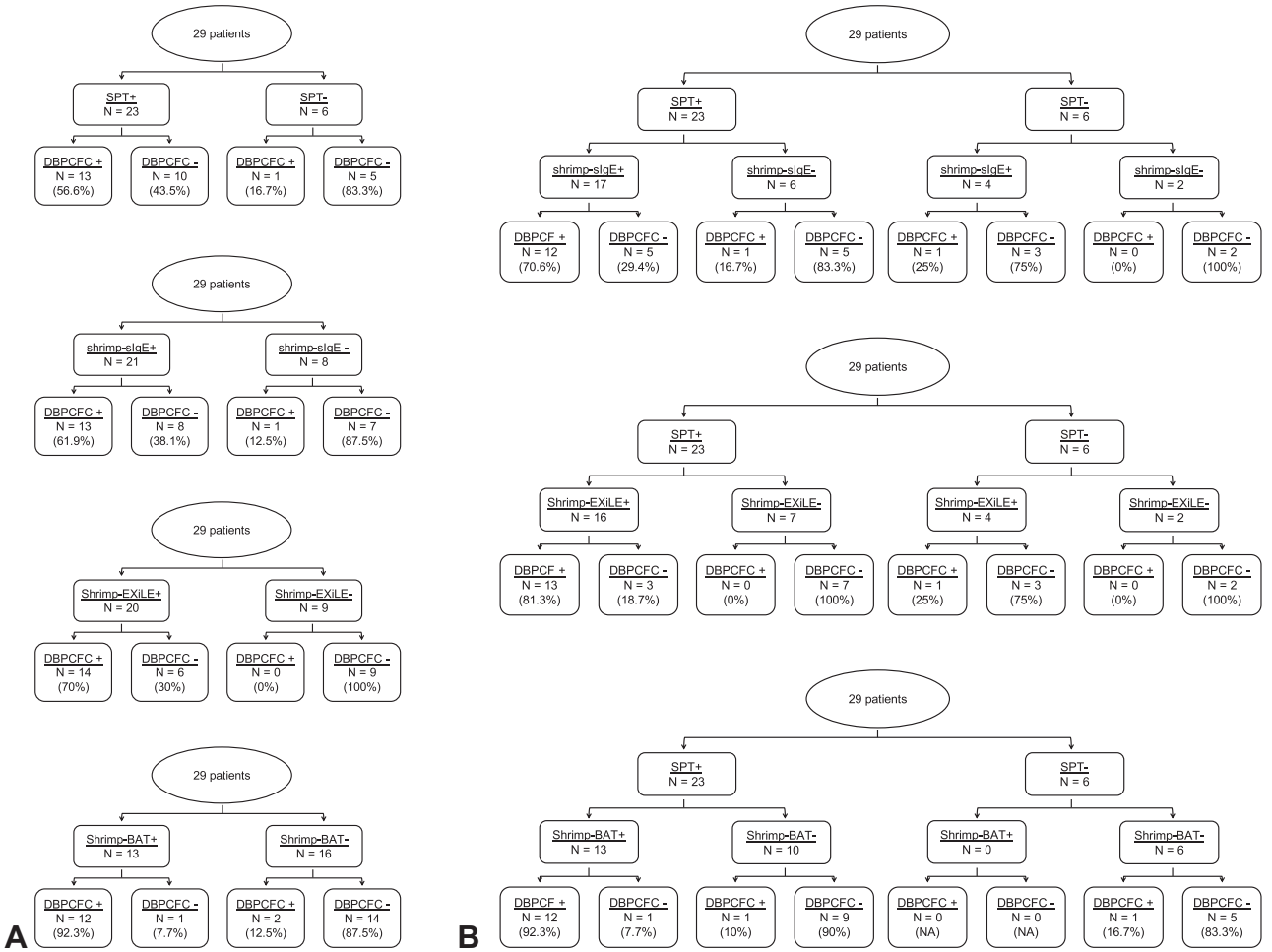

FIGURE 4. Stepwise diagnostic approach for shrimp allergy. **A**, Schematic diagrams depicting the single-step approach for the diagnosis of shrimp allergy. **B**, Schematic diagrams depicting the 2-step approach with the complementary utility of SPT and sIgE level to shrimp, SPT and shrimp-EXiLE, as well as SPT and shrimp-BAT. Cutoff points for SPT, shrimp-sIgE, shrimp-EXiLE, and shrimp-BAT are ≥3 mm wheal size, ≥0.35 kUA/L, ≥1.1-fold change, and 38.8%, respectively. *BAT*, Basophil activation test; *DBPCFC*, double-blind, placebo-controlled food challenge; *EXiLE*, IgE crosslinking–induced luciferase expression; *sIgE*, specific IgE; *SPT*, skin prick test.

diagnostic accuracy was comparable between a single diagnostic step with shrimp-BAT and when adding basophil response as a second diagnostic step to all patients with a positive SPT. Nevertheless, our algorithm clearly illustrated the incomparable discriminative ability of shrimp-BAT as both a rule-in and rule-out test. Our study thus sets the stage for more accurate diagnosis of shrimp allergy and reduction in use of oral food challenges.

Other studies suggested that measuring the tropomyosin-sIgE level could better predict clinical reactivity to shrimp, with independent studies reporting >90% NPV with this parameter.[9,10] Although measuring rPen a 1-sIgE levels generates a specificity of 85% at 0.56 kUA/L decision point in this study, only 8 of 15 (53.3%) of our shrimp-allergic patients were tropomyosin sIgE-positive when compared with those by Pascal et al[25] (82.8%) and Yang et al[10] (71.4%). Although the utilization of shrimp extract–based BAT significantly improved the diagnostic accuracy compared with the ImmunoCAP test with shrimp extract, both allergy tests with shrimp tropomyosin only presented comparable performance (ie, unremarkable differences in diagnostic properties including AUC values, sensitivity, specificity, LH ratio, and variable importance). Consistently, only 7 of 15 shrimp-allergic subjects showed positive basophil response to rPen m 1 at both tested concentrations. Taking together the relatively low IgE sensitization rate to shrimp tropomyosin similarly reported in Thai (34.2%) and Japanese (37%),[26,31] it is possible that tropomyosin might not be the major allergen in Asians. This finding challenged the suitability of tropomyosin as a diagnostic biomarker in these populations.

We noted that both the concentrations of protein extract used and the optimal decision point detected in this study were higher than prior BAT studies in which up to 1000 ng/mL allergen extract was often adopted with cutoff points of 4.78% to 17%.[12] However, these differences might be due to the fact that peanuts and tree nuts had higher basophil activation levels than other allergens.[32] However, we would like to emphasize that our BAT assay could accurately differentiate shrimp-allergic from shrimp-tolerant individuals with high sensitivity and specificity. Considering that our BAT was performed using a commercial kit

with shrimp extract and recombinant allergen being prepared by standardized methods, it would not be difficult for allergy groups in other populations to establish this diagnostic allergy test for shrimp allergy.

BAT results could indeed be interpreted by the absolute percentage of activated basophils, ratio of %CD63$^+$ basophil stimulated by allergen to that by anti-IgE (%CD63$^+$/anti-FcεRI), basophil threshold sensitivity (CD-sens), and effective dose at 50% of maximum dose response (EC50).[14] Santos et al[15] reported that %CD63$^+$/anti-FcεRI was the best basophil marker for predicting symptom severity in peanut allergy. In contrast to their report, we did not detect an improved diagnostic performance when BAT results were interpreted as %CD63$^+$/anti-FcεRI when compared with absolute percentage, with equal AUC values, Youden Indices, PPV, NPV, and LH (data not shown). This might be attributed to similar basophil responses between shrimp-allergic and -tolerant subjects on anti-IgE stimulation, which was different from findings in prior reports.[33] However, this study had not investigated the underlying mechanism behind this difference.

This study had several limitations. The EC50 values and CD-sens of BAT were not assessed due to the lack of the CD63 dose-response curve for all tested samples. Only tropomyosin, being the major shrimp allergen in published studies, was analyzed in this study, whereas a number of other clinically important allergenic components were identified.[34] Our findings revealed that tropomyosin only contributed to approximately 50% of all shrimp sensitization among allergic subjects. Thus, further studies should investigate shrimp-allergy diagnosis using other allergens, such as sarcoplasmic calcium-binding protein and myosin light chain that are associated with clinical reactivity to shrimp as well as arginine kinase and hemocyanin that denote cross-reactivity with tropomyosin.[25] Although BAT with shrimp extract may be a robust test, our present study only compared the magnitude of parameters such as AUC values and Youden Indices without making statistical comparison. Besides, our sample size calculation suggested that 30 participants would allow us to compare AUC values 0.7 to 0.9 for different diagnostic tests (see this article's Online Repository at www.jaci-inpractice.org). Nonetheless, this number might still be insufficient for other diagnostic parameters. Future studies need to recruit a larger sample size and test for more shrimp allergens in the diagnostic algorithms with conventional tests and cell-based functional IgE assays for shrimp allergy.

In conclusion, this is the first study to evaluate the diagnostic utility of BAT in shrimp-allergic patients as defined by DBPCFC. Our results demonstrate the superior diagnostic accuracy of BAT over conventional allergy tests. Further studies are needed to test the combinatorial approach of BAT with other shrimp allergens in the component-resolved diagnosis for shrimp allergy as well as the optimal algorithm with different biomarkers that can minimize the need for oral food challenges.

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ONLINE REPOSITORY

METHODS

Patient selection

Patients aged 5 to 50 years who exhibited documented history of immediate allergic reactions within 2 hours of shrimp consumption on at least 2 occasions over the past 5 years were recruited and assessed in Prince of Wales Hospital in Hong Kong. All patients were evaluated for shrimp IgE sensitization. Skin prick test was performed over patients' volar forearm with commercial shrimp extract (ALK-Abelló, Madrid, Spain) together with histamine (10 mg/mL) and normal saline as positive and negative controls, respectively. The results were read at 15 minutes; wheal sizes were measured and means calculated. The concentrations of total IgE and sIgE to shrimp (f24, a mixture of boiled and raw *Pandalus borealis*, *Penaeus monodon*, *Metapenaeopsis barbata*, and *Metapenaeus joyneri*) and rPen a 1 (f351) were measured by ImmunoCAP (ThermoFisher Scientific, Uppsala, Sweden). Patients were classified into allergic and tolerant groups based on results of double-blind, placebo-controlled food challenge (DBPCFC). The study protocol was approved by the Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee (2018.484) and Kowloon West Research Ethics Committee (127.12), and informed written consent was obtained from all recruited subjects and their parents if subjects were below 18 years old.

Sample size

Sample size was estimated using easyROC (version 1.3.1, Hacettepe University, Ankara, Turkey), which is a web-based tool for receiver operating characteristic curve analysis. A sample size of 30 participants would allow us to compare area under curve values between 0.7 and 0.9 for different diagnostic tests with 80% power and 0.05 type I error.

DBPCFC to shrimp

All subjects underwent DBPCFC for shrimp in the study site where full emergency equipment and drugs were available. DBPCFC was performed in accordance with the EuroPrevall protocol[E1] with slight modifications and under supervision by trained physicians and nurses. Exclusion criteria were intercurrent illness on the day of DBPCFC, and use of antiallergic medications before DBPCFC (eg, second-generation antihistamines within 120 hours and first-generation antihistamines within 72 hours). All subjects underwent full physical examination before each DBPCFC, including measurement of body weight, blood pressure, pulse, and peak expiratory flow. For patients with eczema, SCORing Atopic Dermatitis (SCORAD) as an indicator of their eczema severity was recorded. For DBPCFC, active and placebo provocations were randomly assigned by a dietitian and performed on 2 separate days at least 72 hours apart and within 2 weeks. Shrimp meat (*P. monodon*, black tiger shrimp) was blinded in chicken meat, dried oregano, dried onion, salt, ground black pepper, paprika, carrots, and corn starch. The raw burgers were stored at −20°C for up to 18 days as per previous microbiology tests (Figure E1). Seven blinded doses at 60 and 600 μg, 12 and 120 mg, as well as 1, 3, and 6 g of shrimp protein in shrimp burgers were oven-baked and administered at 20-minute intervals. The occurrence of allergic reactions during challenges were scored in accordance with the American Academy of Allergy, Asthma, and Immunology−European Academy of Allergy and Clinical Immunology PRACTALL consensus report.[E2] Briefly, challenges were discontinued and defined as positive with the occurrence of either objective reactions or persistent and severe subjective reactions during the procedure, within 120 minutes after the final dose or during open challenge. A negative DBPCFC was defined as the absence of objective allergic reaction. Subjects passing DBPCFC were invited for an open challenge with a cumulative dose of 100 g of shrimp meat. Subjects completing open challenge without any objective or significant subjective reactions were regarded as tolerant to shrimp.

Preparation of allergenic extracts and recombinant protein

Shrimp extract and recombinant shrimp tropomyosin were prepared according to standardized methods.[E3] Briefly, frozen black tiger prawn (*P. monodon*) was purchased from local supermarket and peeled shrimp meat was manually homogenized in phosphate-buffered saline (PBS) until a smooth paste was achieved. Protein was then extracted in PBS overnight at 4°C with constant stirring. The protein extract was centrifuged and supernatant was filter-sterilized through a 0.2 μm polyethersulfone membrane. Extract was stored at −20°C. Protein sequence of tropomyosin from *P. monodon* (Pen m 1) was downloaded from the Uniprot database (UniProt ID: A1KYZ2) and reverse translated by MEGA 7.0. The nucleotide sequence coding full-length Pen m 1 was synthesized and cloned into the His-tag expression vector pET30(a)+. His-tagged recombinant Pen m 1 (rPen m 1) was then expressed in *Escherichia coli* BL21 (DE3) (Invitrogen, Carlsbad, Calif) by culturing in MagicMedia (Invitrogen) and purified using the HisPur cobalt spin columns (Thermo Scientific, Rockford, Ill) as per the manufacturer's instructions. Protein concentration and purity of shrimp extract and recombinant Pen m 1 were determined by the bicinchoninic acid assay and on sodium dodecyl sulfate−polyacrylamide gel electrophoresis, respectively. Protein identity and conformation of recombinant Pen m 1 were also confirmed by mass spectrometry and circular dichroism, respectively.[E4,E5] The protein profile of the *P. monodon* extract was comparable with previous studies of the same shrimp species or other commonly edible shrimp species,[E3,E6,E7] whereas the purity of rPen m 1 was >95% (Figure E2) with its identity and conformation validated by mass spectrometry and circular dichroism.

Basophil activation test

In vitro basophil activation was quantified using the Flow CAST kit (BÜHLMANN Laboratories, Schönenbuch, Switzerland) as per the manufacturer's instructions. Basophil responses were quantified as percentage of $CD63^{pos}$basophils as gated in Figure E3, *A*, and were first tested against shrimp protein and rPen m 1 at concentrations ranging from 100 to 10,000 ng/mL on venous blood samples from 5 shrimp-allergic subjects to determine the optimal allergen concentrations to be used in the BAT assay. For high respondent, CD63 expression increased along with higher concentrations of shrimp protein and rPen m 1, and reaching a plateau at 5000 or 10,000 ng/mL (Figure E3, *B* and *E*). For other low-to-intermediate respondents, CD63 expression were detected from 5000 ng/mL of shrimp protein or rPen m 1 (Figure E3, *C* and *F*). Therefore, we used 5000 and 10,000 ng/mL of both shrimp protein and rPen m 1 as the doses for subsequent BAT analyses.

Ethylenediaminetetraacetic acid (EDTA)-anticoagulated venous blood was collected from subjects before DBPCFC. Each

reaction was prepared with 50 μL of allergen (protein extract from *P. monodon* as described above and rPen m 1) at predefined concentrations, 20 μL of staining reagent (anti-CD63-FITC and anti-CCR3-PE antibody mixture) and 50 μL of EDTA whole blood diluted in 100 μL of stimulation buffer containing heparin, CA^{2+} and IL-3 (2 ng/mL). Positive controls were prepared with anti-FcεRI monoclonal antibodies and anti-N-formyl-methionyl-leucyl-phenylalanine, respectively, whereas background reactions were assessed with 50 μL of stimulation buffer. The reaction mixtures were then incubated at 37°C in a water bath for 25 minutes. After the addition of lysis buffer to lyse erythrocytes and stop the stimulation followed by centrifugation, stained cells were acquired on the BD LSRFORTESSA flow cytometer with basophils gated as $CCR3^{pos}/SSC^{low}$. Upregulation of the basophil marker CD63 was calculated based on the percentage of $CD63^{+}$ cells compared with the total number of identified basophils. In each assay, a minimum of 300 events (ie, $CCR3^{pos}$ basophils) were recorded.

IgE crosslinking–induced luciferase expression (EXiLE) test

The human FcεRI-expressing rat mast cell line RS-ATL8 cells cotransfected with the luciferase reporter gene (kindly provided by Professor Ryosuke Nakamura) were maintained in complete Minimum Essential Media (MEM; supplemented with GlutaMAX-1, 10% heat-inactivated fotal bovine serum, 0.5 mg/mL geneticin, and 0.2 mg/mL hygromycin B). The EXiLE test was performed as described by Nakamura et al.[E8,E9] Briefly, the optimal concentration of allergen used in the EXiLE test was determined from a dose-response curve (Figure E3, *D* and *G*). Subsequently in the test, cells were plated onto a clear-bottom white 96-well plate at 5×10^4 cells/50 μL/well and incubated with individual patient serum at a final dilution of 1:100 overnight at 37°C in a CO_2 incubator. After decanting the supernatant and washing the sensitized cells with sterile Dulbecco's phosphate-buffered saline, cells were stimulated with a predetermined concentration of 100 ng/mL *P. monodon* protein extract or rPen m 1 diluted in complete MEM medium (50 μL/well) for 3 hours at 37°C in a CO_2 incubator. Wells with medium only were included as blank control, whereas cells without the addition of extract/allergen were included as nonstimulated control. At the end of the experiment, 50 μL of ONE-Glo luciferase substrate solution (Promega, Madison, Wis) containing cell lysis reagent was added to each well and the chemiluminescence signal was measured using the Victor X4 plate reader (Perkin Elmer, Waltham, Mass). The luciferase expression level was presented as the fold increase of light units calculated as follows: (stimulated cells − blank control)/(nonstimulated cells − blank control). Average readings of duplicate experiments were obtained for subsequent analysis.

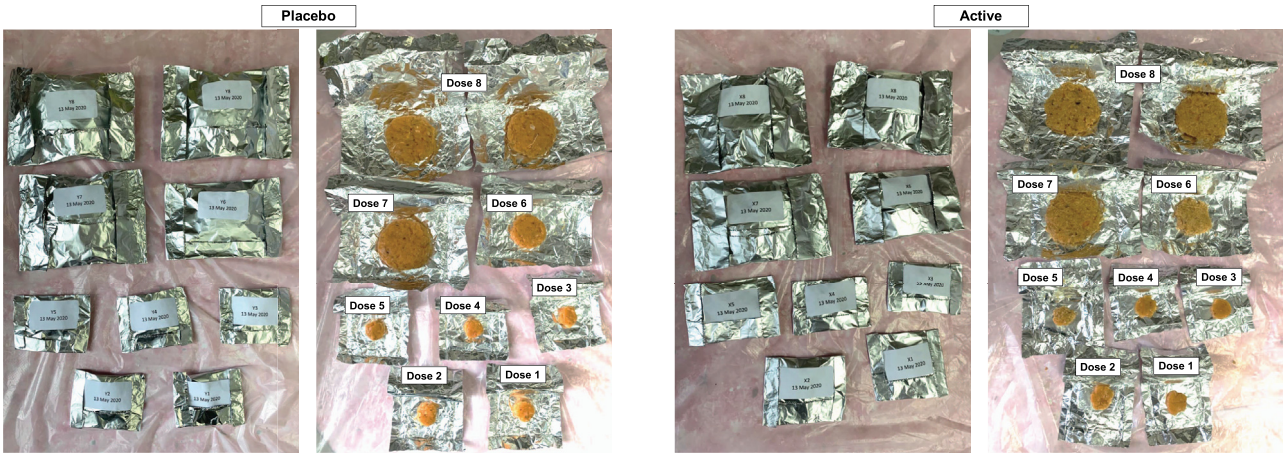

FIGURE E1. Photographs showing the visually indistinguishable placebo and shrimp burgers prepared for DBPCFC in accordance with the EuroPrevall protocol. *DBPCFC*, Double-blind, placebo-controlled food challenge.

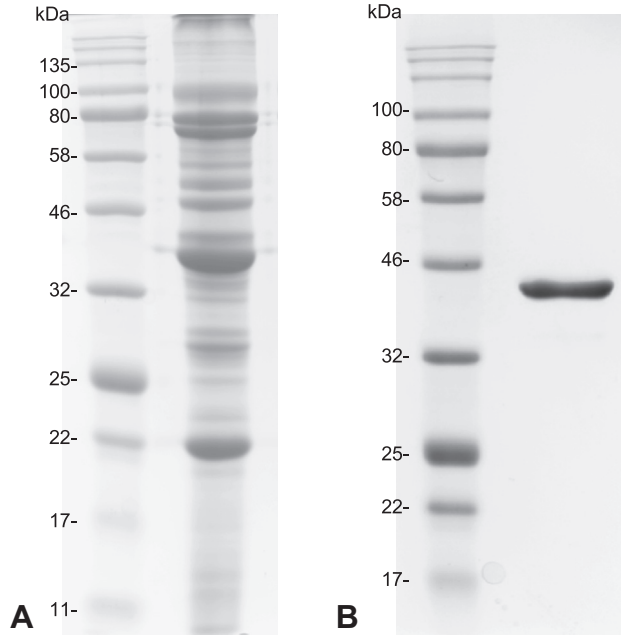

FIGURE E2. SDS-PAGE images. SDS-PAGE of (**A**) *Penaeus monodon* extract and (**B**) purified recombinant tropomyosin (*Pen m* 1). Lane 1, molecular weight markers. The contents of loaded protein were 10 μg. SDS-PAGE was stained with Coomassie blue. *SDS-PAGE*, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

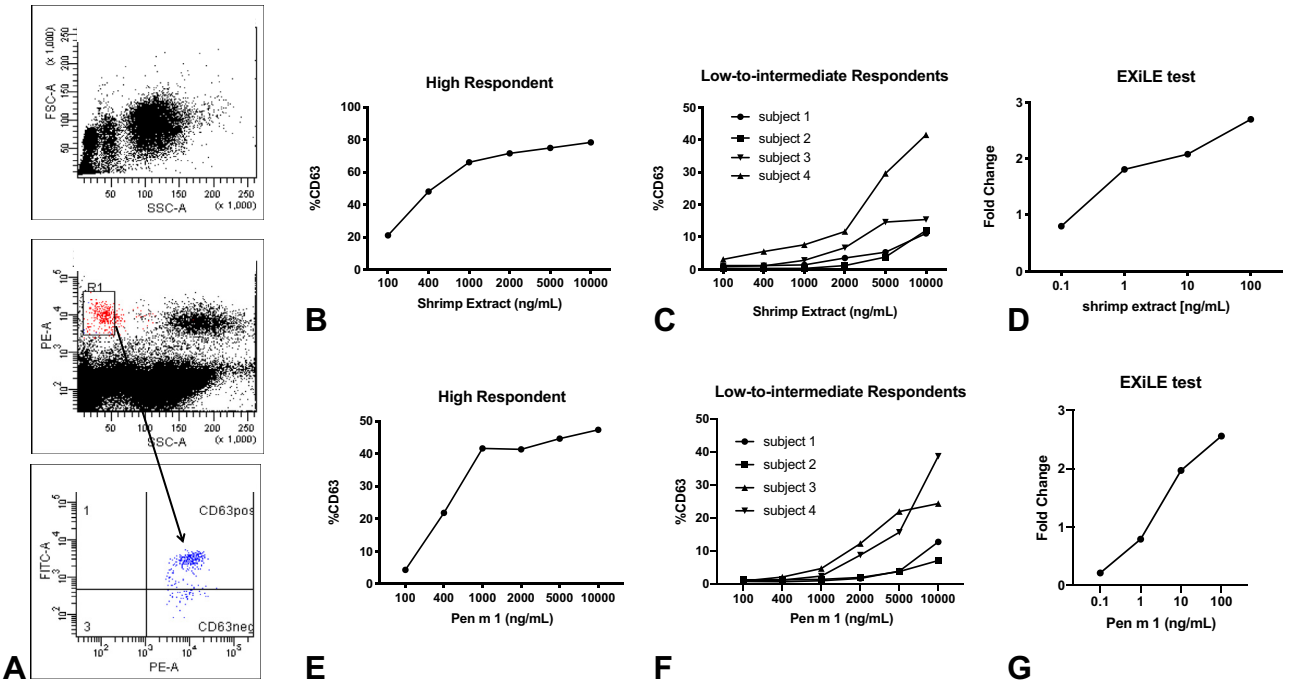

FIGURE E3. Optimization of BAT and EXiLE test. **A**, Flow cytometry gating strategy for basophils. Cells were first gated as PE-CCR3-positive and SSC-low (gate R1), followed by counting the number of FITC-CD63-positive basophils. At least 300 events were counted per sample. Dose-response curves of basophil activity against shrimp extract in shrimp-allergic subjects with (**B**) high and (**C**) low-to-intermediate responses. Each line represents the dose-response to shrimp extract (100-10,000 ng/mL) of an individual patient. **D**, Dose-response curve of luciferase expression in the EXiLE test for shrimp extract. A 1:100 diluted serum pool (sera from 12 shrimp-allergic subjects) was stimulated with 0.1-100 ng/mL of shrimp extract. Dose-response curves of basophil activity against *Pen m* 1 in shrimp-allergic subjects with (**E**) high and (**F**) low-to-intermediate responses. Each line represents the dose-response to shrimp extract (100-10,000 ng/mL) of an individual patient. (**G**) Dose-response curve of luciferase expression in the EXiLE test for *Pen m* 1. A 1:100 diluted serum pool (sera from 12 shrimp-allergic subjects) was stimulated with 0.1-100 ng/mL of shrimp extract. *BAT*, Basophil activation test; *EXiLE*, IgE crosslinking—induced luciferase expression; *FITC*, fluorescein isothiocyanate; *FSC*, forward scatter; *PE*, phycoerythrin; *SSC*, side scatter.

TABLE E1. Details of subjective and objective symptoms at each dose during DBPCFC and open challenge for shrimp-allergic subjects (n = 15)

			Dose	2	3	4	5	6	7	8
Subject	Sex	Age	Provocation	60 μg	600 μg	12 mg	120 mg	1 g	3 g	6 g
1	M	25	S		IB-1	IB-1		IC-1	IIA-3	IB-2 + IVA-1
			P							
2	M	44	S			ID-1	ID-1	ID-1		
			P							
3	M	28	S	IB-1 + IC-1	IB-2 + IIIB-1		IB-2	IB-2	IB-2	IIA-3 + IB-2 + IVA-1
			P			IB-1	IB-1	IB-1		
4	M	34	S	IB-1 + IC-1	IB-1	IC-1		IVA-1	IVA-1	IVA-1
			P							IB-1
5	M	22	S	IB-1 + ID-1 + IVA-1	IB-1 + ID-1+IVA-1	ID-1 + IVA-1	IB-2 + IVA-1	IC-1 + IIA-1 + IVA-1	IC-1 + IIA-2+ IVA-1	
			P							
6	F	35	S							
			P	IB-1						
7	M	17	S							
			P							
8	M	32	S	IB-1	IB-1	IC-1	IB-1 + IIA-3	IB-1 + IIB-3	IB-1	IIB-3
			P	IB-1	IB-1					
9	F	40	S	IVA-1	IVA-1	IVA-1	IVA-1 + IIB-1	IVA-1	IVA-1	
			P		IVA-1					
10	F	5	S							
			P					IB-1	IB-1	IB-1
11	M	13	S				IVA-1 + IIIB-1	IC-2 + IVA-2+IIIB-1	IC-2 + IVA-2 (X)	
			P				IB-1 + ID-1	ID-1	ID-1	ID-1
12	F	31	S	IB-1 + ID-1	ID-1		ID-1	ID-1	IVA-1	IB-1+IC-2+IIIA-3
			P	IB-1	IB-1	IB-1	IB-1	IB-1	IB-1	IB-1
13	M	18	S		IB-1	IB-1 + IC-1	IB-1 + IC-1	IC-2 + IIA-3 X		
			P	IB-1	IB-1	IB-1	IB-1	IB-1	IB-1	IB-1
14	M	19	S				IB-1			
			P							
15	M	7	S			IB-2	IB-1	IB-1 + IC-2	IB-1 + IC-2	IB-1 + IC-2
			P	IB-1		IB-1				

DBPCFC, Double-blind, placebo-controlled food challenge; *OFC*, oral food challenge; *P*, placebo provocation in DBPCFC; *S*, shrimp active provocation in DBPCFC.Symptom scores were calculated as described by Sampson et al.[E2] Skin, upper and lower respiratory, gastrointestinal, and cardiovascular/neurological allergic responses were monitored and assigned scores 0-3 based on symptom severity (0: absent; 1: mild; 2: moderate; and 3: severe). Cumulative score was the sum of the highest scores under each reaction category observed throughout the course of an OFC. The placebo effect was accounted by subtracting the active score by the placebo score; an adjusted score of $\geq$3 was defined as positive.

TABLE E1. CONTINUED

			I. Skin		II. Upper respiratory		III. Lower respiratory		IV. Gastrointestinal		V. Cardio-vascular/neurological		
Open 100 g	Score	OFC score	B. Pruritus	C. Urticaria	C. Lip angioedema	D. Rash	A. Periocular swelling	B. Sniffing/ DOB	A. Wheezing	B. Laryngeal	A. Itchy mouth	B. Nausea	A. Headache
	6	6	X	X			X				X		
	0												
IIA-3 + IIIB-1 +IVA-1	6	6				X	X			X	X		
	0												
	8	7	X	X			X			X	X		
	1												
IB-1 + ID-1 + IVA-1	4	3	X	X		X		X			X		
	1												
	7	7	X	X		X		X			X		
	0												
IB-1 + IC-2 + ID-1	4	3	X	X		X							
	1												
IB-1 + IC-3	4	4	X	X	X								
	0												
	5	4	X	X			X	X					
	1												
IC-2	4	3		X							X		
	1												
IB-2 + IC-2 + IIB-2 + IVB-2	8	7	X	X	X			X				X	
	1												
	5	3			X					X	X		
	2												
	7	6	X	X	X	X	X				X		
	1												
	6	5	X		X		X						
	1												
IB-1 + IC-2 + IVA-1	4	4	X		X						X		
	0												
IB-1 + IVA-1	5	4	X	X							X		
	1												

TABLE E2. Details of subjective and objective symptoms at each dose during DBPCFC and open challenge for the shrimp-tolerant subjects (n = 20)

			Dose	2	3	4	5	6	7	8			
Subject	Sex	Age	Provocation	60 μg	600 μg	12 mg	120 mg	1 g	3 g	6 g	Open 100 g	Score	OFC score
1	M	49	S				IIIB-1					1	1
			P									0	
2	F	32	S									0	0
			P									0	
3	M	27	S									0	0
			P									0	
4	F	40	S								IB-1 + ID-1 + IC-1	3	2
			P							IC-1		1	
5	F	19	S				IC-1 + IIIB-1	IB1 + IC-1	IB1 + IC-1	IB1 + IIIB-1	IIIB0-1	3	1
			P			IB-1 + ID-1		ID-1	ID-1			2	
6	F	35	S				IIIB-1					1	0
			P	IB-1								1	
7	M	14	S	IB-1						IB-2 + ID-1		3	0
			P		IB-2	IB-2	IC-1	IB-2				3	
8	F	10	S									0	−2
			P	IB-1 + ID-1						IB-1 + IC-1		2	
9	M	39	S									0	0
			P									0	
10	M	46	S								IVA-1	1	−2
			P		IB-1 + IVA-1		IIIB-1	IIIB-1	IIIB-1	IIIB-1		3	
11	F	15	S	IVA-1 + IC-1	IB-1	IB-1	IB-1	IB-1	IB-1	IC-1 + IB-1	IC-1 + IVA-1	3	0
			P	IVA-1	IB-1 + IVA-1	ID-1	IVA-1	IVA-1	IB-1 + IC-1	IB-1		3	
12	M	31	S							IC-1	IB-2+IC-2	4	1
			P		IA-0	IA-0	IB-1	IB-1 + IC-2	IA-0 + IB-2	IB-1		3	
13	F	18	S	IVA-1						IB-1 + IC-1		2	0
			P	IVA-1					IC-1	IVA-1		2	
14	F	30	S	ID-1	ID-1	ID-1	ID-1	ID-1	ID-1	ID-1		1	−1
			P	ID-1	IVA-1	IVA-1	IVA-1	IVA-1	IVA-1	IVA-1		2	
15	F	14	S									0	0
			P									0	
16	F	39	S				IB-1	1B-1		ID-1	IB-1 + ID-1	2	1
			P					ID-1				1	
17	M	36	S	IB-1 + IC-1	IB-1 + IC-1	IB-1 + IC-1	IB-1 + IC-1	IB-1 + IC-1	IB-1 + IC-1	IB-1 + IC-1	IB-1	2	0
			P	IB-1 + ID-1	IB-1 + ID-1							2	
18	M	24	S		IB-1	IB-1	IB-1	IB-1	IB-1	IB-1	IB-1 + IVA-1	2	1
			P		IB-1	IB-1						1	

(continued)

TABLE E2. *(Continued)*

			Dose	2	3	4	5	6	7	8			
Subject	Sex	Age	Provocation	60 μg	600 μg	12 mg	120 mg	1 g	3 g	6 g	Open 100 g	Score	OFC score
19	F	34	S	ID-1 + IIIB-1	IIIB-1	IIIB-1	IIIB-1	IIIB-1	IIIB-1	IC-1 + IIIB-1	IIIB-1	3	1
			P			ID-1	IB-1 + ID-1					2	
20	M	9	S		V-1	V-1	IB-1	IB-1	IB-1	IB-1		2	0
			P			IB-1 + ID-1	IB-1 + ID-1					2	

DBPCFC, Double-blind, placebo-controlled food challenge; *OFC*, oral food challenge; *P*, placebo provocation in DBPCFC; *S*, shrimp active provocation in DBPCFC.

Symptom scores were calculated as described by Sampson et al.[E2] Skin, upper and lower respiratory, gastrointestinal, and cardiovascular/neurological allergic responses were monitored and assigned scores 0-3 based on symptom severity (0: absent; 1: mild; 2: moderate; and 3: severe). Cumulative score was the sum of the highest scores under each reaction category observed throughout the course of an OFC. The placebo effect was accounted by subtracting the active score by the placebo score. All tolerant subjects present an adjusted symptom score of <2 and were defined as negative.

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