SHRIMP ALLERGY: ANALYSIS OF COMMERCIALLY AVAILABLE EXTRACTS FOR IN-VIVO DIAGNOSIS

Short title: Analysis of commercial shrimp extracts for SPT

Asero R (1), Scala E (2), Villalta D (3), Pravettoni V (4), Arena A (5), Billeri L (6), Colombo G (7), Cortellini (8), Cucinelli F (9), De Cristofaro ML (10), Farioli L (11) Iemoli E (12), Lodi Rizzini F (13), Longo R (14), Losappio L (15), Macchia D (16), Maietta G (17), Minale P (18), Murzilli F (9), Nebiolo F (19), Pastorello EA (15), Ventura MT (20), Voltolini (18), Amato S (21), Mistrello G (21).

1) Ambulatorio di Allergologia, Clinica San Carlo, Paderno Dugnano (MI), Italy

2) Istituto Dermopatico dell'Immacolata – IRCCS, Roma, Italy

3) SSD di Allergologia e Immunologia Clinica, Azienda Ospedaliera S. Maria degli Angeli, Pordenone, Italy

4) UOC Clinical Allergy and Immunology, IRCCS Foundation Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy.

5) Ambulatorio Allergologia, Azienda Usl 5 di Messina, Italy

6) Department of Laboratory Medicine, University Hospital, Padova, Italy

7) Allergy and Clinical Immunology Unit, IRCCS San Raffaele Hospital, Milan, Italy

8) UO di Medicina Interna e Reumatologia, Azienda Sanitaria della Romagna, Rimini, Italia.

9) U.O.S.D. di Allergologia, Ospedale SS. Filippo e Nicola di Avezzano (AQ), Italy

10) Ambulatorio di Allergologia, Ospedale San Timoteo, Termoli (CB), Italy

11) Department of Laboratory Medicine, ASST Grande Ospedale Metropolitano Niguarda, Milan, Italy

12) Allergy and Clinical Immunology Unit, ASST Fatebenefratelli/Sacco, Milano, Italy

13) S.S.V.D. Allergologia, A.O. Spedali Civili di Brescia, Italy

14) Ambulatorio Territoriale di Allergologia, ASP Vibo Valentia, Italy

15) Department of Allergology and Immunology, ASST Grande Ospedale Metropolitano Niguarda, Milan, Italy

16) Allergologia Immunologia Clinica, P.O. S. Giovanni di Dio, Firenze, Italy

17) Ambulatorio di Allergologia Accreditato ASL, Lecce, Italy

18) U.O.C. Allergologia IRCCS San Martino-IST, Genova, Italy

19) Ambulatorio di Allergologia e Immunologia, AO Ordine Mauriziano, Torino, Italy

20) Dipartimento di Medicina Interna, Immunologia e Malattie Infettive, Università di Bari, Policlinico, Bari, Italy.

21) R & D, Lofarma, Milano, Italy

Conflict of Interest: The authors declare the absence of any conflict of interest

Financial sources: None to declare.

Corresponding Author:

Dr Riccardo Asero, Ambulatorio di Allergologia, Clinica San Carlo

Via Ospedale 21, 20037 Paderno Dugnano (MI), Italia

e-mail: r.asero@libero.it

ABSTRACT

Background: SPT with commercial extracts represent the first step of the diagnosis of shrimp allergy but their clinical efficiency is undefined.

Objective: To analyze the clinical usefulness of all commercial extracts of crustaceans for SPT available in Italy.

Methods: One hundred fifty-seven shrimp-allergic patients underwent SPT with five commercial extracts of crustaceans and with house dust mite (HDM) extract in a multicenter study. Commercial extracts were analyzed by SDS-PAGE and compared with a freshly prepared in house shrimp extract. IgE to Pen a 1/Pen m 1; Pen m 2, and Pen m 4 were detected and immunoblot analysis was carried on a large number of sera.

Results: Commercial crustaceans extracts gave extremely inhomogeneous skin reactions resulting in 32 different clinical profiles, showed marked differences in protein content, and sometimes lacked proteins at molecular weights corresponding to those of major shrimp allergens. Only strong Pen a 1/Pen m 1 reactors reacted to both HDM and all 5 commercial extracts on SPT. Most patients, including tropomyosin-negative ones, reacted to HDM. Patients reacted to a variable and large array of proteins and IgE reactivity at high molecular weights (> 50 kDa) was frequently detected.

Conclusions: The in-vivo diagnosis of shrimp allergy must be still based on SPT with fresh material. Shrimp-allergic patients frequently react to a numberof ill-defined high molecular weight allergens which makes currently available molecules for the component-resolved diagnosis largely insufficient. Mites and crustaceans probably share several allergens other than tropomyosin.

KEY WORDS: Allergens; Allergy diagnosis; Food allergy; Shrimp allergy; Skin testing.

RESUMEN

Introducción: Las pruebas cutáneas con extractos comerciales representan el primer paso en el diagnóstico de alergia a gamba, si bien, su eficacia clínica no está bien definida.

Objetivos: El objetivo de este estudio fue analizar la utilidad clínica de todos los extractos comerciales disponibles en Italia frente a crustáceos en pruebas cutáneas.

Métodos: En un estudio multicéntrico, se incluyeron 157 pacientes alérgicos a gamba a los que se realizaron pruebas cutáneas con cinco extractos comerciales de crustáceos y con ácaros del polvo doméstico. Los extractos comerciales fueron analizados mediante SDS-PAGE y comparados con un extracto de gamba preparado en fresco. Se determinó IgE frente a Pen a 1/Pen m 1; Pen m 2, y Pen m 4; y el análisis mediante inmunoblotting se realizó en un amplio número de sueros.

Resultados: Los extractos de gamba comercializados dieron lugar a reacciones cutáneas muy poco homogéneas en 32 perfiles clínicos diferentes; así mismo, mostraron grandes diferencias en contenido proteico y, en algunos casos, a falta de proteína a pesos moleculares correspondientes a alérgenos mayoritarios de gamba. Únicamente los reactores más fuertes a Pen a1 /Pen m 1 reaccionaron tanto a ácaros del polvo de casa como a los cinco extractos comerciales en pruebas cutáneas. La mayoría de los pacientes, incluyendo los negativos a tropomiosina, reaccionaron a los ácaros del polvo. Los pacientes reaccionaron a un amplio y variable array de proteínas y se detectó con frecuencia reactividad de IgE en pesos moleculares altos (>50 kDa).

Conclusiones: El diagnóstico in vivo de alergia a gamba todavía debe estar basado en pruebas cutáneas prick with producto fresco. Los pacientes alérgicos a gamba a menudo reaccionan a un número de alérgenos de peso molecular alto poco definido lo que hace que las moléculas disponibles hoy en día para el diagnóstico por componentes sean muy insuficiente. Ácaros y crustáceos probablemente comparten varios alérgenos además de la tropiomiosina.

PALABRAS CLAVE: Alérgenos. Diagnóstico alergológico. Alergia alimentaria. Alergia a gamba. Pruebas cutáneas.

INTRODUCTION

Crustaceans are one of the most prevalent causes of food allergy worldwide including Italy where they represents the second cause of type 1 food allergy after plant foods [1] and the second cause of food-induced anaphylaxis after lipid transfer protein, particularly in adults [2]. The complexity of the allergenic profile of shrimp and other crustaceans has been increasingly recognized over the last 10 years. The muscle protein tropomyosin has been the first shrimp allergen detected [3-5]. Its presence in a conserved form throughout invertebrates family has led to consider it as a panallergen [5-7]. Tropomyosin (Pen m 1/Pen a 1; m.w. 38-41 kDa) has been considered as the major shrimp allergen ever since although recent multicenter studies carried out in Italy found that less than 50% of shrimp allergic patients from this geographic area react to this allergen [8]. Several shrimp allergens other than tropomyosin have been detected and characterized in recent years. Some of them, namely arginine kinase (Pen m 2; 40 kDa) [9, 10] and sarcoplasmic calcium binding protein (Pen m 4; 20-22 kDa) [11,12] are currently available for in-vitro diagnosis, whereas others such as myosin light chain (Lit v 3; 20 kDa) [13] are not. The number of shrimp allergens that have been characterized has been recently increased by hemocyanin (75 kDa) [14], troponin C (Cra c 6; 21 kDa), triose phosphate isomerase (Cra c 8; 28 kDa), paramyosin (100 kDa) [15], myosin heavy chain (225 kDa), alpha-actin (31-42 kDa), smooth endoplasmic reticulum Calcium ATPase (113 kDa), glyceraldehyde phosphate dehydrogenase (37 kDa) (reviewed in [16]), pyruvate kinase, thioredoxin, and enolase, but it is very likely that the spectrum of crustacean allergens is much wider, both in the low [17,18] and the high molecular weight range [8,14].

In the clinical practice the diagnosis of crustaceans allergy is based on clinical history, skin prick tests (SPT) with either fresh food or commercial extracts, serum specific IgE, and (where possible) oral food challenges. While the clinical diagnosis of shrimp allergy is virtually always possible by the use of a combination of the tools listed above, only 3 shrimp allergens are currently available for component-resolved diagnosis of this condition, all of which on the ISAC microarray platform (Pen m 1, Pen m 2, Pen m 4) (Thermo Fisher Scientific), and only tropmyosin IgE can be measured by the singleplex ImmunoCAP (Pen a 1) (Thermo Fisher Scientific). Very little is known about the sensitivity of commercial extracts for SPT that are commonly used as a first step for in-vivo diagnosis of shrimp allergy by virtually the totality of clinical allergologists. In the present multicenter study the sensitivity of all commercial shrimp extracts for SPT available in Italy was analyzed in a large group of shrimp-allergic patients.

MATERIALS AND METHODS

Patients

Eighteen allergy centers scattered throughout Italy participated to this multicenter study (no patients were supplied by the Centre #4 which participated in writing the paper). Participating doctors enrolled all the

doi: 10.18176/jiaci.0127

shrimp allergic patients routinely diagnosed during 2015 by the following criteria: 1) unequivocal clinical history of allergy to shrimp (either oral allergy syndrome, urticaria with or without angioedema, asthma, or anaphylaxis); and 2) unequivocally positive skin test with fresh shrimp by the prick-prick technique as defined by the EAACI criteria [19]. Since virtually only seawater shrimps are consumed in Italy, in view of the possible allergenic differences with freshwater shrimps [20], only seawater animals belonging to the *Penaeides* family (*Aristeus antennatus, Parapenaeus longirostris, Parapeneopsis cornuta, Melicertus kerathurum*) were employed to carry out SPT with fresh shrimp and to prepare the extracts for in-vitro analyses.

All patients provided a written informed consent to the participation to the study. Blood samples were taken from all subjects; after centrifugation, properly coded sera were kept at -20°C until their use in in-vitro analyses.

Skin tests

All patients underwent SPT with all five commercial extracts of crustaceans currently available in Italy, namely ALK-Abellò, Madrid, Spain (shrimp), Stallergenes, Anthony, France (shrimp), Lofarma, Milano, Italy (lobster), Allergopharma, Reinbeck, Germany (shrimp), and Anallergo, Firenze, Italy ("crustaceans"). These skin tests were carried out in parallel in every single patient, and read as previously described [19]. SPTs with commercial house dust mite extracts (both *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*) were carried out as well. Clinically relevant mite-induced respiratory allergy was not recorded in this study.

In vitro analyses

Commercial extracts for SPT were dialyzed overnight at 4°C against PBS buffer to eliminate glycerol. Raw seawater shrimps were homogenized and extracted (5%) in 0.1 M phosphate-buffered saline (PBS), pH 7.4 under shaking for 2 h at 4°C. The protein content measured after Bradford [21] was 1,2 mg/ml. The extract was divided into aliquots and stored freeze-dried at -20°C until use. An SDS PAGE of the shrimp extract as well as of the commercial extracts for SPT was carried out.

Patients' sera underwent immunoblot (IB) analysis at Lofarma Laboratories (Milan, Italy). Immunoblots were carried out under reducing and non-reducing conditions. The extract was mixed with LDS sample buffer (Nupage Bis-Tris; Novex, Prodotti Gianni, Milano, Italy) and 5% β -mercaptoethanol (without β-mercaptoethanol in experiments carried out under non-reducing conditions). The samples were then denaturized by heating at 100°C for 10 min. Electrophoresis of shrimp extracts (25 µg/lane) was carried out in a 10% polyacrylamide precast gel (Nupage Bis-Tris, Novex, Prodotti Gianni, Milano, Italy) at 180 mA for 1

doi: 10.18176/jiaci.0127

h. The SDS-PAGE gel was stained with Comassie colloidal blue according to manufacturer's instructions (Invitrogen, Milan, Italy). In IB analysis the resolved proteins were transferred for 1 h onto a nitrocellulose membrane according to Towbin et al [22]. The membrane was saturated with 0,1 mol/L tris-buffered saline containing 5% fat free milk powder and incubated for 16 h at 4°C with patients' sera (diluted 1:10 in saturation buffer). After 3 washings bound specific IgE were detected by peroxidase-conjugated anti-human IgE antibodies from goat (1:5000 in saturation buffer; Biospacific, CA, USA) and using an ECM western blotting kit (Amersham, Milano, Italy) as substrate.

Shrimp tropomyosin specific IgE were measured in a proportion of patients by ImmunoCAP (Pen a 1; Thermo Fisher Scientific; Uppsala, Sweden) whereas in other patients IgE to Pen m 1; Pen m 2, and Pen m 4 were detected by ImmunoCAP-ISAC 112 microarray (Thermo Fisher) platform following manufacturer's recommendations. Results were expressed in KUA/L or ISU-E/L, respectively, and levels < 0,1 and < 0,3, respectively, were considered negative.

Statistics

Pearson's Chi squared test or Fisher's exact test (used for two-by-two contingency tables with less than 50 cases) were used to assess if different allergen profiles and IgE specific for Pen m 1, Pen m 2, and Pen m 4, were independent of each other. The associations between independent covariates and dependent variables were further analyzed by binary logistic regression (Spearman test). Probability values less than 5% were considered statistically significant.

Ethics

The study was approved by the reference Ethical Committee of the leading center (Clinica San Carlo, Paderno Dugnano) with the code 306-052015.

RESULTS

One hundred-fifty-seven (M/F: 79/78; mean age 36,3 years, range 5-69) shrimp-allergic patients were eventually enrolled by the participating centers. Shrimp ingestion induced local symptoms (oral allergy syndrome/angioedema of the lips and/or pharynx [n=40], gastrointestinal symptoms [n= 7], and isolated dyspnea [n=3]) as well as systemic symptoms (urticaria/angioedema [n=87], anaphylaxis [n=16], food-dependent exercise-induced anaphylaxis [n=4]).

Skin tests

One hundred forty-five/157 (92%) patients scored positive on SPT with at least one of the five commercial extracts of crustaceans which, however, gave extremely inhomogeneous results, and 132/157 (84%) patients scored positive on SPT with commercial house dust mite extract. Combining the results of SPT with mite and crustaceans extracts in the study population 32 different allergenic profiles were detected (Table 1). These were grouped as follows: A= HDM positive/All SPT negative; B= HDM positive/All SPT positive; C= HDM negative/All SPT positive; D=HDM negative/All SPT negative; E= HDM positive/SPT partially positive; F= HDM negative/SPT partially positive.

SDS-PAGE

The SDS-PAGE profiles of both the five commercially available extracts of crustaceans for SPT and the freshly prepared shrimp extract used to carry out the immunoblot analyses are shown in figure 1. The fresh extract showed a large number of protein bands whereas commercial extracts of crustaceans showed marked differences one from the other, with a frequent lack of proteins at molecular weights corresponding to those of the major shrimp allergens. Commercial extract #2 appeared as the one containing the largest number of proteins on SDS-PAGE.

Specific IgE measurements

In 72 patients the doctors measured specific IgE to Pen a 1/Pen m 1; 41 (57%) of them scored positive. Plotting these results against the allergenic profile of the corresponding patients a clear association between IgE reactivity to tropomyosin and allergenic profile B (skin reactivity to HDM and all 5 commercial extracts of crustaceans) appeared ($p< 0,001$; table 2). Analyzing the effect of specific IgE levels in the 23 Pen m 1-monosensitized patients (defined as Pen m 1-pos; Pen m 2-neg; Pen m 4-neg) on the allergenic profile obtained by SPT with commercial extracts of crustaceans, it was found that the 16 subjects showing low IgE levels (arbitrarily defined as < 2.0 ISU/L) showed a variable allergenic profile including profile B (n= 8), E20

(n= 2), C, E6, E17, E19, and E21 (n=1 each), whereas all 7 strong responders showing IgE levels > 2.0 ISU/L showed a profile B. Interestingly, 24/31 (77%) patients that did not show Pen a 1/Pen m 1 hypersensitivity scored positive on SPT with HDM. SPT with extract #2, notably the commercial extract showing the highest number of protein bands on SDS-PAGE (figure 1), scored positive in patients showing 7 of the 14 (50%) profiles negative for tropomyosin hypersensitivity (table 2). Such proportion for the other commercial extracts was 5/14 (commercial extracts # 1 and #5), 4/14 (extract #3), and 3/14 (extract #4).

IgE to Pen m 2 and Pen m 4 were measured by ISAC microarray in 46 patients. IgE reactivity to Pen m 2 and Pen m 4 was detected in 11 and 8 cases respectively; two patients showed a Pen m 2/Pen m 4 co-sensitization. Although the results, plotted against the different allergenic profiles, showed that in many cases the two allergens were recognized by patients showing the allergenic profile B (i.e., the profile associated with tropomyosin hypersensitivity), a statistically significant association between profile E19 (i.e., skin reactivity to HDM and all commercial extracts of crustaceans but #1) and Pen m 2 hypersensitivity was detected ($p < 0.05$) (table 3). In effect, all patients monosensitized to Pen m 2 (i.e. Pen m 2+, Pen m 1-Pen m 4-) showed an E19 profile. The two patients monosensitized to Pen m 4 (i.e. Pen m 4+, Pen m 1, Pen m 2-) showed an E19 and F7 profile, respectively. Interestingly, one Pen m 4 "monoreactor" (F7 profile) scored positive on SPT with extracts # 3, 4 and 5, suggesting the presence of such molecule only in some commercial extracts.

Analysis of SPT extracts in the light of specific IgE measurements

SPT with commercial extract #2 scored negative in patients showing weak Pen m 1 monoreactivity (profile E 20 in table 1). SPT with commercial extract #1 scored negative in patients monosensitized to Pen m 4 or Pen m 2 (profile E19, Table 1) and only weakly positive in one patient reactive to both these allergens but not to Pen m 1 (profile E17). However, it has to be considered that most patients showed low specific IgE levels. In two strong Pen m 2 reactors (18,5 and 25,0 ISU/L, respectively, both belonging to profile B) SPT with extract #1 scored positive.

Four patients did not react to Pen m 1, Pen m 2, and Pen m 4 on ISAC microarray; on SPT, three scored positive with commercial extract #2 (in one case alone [profile E 12, table 1], and in two cases associated with weak reactivity to extracts #1 and #5, respectively [profiles E5 and E2, table 1]), whereas in one case all commercial extracts scored negative (profile A, table 1).

Immunoblot analysis

Sera from 59 patients underwent immunoblot analysis. As expected, IgE reactivity showed much variability from one patient to another (figure 2). No differences were observed between immunoblots carried out

doi: 10.18176/jiaci.0127

using the same sera under reducing and non-reducing conditions (data not shown). Results are summarized in table 4 where they are plotted against the allergenic profiles observed by SPT to HDM and commercial extracts of crustaceans. A highly significant association was found between profile B and IgE reactivity at 35 kDa (presumably tropomyosin) ($p< 0.001$). Notably, a large number of patients' sera showed IgE reactivity at high molecular weights (> 50 kDa), whereas no serum appeared to react at 40 kDa, the molecular weight of shrimp arginine kinase (Pen m 2).

IgE reactivity at 20 kDa on immunoblot analysis was detected in 5/8 Pen m 4 reactors, whereas the remaining 3 sera scored negative.

DISCUSSION

Skin prick tests with commercial allergenic extracts are the mainstay of clinical allergy diagnosis, and are used virtually in all clinical settings dealing with allergic disorders. Crustaceans are one of the most frequent causes of food allergy, and this study investigated for the first time the sensitivity of a series of different commercial extracts of shrimp/lobster. To this end all five commercial extracts currently available in Italy for the diagnosis of allergy to crustaceans were analyzed for their protein content by SDS-PAGE and used to perform SPT in more than 150 shrimp-allergic patients. Further, sera from a large number of study patients underwent the detection of IgE to all currently available shrimp allergens, namely Pen a 1/Pen m 1, Pen m 2, and Pen m 4, and immunoblot analysis in order to assess their IgE reactivity profile. Finally, house dust mite (both *Dermatophagoides pteronissynus* and *farinae*) hypersensitivity was detected in the whole study population. In-vivo tests produced an extremely complex picture, including 32 different profiles that were combined with the results of in-vitro studies.

There are several aspects of this study that deserve to be discussed. One impressive finding was the high proportion of HDM-hypersensitive patients (84% of the study population) that changed very little (77%) in patients that did not react to Pen a 1/Pen m 1, suggesting that mites and crustaceans might share also allergens other than tropomyosin. In effect, in a previous study [8] we were able to demonstrate the allergenic cross-reactivity between high molecular weight allergens in shrimp and mites; further, as long as 15 years ago Binder et al. reported the cross reactive nature of arginine kinase in different invertebrates [23]. Another interesting point is that tropomyosin hypersensitivity did not correlate fully with HDM hypersensitivity; in fact more than 10% of Pen m 1 reactors were negative on SPT with house dust mites (Table 3). Whether this depends on a low concentration of Der p 10, the HDM tropomyosin, in commercial mite extracts for SPT or to allergenic differences between tropomyosins from different invertebrates has to be established. Anyway, this study shows that a negative SPT with HDM in a shrimp-allergic patient does not rule out tropomyosin hypersensitivity.

Surprisingly enough, in comparison with the fresh shrimp extract, commercial extracts for SPT showed a dramatic loss of protein bands which was in some cases so pronounced to suggest that the diagnosis of shrimp hypersensitivity by the use of certain extracts would have been severely hampered. In effect, the commercial extract that showed the poorest protein profile on SDS-PAGE (extract #4) was the one that scored negative in the largest proportion (> 40 %) of allergic patients.

In view of the large differences in protein content of commercial extracts it is not surprising that SPTs eventually produced so many different allergenic profiles with all possible combinations ranging from “all negative” to “all positive”. Interestingly, although some extracts appeared to lack proteins at about 35 kDa, the molecular weight of tropomyosin, all of them gave positive results in subjects showing strong hypersensitivity to Pen m 1. Some commercial extracts seemed to lack specific allergens; for instance, extract #1 showed low sensitivity in the detection of Pen m 2 hypersensitivity. However, this was the case also for the immunoblot analysis that was carried out using a fresh, in-house shrimp extract. Since the commercial extract #1 and fresh shrimp extract for immunoblot were prepared by the same laboratories one might hypothesize that certain specific features of extraction procedures may lead to the loss of arginine kinase in the final product. Further studies are needed to clarify this point.

The immunoblot analysis confirmed that shrimp allergic patients frequently react to high molecular weight allergens. We recently detected hemocyanin as a high m.w. shrimp allergen [14] but other allergens, including paramyosin (100 kDa), myosin heavy chain (225 kDa), and calcium ATPase (113 kDa) [15,16], have been described as well. Although in our patients hypersensitivity to these high m.w. allergens was frequently found in association with IgE reactivity to tropomyosin or other low m.w. allergens, they seem clinically relevant (e.g., allergenic profiles A, E2, or E5). Our next studies will point to a better characterization of these allergenic proteins. The immunoblot analysis scored negative in a significant proportion of shrimp-allergic patients; this has been observed also in a number of previous studies, and is probably due to the fact that immunoblot shows a lower sensitivity than SPT or specific IgE measurement.

In conclusion, the in-vivo diagnosis of shrimp allergy must be still based on SPT with fresh material as the sensitivity of current commercial extracts of crustaceans may be rather low and their allergen content highly variable. Nonetheless, performing SPT with all available commercial extracts in parallel with SPT with fresh material and with the detection of IgE to the few recombinant shrimp molecules currently available, may provide useful information about hypersensitivity to minor shrimp allergens. In the clinical practice the use of extract #2 in combination with extract #1 led to a slightly better sensitivity of commercial SPTs as the majority of the allergenic profiles were detected (Table 1).

REFERENCES

1) Asero R, Antonicelli L, Arena A, Bommarito L, Caruso B, Crivellaro M, De Carli M, Della Torre E, Della Torre F, Heffler E, Lodi Rizzini F, Longo R, Manzotti G, Marcotulli M, Melchiorre A, Minale P, Morandi P, Moreni B, Moschella A, Murzilli F, Nebiolo F, Poppa M, Randazzo S, Rossi G, Senna GE. EpidemAAITO: Features of food allergy in Italian adults attending allergy clinics: a multicenter study. Clin Exp Allergy 2009;39:547-55.

2) Asero R, Antonicelli L, Arena A, Bommarito L, Caruso B, Colombo G, Crivellaro M, De Carli M, Della Torre E, Della Torre F, Heffler E, Lodi Rizzini F, Longo R, Manzotti G, Marcotulli M, Melchiorre A, Minale P, Morandi P, Moreni B, Moschella A, Murzilli F, Nebiolo F, Poppa M, Randazzo S, Rossi G, Senna GE. Causes of food-induced anaphylaxis in Italian adults: a multi-centre study. Int Arch Allergy Immunol 2009;150:271-77.

3) Leung PS, Chu KH, Chow WK, Ansari A, Bandea CI, Kwan HS, Nagy SM, Gershwin ME. Cloning, expression, and primary structure of *Metapenaeus ensis* tropomyosin, the major heat-stable shrimp allergen. J Allergy Clin Immunol 1994;94:882-90.

4) Daul CB, Slattery M, Reese G, Lehrer SB. Identification of the major brown shrimp (*Penaeus atzecus*) allergen as the muscle protein tropomyosin. Int Arch Allergy Immunol 1994;105:49-55.

5) Witteman AM, Akkerdaas JH, van Leeuwen J, van der Zee JS, Aalberse RC. Identification of a cross-reactive allergen (presumably tropomyosin) in shrimp, mite and insects. Int Arch Allergy Immunol 1994;105:56-61.

6) Leung PS, Chow WK, Duffey S, Kwan HS, Gershwin ME, Chu KH. IgE reactivity against a cross-reactive allergen in Crustacea and Mollusca: evidence for tropomyosin as the common allergen. J Allergy Clin Immunol 1996;98:954-61.

7) Reese G, Ayuso R, Lehrer SB. Tropomyosin: an invertebrate pan-allergen. Int Arch Allergy Immunol 1999;119:247-58.

8) Asero R, Mistrello G, Amato S, Ariano R, Colombo G, Conte ME, Crivellaro M, De Carli M, Della Torre F, Emiliani F, Lodi Rizzini F, Longo R, Macchia D, Minale P, Murzilli F, Nebiolo F, Quercia O, Senna GE, Villalta D. Shrimp allergy in Italian adults: a multicenter study showing a high prevalence of sensitivity to novel high molecular weight allergens. Int Arch Allergy Immunol 2012;157:3-10.

9) Yu CJ, Lin YF, Chiang BL, Chow LP. Proteomics and immunological analysis of a novel shrimp allergen, Pen m 2. J Immunol 2003; 170: 445-453.

10) Garcia-Orozco KD, Aispuro-Hernandez E, Yepiz-Plascencia G, Calderon de la Barca AM, Sotelo-Mundo RR. Molecular characterization of arginine chinase, an allergen from the shrimp *Litopenaeus vannamei*. Int Arch Allergy Immunol 2007;144:23-28.

11) Shiomi K, Sato Y, Hamamoto S, Mita H, Shimakura K. Sarcoplasmic calcium binding proteins: identification as a new allergen of the black tiger shrimp *Penaeus monodon*. Int Arch Allergy Immunol 2008;146:91-98.

12) Ayuso R, Grishina G, Ibáñez MD, Blanco C, Carrillo T, Bencharitiwong R, Sánchez S, Nowak-Wegrzyn A, Sampson HA. Sarcoplasmic calcium binding protein is an EF-hand-type protein identified as a new shrimp allergen. J Allergy Clin Immunol 2009;124:114-20.

13) Ayuso R, Grishina G, Bardina L, Carrillo T, Blanco C, Ibanez MD, Sampson HA, Beyer K. Myosin light chain is a novel shrimp allergen, Lit v 3. J Allergy Clin Immunol 2008;122:795-802.

14) Giuffrida MG, Villalta D, Mistrello G, Amato S, Asero R. Shrimp allergy beyond tropomyosin in Italy: clinical relevance of arginine kinase, sarcoplasmic calcium binding protein and hemocyanin. Eur Ann Allergy Clin Immunol 2014;46:172-77.

15) Suzuki M, Kobayashi Y, Hiraki Y, Nakata H, Shiomi K. Paramyosin of the disc abalone Haliotis discus discus: identification as a new allergen and cross-reactivity with tropomyosin Food Chem 2011;124:921-26.

16) Pedrosa M, Boyano-martinez T, Garcia-Ara C, Quirce S. Shellfish allergy: a comprehensive review. Clin Rev Allerg Immunol 2015;49:203-16.

17) Villata D, Tonutti E, Visentini D, Bizzarro N, Roncarolo D, Amato S, Mistrello G. Detection of a novel 20 kDa shrimp allergen showing cross-reactivity to house dust mites. Eur Ann Allergy Clin Immunol 2010;42:20-24.

18) Alonso RE, Zavala BB, Escoda JM. Mantis shrimp allergy. J Investig Allergol Clin Immunol. 2006;16:394-6.

19) Dreborg S, Frew A. Allergen standardization and skin tests. EAACI position paper. Allergy 1993;48:49-75.

20) Jirapongsananuruk O1, Sripramong C, Pacharn P, Udompunturak S, Chinratanapisit S, Piboonpocanun S, Visitsunthorn N, Vichyanond P. Specific allergy to *Penaeus monodon* (seawater shrimp) or *Macrobrachium rosenbergii* (freshwater shrimp) in shrimp allergic children. Clin Exp Allergy 2008;38:1038-47.

21) Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principles of protein-dye binding. Anal Biochem 1976;72:248-54.

22) Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. Proc Natl Acad Sci USA 1979;76:4350-54.

23) Binder M, Mahler V, Hayek B, Sperr WR, Schöller M, Prozell S, Wiedermann G, Valent P, Valenta R, Duchêne M. Molecular and immunological characterization of arginine kinase from the Indianmeal moth, Plodia interpunctella, a novel cross-reactive invertebrate pan-allergen. J Immunol. 2001;167:5470-77.

Table 1. Allergenic profiles detected in 157 shrimp-allergic patients based on hypersensitivity to HDM and skin reactivity to 5 commercial extracts of crustaceans.

Allergenic Profile	HDM	Extract #1	Extract #2	Extract #3	Extract #4	Extract # 5	no.
A	*						10
B	*	*	*	*	*	*	67
C		*	*	*	*	*	11
D							2
E1	*	*					2
E2	*		*			*	5
E3	*					*	7
E4	*	*			*		2
E5	*	*	*				1
E6	*			*			1
E7	*	*	*		*		1
E8	*	*		*			1
E9	*	*				*	1
E10	*		*	*		*	2
E11	*		*	*			1
E12	*		*				6
E13	*	*	*	*			2
E14	*		*	*	*		1
E15	*				*		1
E16	*	*	*			*	1
E17	*	*	*	*		*	10
E18	*			*		*	1
E19	*		*	*	*	*	6
E20	*	*		*	*	*	2
E21	*	*		*	*		1
F1			*			*	3
F2			*	*			2
F3		*		*			1
F4			*				2

F5		*	*	*		*	2
F6			*	*		*	1
F7				*	*	*	1
Total	132	105	124	113	93	120	157
Sensitivity		66,8	78,9	71,9	59,2	76,4	

Legend: *= SPT positive

Table 2. IgE reactivity to Pen a 1 or Pen m 1 in 72 shrimp-allergic patients showing different allergenic profiles

Allergenic Profile	HDM	Extract #1	Extract #2	Extract #3	Extract #4	Extract #5	no.	Pen a 1/Pen m 1 IgE +
A	*						3	0
B	*	*	*	*	*	*	33	30
C		*	*	*	*	*	3	3
D							1	0
E1	*	*					1	0
E2	*		*			*	3	0
E3	*					*	2	0
E5	*	*	*				1	0
E6	*			*			1	1
E7	*	*	*		*		1	0
E8	*	*		*			1	0
E10	*		*	*		*	1	0
E12	*		*				3	1
E13	*	*	*	*			1	0
E15	*				*		1	0
E17	*	*	*	*		*	3	1
E19	*		*	*	*	*	6	1
E20	*	*		*	*	*	2	2
E21	*	*		*	*		1	1
F1			*			*	1	0
F4			*				1	0
F5		*	*	*		*	1	1
F7				*	*	*	1	0
Total							72	41

Legend: See table 1. A strong association between Profile B and Pen m 1 IgE is present ($p < 0.001$)

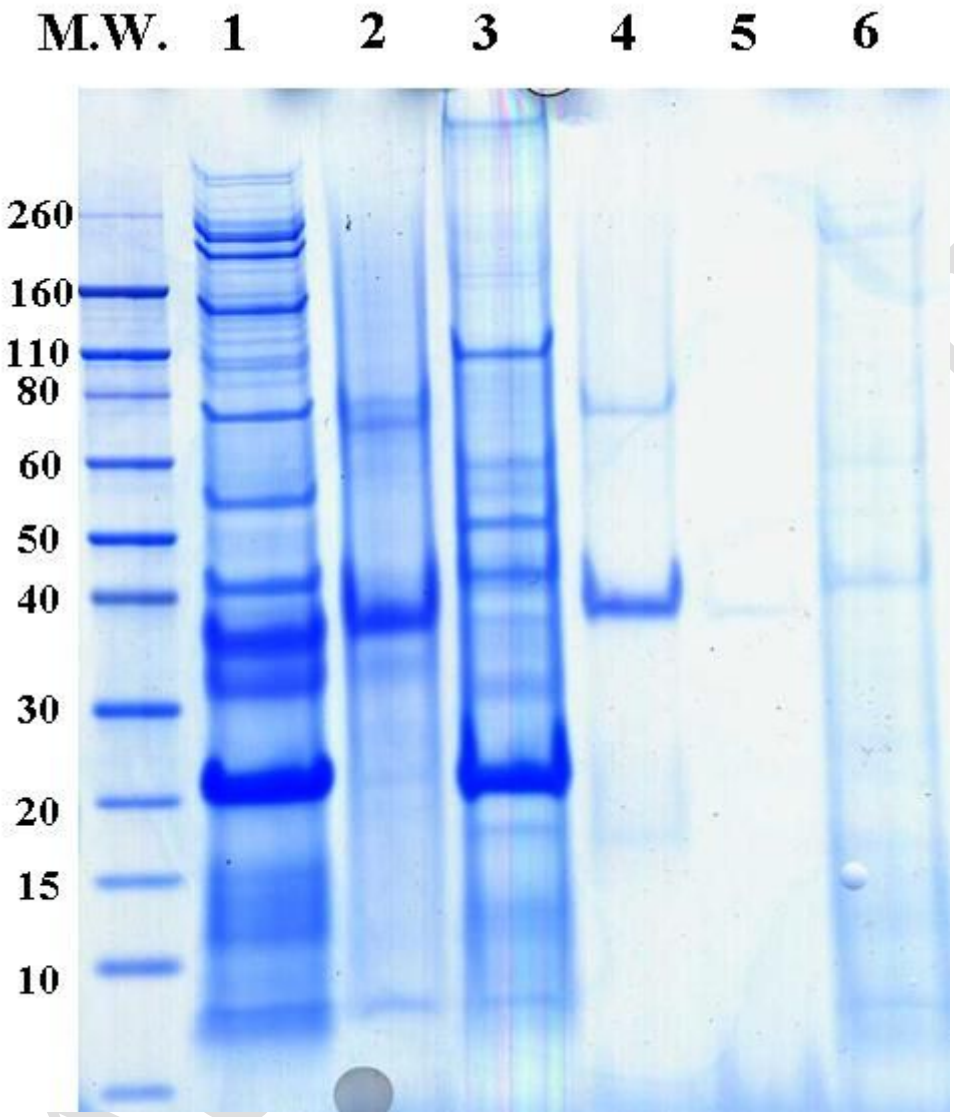
Table 3. IgE reactivity to Pen m 1, Pen m 2, and Pen m 4 in 42 shrimp allergic patients showing different allergenic profiles

Profile	HDM	Extract #1	Extract #2	Extract #3	Extract #4	Extract #5	no.	Pen m 1 +	Pen m 2 +	Pen m 4 +
A	*						1	0	0	0
B	*	*	*	*	*	*	25	25	6	4
C		*	*	*	*	*	3	3	0	1
E2	*		*			*	1	0	0	0
E5	*	*	*				1	0	0	0
E6	*			*			1	1	0	0
E12	*		*				1	0	0	0
E17	*	*	*	*		*	2	1	1	1
E19	*		*	*	*	*	6	1	4	1
E20	*	*		*	*	*	2	1	0	0
E21	*	*		*	*		1	2	0	0
F5		*	*	*		*	1	1	0	0
F7				*	*	*	1	0	0	1
Total							46	35	11	8

Table 4: Immunoblot analysis results with sera from patients showing different allergenic profiles.

Profile	HDM	Extract #1	Extract #2	Extract #3	Extraxct #4	Extract #5	no. tested	<20 kDa	20 kDa	30 kDa	35 kDa	50 kDa	60 kDa	70 kDa	80 kDa	90 kDa	100 kDa	120 kDa	> 120 kDa	WB negative
A	*						7/10			3		2	2	2	3		3	3		1
B	*	*	*	*	*	*	31/67	7	7	1	14	2	4	3	7	6	5	7	2	3
C		*	*	*	*	*	5/11		1	3		1			2		1	1		2
E1	*	*					1/2													1
E2	*		*			*	2/5			1			1	1	1		2	1		0
E10	*		*	*		*	1/2													1
E12	*		*				2/6			1			1							1
E15	*				*		1/1													1
E17	*	*	*	*		*	2/10													2
E19	*		*	*	*	*	4/6	1									1			3
F5		*	*	*		*	2/2								2		1	2		0
F7				*	*	*	1/1													1
Total							59													

Legend to figure 1: SDS- PAGE of the fresh shrimp extract (lane 1) and of commercial crustacean extracts for SPT : lane 2: commercial extract #1 (Lofarma); lane 3: commercial extract #2 (ALK); lane 4: commercial extract #3 (Allergopharma); lane 5: commercial extract #4 (Anallergo); lane 6: commercial extract #5 (Stallergenes).



Legend to figure 2: Selected immunoblots showing the variety of shrimp proteins recognized by different patients. Lane M.W.: molecular weight markers. Lanes 1-6: six different patients. Last lane: negative control serum.