Original Article

Barnacle allergy: allergen characterization and cross-reactivity with mites

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Abstract. *Background:* Barnacles are a type of seafood with worldwide distribution and abundant along the shores of temperate seas. They are particularly appreciated and regularly consumed in Portugal as well as in Spain, France and South America, but barnacle allergy is a rare condition of which there is only one reference in the indexed literature. The molecular allergens and possible cross-reactivity phenomena implicated (namely with mites) have not been established.
Objective: To demonstrate the IgE-mediated allergy to barnacle and to identify the proteins implicated as well as possible cross-reactivity phenomena with mites.
Methods: We report the clinical and laboratory data of five patients with documented IgE-mediated allergy to barnacle. The diagnosis was based on a suggestive clinical history combined with positive skin prick tests (SPT) to barnacle – prick to prick method. Two barnacle extracts were prepared (raw and cooked barnacle) and sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and IgE-immunoblotting were performed. An immunoblotting inhibition assay with *Dermatophagoides pteronyssinus* was also done in order to evaluate cross-reactivity.
Results: All patients had mite-related asthma and the allergic rhinoconjunctivitis; they all experienced mucocutaneous symptoms. All of them had positive SPT to barnacle, and the immunoblotting showed several allergenic fractions with a wide molecular weight range (19 – 94 kDa). The *D. pteronyssinus* extract inhibited several IgE-binding protein fractions in the barnacle extract.
Conclusions: We describe five patients with IgE-mediated barnacle allergy. We also describe a group of IgE-binding+proteins between 30 and 75 kDa as the allergenic fractions of this type of Crustacea. Cross-reactivity with *D. pteronyssinus* was demonstrated in two cases.

Key words: food allergy, barnacle, cross-reactivity, Crustacea, mites, *Pollicipes pollicipes*.

Resumen. *Introducción:* El percebe es un tipo de crustáceo distribuido en todo el mundo que es abundante en las costas de agua templada. Es especialmente apreciado y se consume con regularidad en Portugal, así como en España, Francia y Sudamérica, pero la alergia al percebe es una afección rara de la que sólo existe una referencia en la literatura indexada. No se han establecido los alérgenos moleculares ni los posibles fenómenos de reactividad cruzada implicados (especialmente con los ácaros).
Objetivo: Demostrar la alergia mediada por IgE al percebe e identificar las proteínas implicadas y los posibles fenómenos de reactividad cruzada con los ácaros.
Métodos: Se notificaron los datos clínicos y de laboratorio de cinco pacientes con alergia mediada por IgE al percebe documentada. El diagnóstico se basó en una historia clínica indicativa combinada con pruebas cutáneas positivas al percebe (método *prick to prick*). Se prepararon dos extractos de percebe (percebe crudo y cocido) y se llevaron a cabo las pruebas SDS-PAGE (electroforesis en gel de poliacrilamida con dodecil-sulfato de sodio) e inmunotransferencia-IgE. También se efectuó un ensayo de inhibición por inmunotransferencia con *Dermatophagoides pteronyssinus* a fin de evaluar la reactividad cruzada.

Resultados: Todos los pacientes presentaban asma y rinoconjuntivitis alérgicas relacionadas con los ácaros; todos experimentaron síntomas mucocutáneos. Todos ellos obtuvieron resultados positivos en la prueba de punción cutánea al percebe, y la inmunotransferencia mostró distintas fracciones alergénicas con una gran variedad de pesos moleculares (19–94 kDa). El extracto de *D. pteronyssinus* inhibió varias fracciones proteicas de unión a IgE en el extracto de percebe.
Conclusiones: Se describen 5 pacientes con alergia al percebe mediada por IgE. También se describe un grupo de proteínas de unión a IgE entre 30 y 75 kDa, como fracciones alergénicas de este tipo de crustáceo. Se demostró reactividad cruzada con *D. pteronyssinus* en dos casos.

Palabras clave: alergia alimentaria, percebe, reactividad cruzada, crustáceos, ácaros, *Pollicipes pollicipes*.

Introduction

Crustacea are recognized as a common cause of food hypersensitivity reactions, and Crustacea allergy is a problem of increasing prevalence and a significant health concern (also related to the increasing popularity and consumption of this type of shellfish) [1-2].

Seafood is usually associated with potentially severe reactions, though the pattern of immediate allergic reactions to Crustacea is similar to that reported for other foods [3-6]. Barnacles belong to the phylum *Arthropoda*, class *Crustacea*, subclass *Cirripedia*. They are the only sessile group of Crustacea, with free swimming larvae. The adults have antennae that are used as an attachment organ and there are six pairs of thoracic legs. The body is surrounded by pairs of fixed calcareous plates and is protected by another pair of plates which close the opening. Barnacles are divided into two groups: the acorn barnacles (*Balanomorpha*) in which the plates attach directly to the rock, and goose barnacles (*Lepadomorpha*) which are attached on the end of a stalk. They have a worldwide distribution and are abundant along the shores of warm seas, such as the coasts of Mediterranean countries where they are regarded as a delicacy (*Pollicipes pollicipes* is the most consumed species).

Barnacles are a seafood particularly appreciated and regularly consumed in Portugal, as well as in Spain, France and South America, but barnacle allergy is a rare condition of which there is only one reference in the indexed literature [7]. The molecular allergens and possible cross-reactivity phenomena implicated (namely with mites) have not been established.

We report 5 cases of documented IgE-mediated allergy to barnacle – evidencing specific IgE by *in vivo* and *in vitro* methods- and describe the IgE-binding+proteins implicated in the reactivity phenomena to this type of Crustacea, as well as cross-reactivity with *Dermatophagoides pteronyssinus*.

Material and methods

Five patients, 3 male and 2 female, whose ages ranged from 2 to 29 years, were included in the study. All patients suffered from mucocutaneous manifestations upon the ingestion of cooked barnacles.

The diagnosis of IgE-mediated barnacle allergy was based on a suggestive clinical history combined with positive skin prick tests (SPT) to raw and/or cooked barnacle – prick to prick method. The study also included SPT to a standard battery of inhalant and other seafood allergens (according to the clinical history) and the determination of serum specific IgE to raw and cooked barnacle extract, to shrimp recombinant tropomyosin and to other inhalant and food allergens (using the UniCap System®, Pharmacia). An oral provocation challenge was proposed to 4 patients (with the exception of patient 4, due to the severity of her clinical manifestations), but they/their parents did not accept the test, and the procedure was discarded, also based on the fact that barnacles are not an important food in our diet and are usually eaten as a delicacy.

Two barnacle extracts (*Pollicipes pollicipes*) were prepared. One was prepared with raw barnacles and for the other the barnacles were heated for 15 minutes at 75ºC in order to reproduce the conditions in which they are consumed. After this, 2 g of each ground barnacle meat were mixed with 20 ml of phosphate-buffered saline (PBS) and kept in agitation overnight at 4º C. Then, the extracts were centrifuged at 1000 g for 10 minutes; the supernatant was recovered and sterilized by passing it through a Millipore filter (size 0.22 μm). These supernatants were dialyzed and lyophilized and the protein concentration was determined by the Bicinchoninic Acid method (Sigma). For the development of the *in vitro* tests - specific IgE determination (UniCAP System®, Pharmacia), sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and IgE-immunoblotting - the protein concentration was adjusted to 4 mg/ml.

Allergens commercially not available (raw and cooked barnacle) were coupled to Streptavidin Immunocaps as solid phase, and specific IgE was measured using the UniCap System® according to Sander *et al.* [8].

Immunoblotting was carried out with the individual sera from the five patients to analyze the allergenic components implicated in the sensitization.

The barnacle extracts were separated in 12% SDS-PAGE and transferred to an Immobilon-P (PVDF) membrane (Millipore, Bradford, MA) by electroblotting at 0.24 A for 45 minutes. The immunoblotting was performed as previously described [9,10]. Briefly, 1 ml of each patient serum was incubated with the membrane overnight at 4ºC in agitation. After five washes with Phosphate Buffer Saline (PBS), 1 ml of a 1:10.000 dilution rabbit anti-human IgE peroxidase conjugate (DAKO P0295)

Table 1. Skin prick test and specific IgE results.

	PATIENTS									
	1		2		3		4		5	
Patients characteristics	3 years, ♂		6 years, ♀		9 years, ♂		20 years, ♀		29 years 2♂	
Allergens	SPT	IgE	SPT	IgE	SPT	IgE	SPT	IgE	SPT	IgE
D. pteronyssinus	13	>100	5	>100	5	>100	10	>100	8.5	3.24
D. farinae	7.5	>100	9	>100	3.5	>100	8	>100	8	2.56
L. destructor	nd	<0.35	nd	<0.35	nd	69.5	nd	5.82	nd	0.53
B. germanica	nd	<0.35	nd	<0.35	4	37.3	nd	<0.35	4	<0.35
P. americana	nd	<0.35	nd	<0.35	3.5	11.2	nd	<0.35	5	0.52
B. orientalis	nd	<0.35	nd	<0.35	2.5	9.7	nd	<0.35	7.5	<0.35
Raw	5	<0.35	nd	<0.35	9.5	0.82	nd	<0.35	2	<0.35
/Cooked barnacle										
(PP/prepared extract)	10	<0.35	8.5	<0.35	9.5	3.65	7.5	<0.35	6	<0.35
rTropomyosin	–	<0.35	–	<0.35	–	86.0	–	<0.35	–	<0.35
Shrimp (PP)	nd	–	nd	–	7	–	1.5	–	2	–
(CE)	nd	<0.35	Neg	<0.35	nd	85.2	Neg	<0.35	3	<0.35
Snail (PP)	nd	–	nd	–	8.5	–	nd	–	Neg	–
(CE)	nd	nd	nd	nd	nd	20.0	nd	nd	Neg	<0.35
Clam (PP)	nd	–	nd	–	7	-	nd	–	Neg	–
(CE)	Neg	<0.35	Neg	nd	1.5	>100	nd	nd	Neg	<0.35
Squid (PP)	nd	–	nd	–	9	–	nd	–	nd	–
(CE)	Neg	nd	Neg	nd	nd	6.41	nd	nd	Neg	<0.35
Octopus (PP)	nd	–	Neg	–	9	–	nd	–	nd	–
(CE)	nd	nd	nd	nd	nd	38.6	nd	nd	nd	<0.35
Cuttlefish (PP)	nd	–	nd	–	6	–	nd	–	nd	–

Skin prick test (SPT) results: wheal mean diameter in mm; specific IgE results in kU/l; CE = commercial extract; PP = prick to prick skin test; Neg = negative; nd = not determined.

was used as second antibody. It was incubated with the membrane for 1 hour at room temperature and in agitation (100 r.p.m.). For the development of the reaction, ECL plus Western Blotting Detection System (Amersham Biosciences RPN2132) was used according to the manufacturer indications. The measurement of the molecular masses was performed using the program QuantityOne, BioRad.

In order to study possible cross-reactivity between barnacle and house-dust mite, we performed an immunoblotting inhibition assay where the sera were preincubated with a *D. pteronyssinus* extract, and a new immunoblot with the raw barnacle extract was obtained afterwards. Two of the patients (2 and 4) refused to provide further serum for the assay, and no inhibition immunoblotting was performed.

For the immunoblotting inhibition assay, 0.5 ml of the patients 1, 3 and 5 sera at 1:1 dilution in PBS, were preincubated with either 1 mg of *D. pteronyssinus* extract or 1 mg of the raw barnacle extract (control), overnight at 4ºC in agitation. After centrifuging each sample at 10.000 r.p.m during 5 minutes, the sera were recovered and the immunoblotting assay was carried out under the same conditions as described before.

Due to the similarity of protein fractions obtained in the immunoblotting assay using either the raw or the cooked barnacle extracts, the inhibition assay was only performed with one of these (raw barnacle extract).

Results

All patients had mite-related asthma and allergic rhinoconjunctivitis. None of them had received mite specific immunotherapy previously. They all had mucocutaneous symptoms (generalized urticaria and angioedema of the face and hands) starting between 10 and 90 minutes after the ingestion of cooked barnacle. Two patients had oropharyngeal pruritus and one (patient 4) had laryngeal edema with dysphonia and stridor.

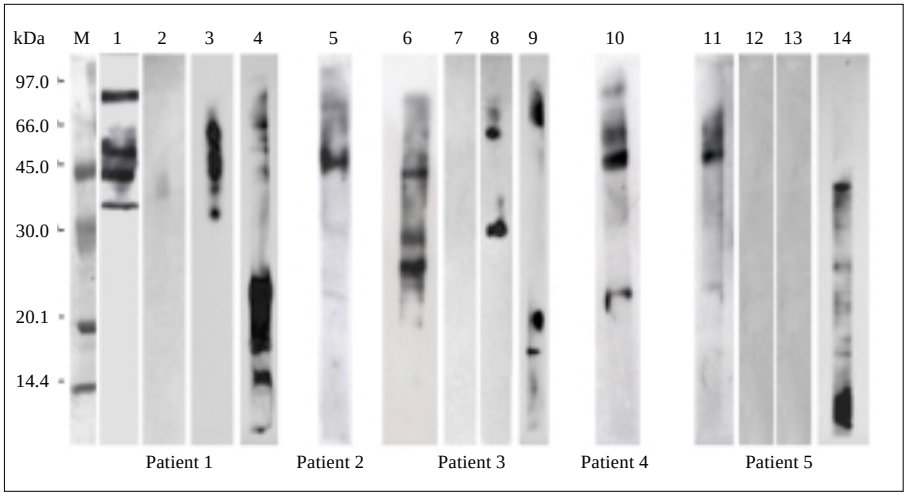

Figure 1. IgE immunoblotting to barnacle and IgE-immunoblotting-inhibition using raw barnacle extract in solid phase and *D. pteronyssinus* extract as inhibitor.
M: Molecular mass marker. Lanes 1, 5, 6, 10 and 11: immunoblotting to barnacle; Lanes 2, 7 and 12; immunoblotting-inhibition control: sera preincubated with barnacle extract; Lanes 3, 8 and 13: immunoblotting-inhibition: sera preincubated with *D. pteronyssinus* extract; Lanes 4, 9 and 14: immunoblotting to *D. pteronyssinus.*

Patients 1 to 3 were all children who had symptoms upon their first ingestion of barnacle.

Patient 3 also had symptoms related to the ingestion of shrimp, snail, squid and cuttlefish; patient 4 also experienced anaphylaxis with shrimp, and patient 5 had symptoms upon the ingestion of all other Crustacea, snail and octopus. All of them tolerated bivalves. Patients 1 and 2 tolerated all other Crustacea and molluscs. They were not exposed to any Crustacea afterwards.

All of the patients had positive SPT to barnacle (prick to prick), though in only one serum could barnacle-specific IgE be determined. They were all also sensitized to mites (on SPT and IgE). Specific IgE to recombinant tropomyosin was only positive in patient 3 (86.0 kU/l). The results of SPT and specific IgE determinations of each patient are shown in Table 1.

The IgE-immunoblotting revealed several allergenic bands (Figure 1 and Table 2) that were very similar by using either raw or cooked barnacle extract. We point out three IgE-binding bands around 37 kDa, 52 kDa and 60 kDa, that were recognized by all patients.

Table 2. Molecular masses (kDa) of the antigenic fractions recognized by each patient in the barnacle IgE-Immunoblotting and Immunoblotting inhibition assays.

Patient 1			Patient 2		Patient 3			Patient 4		Patient 5		
Barnacle Immuno--blotting		Immuno--blotting inhibition (Dpt)	Barnacle Immuno--blotting		Barnacle Immuno--blotting		Immuno--blotting inhibition (Dpt)	Barnacle Immuno--blotting		Barnacle Immuno--blotting		Immuno -blotting inhibition (Dpt)
RB	CB	RB	RB	CB	RB	CB	RB	RB	CB	RB	CB	RB
				94								
						87.9						
				79.7							83	
76.5	75	75		76.6	74							
				72					68.8	72.2	70	
				65		63.4		62				
59	**57.8**	**60**	**59**	**60**	**59**	**60**	**60**	**55**	**59**	**61.5**	**60**	
54			55		56							
50	**51.5**	**52**	**50.8**	**51.9**	**49**	**50.2**			**52.2**		**51.5**	
43	47.4	47	45		44	45.3				45.5	42.6	
40	43.7	43	41		40.3				41			
35.7	**35**	**35**	**38**	**36.4**	**36.8**	**37**			**37.5**	**36**	**38.1**	
						34.3						
	33	33				32.5						
					30	30.5	30					
						27.6			27.6		26.5	
					25	25.6						
					23.5	24						
					22	23						
					19	20						

Dpt = *Dermathophagoides pteronyssinus* extract; RB = Raw barnacle extract (solid phase); CB = Cooked barnacle extract (solid phase).

In the inhibition assay, the results were different for each studied patient. Only in patient 5 was the inhibition complete. In patient 1 there was no inhibition, and in patient 3 the *D. pteronyssinus* extract inhibited several IgE-binding molecules between 19 and 74 kDa in the barnacle immunoblotting assay, two fractions of 30 and 60 kDa remaining that were not inhibited and may be barnacle specific allergens (Figure 1 and Table 2).

Discussion

Although IgE-mediated allergic reactions to Crustacea are frequent and barnacles are highly appreciated in Portugal, as well as in other countries, only one report describes barnacles as causal agents, probably related to their limited consumption.

It is noteworthy that all five of our patients, as well as most cases reported in the literature, were sensitized to house-dust mites. This prompted us to evaluate the presence of cross-reactivity between barnacle and *D. pteronyssinus*, demonstrating common IgE-binding epitopes. Our cases are included in the context of cross-reactivity between invertebrates.

The SDS-PAGE immunoblotting analysis revealed three relevant thermostable proteins, recognised by all the patients: one with a molecular mass of around 37 kDa that might correspond to tropomyosin, and two other protein fractions with molecular masses of around 52 kDa and 60 kDa, respectively.

In the only other published paper describing barnacle allergy, by Moreno Escobosa et al [7], including 5 patients (three of them sensitized to mites), the authors also performed barnacle IgE-immunoblotting with raw and cooked extracts; no inhibition assays were performed. Their results showed IgE-binding bands with molecular masses ranging between 37-39 kDa and 58-68 kDa. The first band could be tropomyosin (in accordance with our results), and the second one could correspond to the protein fraction around 60 kDa identified in our study. There is no previous reference to an IgE-binding fraction with a molecular mass of around 52 kDa.

In our study, the immunoblotting inhibition assay showed no inhibition in patient 1, partial inhibition of the barnacle extract after preincubation with the *D. pteronyssinus* extract in patient 3, and total inhibition in patient 5. Only two fractions of 30 and 60 kDa were not inhibited in patient 3 and might be specific of the barnacle extract.

Allergy to Crustacea has been extensively studied and there are a number of papers referring to the major allergen involved in the reactivity to several species of this class: tropomyosin. This molecule, with a molecular mass of 36 kDa, belongs to a family of highly conserved proteins with multiple isoforms, found in both muscle and non-muscle cells and ubiquitous in the animal kingdom. Because of its high degree of conservation and significant sequence homology amongst the invertebrates, tropomyosin is pointed out as the homologous pan-allergen implicated in clinically important strong IgE cross-reactivity reactions among several invertebrate species, from Crustacea (shrimp, lobster, crab) to molluscs (snail, squid, clam), arachnids (mites), insects (cockroaches) and even nematodes [1;11-16]. Nevertheless, in our study, it is not clear that tropomyosin plays a relevant role in the cross-reactivity except in patient 3 (although further studies would be necessary to confirm this hypothesis).

Recently, a new allergen of 39.9 kDa from the shrimp *Penaeus monodon* has been identified using sera from patients with shrimp allergy - Pen m 2. This protein revealed extensive similarity with arginine kinase from Crustacea, and it was identified as a novel cross-reactive Crustacea allergen [17].

In light of the cases presented, it seems plausible that cross-reactivity phenomena are implicated in patients 3 and 5, though we may also be in the presence of co-sensitization or cross-reactivity with other Crustacea (especially considering patient 3, who presented symptoms upon his first ingestion of barnacles). Regarding patient 1, who also presented symptoms upon his first ingestion of barnacles, though he has no allergy to other Crustacea or molluscs and in whom cross-reactivity with mites was not documented, we speculate that he may have sensitized to barnacle proteins through breastfeeding or via transplacental transference of allergens. The mother referred regular consumption of barnacles throughout pregnancy and breastfeeding, and the child was breastfed for 6 months.

A link between allergen immunotherapy with *D. pteronyssinus* and development of food reactions to molluscs and Crustacea has been reported [18]. However, none of our patients had received immunotherapy with *D. pteronyssinus*, weakening the role of this type of treatment as a predisposing factor in Crustacea allergy.

Our study demonstrates the presence of two IgE-binding proteins of 30 and 60 kDa in the barnacle extract that could be barnacle specific allergens since: they were the only remaining fractions in the inhibition assay with *D. pteronyssinus* in patient 3; and no IgE-binding bands with the same molecular masses have been revealed in Immunobloting assays of other Crustacea or molluscs extracts, according to the literature [1,14,19]. However, further experiments using a larger variety of mollusc and Crustacea extracts should be carried out to confirm this hypothesis.

We conclude that the food habits of a population strongly influence the patterns of food allergy; and that the cross-reactivity phenomena with mites should be considered in the diagnosis of barnacle allergy, as was described for other Crustacea.

References

1. Musmand JJ, Daul CB, Lehrer SB. Crustacea allergy. Clin Exp Allergy 1993, 23:722-732.
2. Sicherer SH, Munoz-Furlong A, Sampson HA. Prevalence of seafood allergy in the United States determined by a random telephone survey. J Allergy Clin Immunol 2004,

114:159-165.
3. Atkins FM, Steinberg SS, Metcalfe DD. Evaluation of immediate adverse reactions to foods in adult patients. II. A detailed analysis of reaction patterns during oral food challenge. J Allergy Clin Immunol 1985, 75:356-363.
4. Atkins FM, Steinberg SS, Metcalfe DD. Evaluation of immediate adverse reactions to foods in adult patients. I. Correlation of demographic, laboratory, and prick skin test data with response to controlled oral food challenge. J Allergy Clin Immunol 1985, 75:348-355.
5. Sampson HA. Anaphylaxis and emergency treatment. Pediatrics 2003, 111:1601-1608.
6. Moneret-Vautrin DA, Kanny G, Morisset M, Rance F, Fardeau MF, Beaudouin E. Severe food anaphylaxis: 107 cases registered in 2002 by the Allergy Vigilance Network. Allerg Immunol (Paris) 2004, 36:46-51.
7. Moreno Escobosa MC, Alonso LE, Sanchez AA, Mendez AJ, Rico Diaz MA, Garcia AG, Bartolome ZB. Barnacle hypersensitivity. Allergol Immunopathol (Madr) 2002, 30:100-103.
8. Sander I, Kespohl S, Merget R, Goldscheid N, Degens PO, Bruning T, Raulf-Heimsoth M. A new method to bind allergens for the measurement of specific IgE antibodies. Int Arch Allergy Immunol 2005, 136:39-44.
9. Towbin H, Stahelin I, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proceedings of the National Academy of Sciences of the United States of America 1979, 76:4350-4354.
10. Shen HD, Wang SR, Tang RB, Chang FZN, Su SN, Han SN. Identification of allergens and antigens of Bermuda grass (Cynodon dactylon) pollen by immunoblot analysis. Clinical Allergy 1988, 18:401-409.
11. Reese G, Ayuso R, Carle T, Lehrer SB. IgE-binding epitopes of shrimp tropomyosin, the major allergen Pen a 1. Int Arch Allergy Immunol 1999, 118:300-301.
12. Reese G, Ayuso R, Lehrer SB. Tropomyosin: an invertebrate pan-allergen. Int Arch Allergy Immunol 1999, 119:247-258.
13. Ayuso R, Reese G, Leong-Kee S, Plante M, Lehrer SB. Molecular basis of arthropod cross-reactivity: IgE-binding cross-reactive epitopes of shrimp, house dust mite and cockroach tropomyosins. Int Arch Allergy Immunol 2002, 129:38-48.
14. Lehrer SB, Ayuso R, Reese G. Seafood allergy and allergens: a review. Mar Biotechnol (NY) 2003, 5:339-348.
15. Fernandes J, Reshef A, Patton L, Ayuso R, Reese G, Lehrer SB. Immunoglobulin E antibody reactivity to the major shrimp allergen, tropomyosin, in unexposed Orthodox Jews. Clin Exp Allergy 2003, 33:956-961.
16. Ferreira F, Hawranek T, Gruber P, Wopfner N, Mari A. Allergic cross-reactivity: from gene to the clinic. Allergy 2004, 59:243-267.
17. 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.
18. van Ree R, Antonicelli L, Akkerdaas JH, Garritani MS, Aalberse RC, Bonifazi F. Possible induction of food allergy during mite immunotherapy. Allergy 1996, 51:108-113.
19. Leung PS, Chu KH. Molecular and immunological characterization of shellfish allergens. Front Biosci 1998, 15:306-312.

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