

Monosensitivity to Pangasius and Tilapia Caused by Allergens Other Than Parvalbumin

DG Ebo,¹ A Kuehn,² CH Bridts,¹ C Hilger,² F Hentges,² WJ Stevens¹

¹Department of Immunology–Allergology–Rheumatology, University Hospital Antwerp, University of Antwerp, Belgium

²Laboratory of Immunogenetics and Allergology, CRP-Santé, Luxembourg

■ Abstract

Fish allergy is one of the most common food allergies in populations where fish is a major part of the diet. Most fish-allergic patients react to the panallergen parvalbumin present in multiple fish species. Our aim was to investigate the clinical case of a patient with oral allergy syndrome to pangasius and Nile tilapia but tolerance of other fish and seafood. The temporal relationship between fish consumption and allergic symptoms, the positive skin prick tests, and the basophil activation test results for both fish species strongly supported the diagnosis of an immunoglobulin (Ig) E-mediated allergy. This was confirmed by the detection of specific IgE to 18-kDa and 45-kDa proteins in immunoblot analysis. Notably, the patient was not sensitized to parvalbumin, as shown by enzyme-linked immunosorbent assay using purified allergens.

Cross-reactivity between fish species can result from sensitization to allergens other than parvalbumin. This case report emphasizes the applications of flow cytometry–assisted analysis in the diagnosis of food allergy.

Key words: BAT assay. Fish allergy. Monosensitivity. Pangasius. Tilapia.

■ Resumen

La alergia al pescado es una de las alergias alimentarias más habituales en poblaciones donde el pescado forma parte importante de la alimentación. La mayoría de los pacientes alérgicos al pescado reaccionan al panalérgeno parvalbúmina presente en muchas especies de peces. El objetivo del presente estudio fue investigar el caso clínico de un paciente con síndrome de alergia oral al pangasio y a la tilapia del Nilo, pero que presentaba tolerancia a otras clases de pescado y marisco. La relación temporal entre el consumo de pescado y los síntomas alérgicos, las pruebas de punción positivas y los resultados del test de activación de basófilos para ambas especies de pescado corroboraron firmemente el diagnóstico de una alergia mediada por la inmunoglobulina E (IgE). Esto se confirmó tras la detección de IgE específica a proteínas de 18 kDa y 45 kDa en los análisis de inmunotransferencia. En particular, el paciente no mostró sensibilización a la parvalbúmina, según se demostró por medio de un ensayo de inmunoadsorción (ELISA) con alérgenos purificados.

La reactividad cruzada entre especies de peces puede deberse a la sensibilización a alérgenos distintos de la parvalbúmina. Este caso clínico pone el énfasis en las aplicaciones del análisis por citometría de flujo en el diagnóstico de la alergia alimentaria.

Palabras clave: Test de activación de basófilos. Alergia al pescado. Monosensibilidad. Pangasio. Tilapia.

Introduction

The majority of patients with fish allergy develop clinical symptoms to a range of fish species. The reason for this broad immunoglobulin (Ig) E cross-reactivity is sensitization to the fish panallergen parvalbumin [1,2]. Parvalbumins are low-molecular-weight, calcium-binding muscle proteins that are highly conserved across fish species. In immunological and

molecular studies, parvalbumins have been characterized from a range of common fishes such as cod, salmon, and carp [3-5]. Fish allergens other than parvalbumin have also been reported. Examples are aldehyde phosphate dehydrogenase (~41 kDa) from codfish and fish collagen (~110 kDa) and transferrin-like protein (~94 kDa) from tuna [6-8]. Putative fish allergens such as IgE-reactive proteins of different molecular weights have also been described in swordfish, sole, eel, and snapper [9-12].

In recent years, different fish species from African and Asian countries have gained in importance on the European market. Pangasius (*Pangasius hypophthalmus*) and Nile tilapia (*Oreochromis niloticus*) account for a considerable market share as they are attractive alternatives to traditional and pricey fish species [13]. To date, no information has been published on species-specific fish allergy to pangasius and Nile tilapia.

In the present study, we analyzed the IgE-binding profile of a fish-allergic patient exclusively sensitized to pangasius and Nile tilapia in order to detect the causative allergen(s).

Case Description

We report on a patient with oral allergy syndrome characterized by itching and swelling of the lips, tongue, and oropharynx to both pangasius and Nile tilapia. The patient, a 27-year-old atopic woman with a history of house dust mite (HDM) allergy and documented allergy to sesame, had experienced several episodes of itching and swelling of the oropharynx within minutes of eating both fish species. She tolerated other seafood including cod, salmon, tuna, crustaceans, and molluscs.

Total IgE was 69 kU/L. Specific IgE was 1.87 kUa/L for HDMs, 0.43 kUa/L for sesame, 0.41 kUa/L for tilapia, and 0.46 kUa/L for cod. Specific IgE was negative for lobster, crab, oyster, mussel, and recombinant parvalbumin from carp (rCyp c 1) (<0.35 kUa/L, ImmunoCAP FEIA, Phadia, Uppsala, Sweden).

Skin prick tests (SPTs) were performed with fish extracts from pangasius, tilapia, and cod, which were also used for stimulation in the basophil activation tests (BATs). The skin reaction was positive with a clear wheal and flare of 14/40 mm for pangasius and 3/7 mm for tilapia. The skin test for cod was negative.

Basophil Activation Test

Flow cytometric analysis (FACScan, BD Immunocytometry Systems, Erembodegem, Belgium) of activated basophils was performed using Alexa 448-coupled anti-IgE (Sigma-Aldrich, Chemic GmbH, Steinheim, Germany) and phycoerythrin-conjugated anti-CD63 (Pharmingen, BD Biosciences, Erembodegem, Belgium) double labeling. Basophil activation tests involved a negative control (spontaneous CD63 expression without any allergen), a positive control (anti-IgE), and dialyzed extracts from pangasius, tilapia, and cod (serial dilutions of 0.01, 0.1, 1 and 10 $\mu\text{g/mL}$). For this purpose the fish extracts were prepared as described by Alenius et al [14].

The BAT results are summarized in Figure 1.

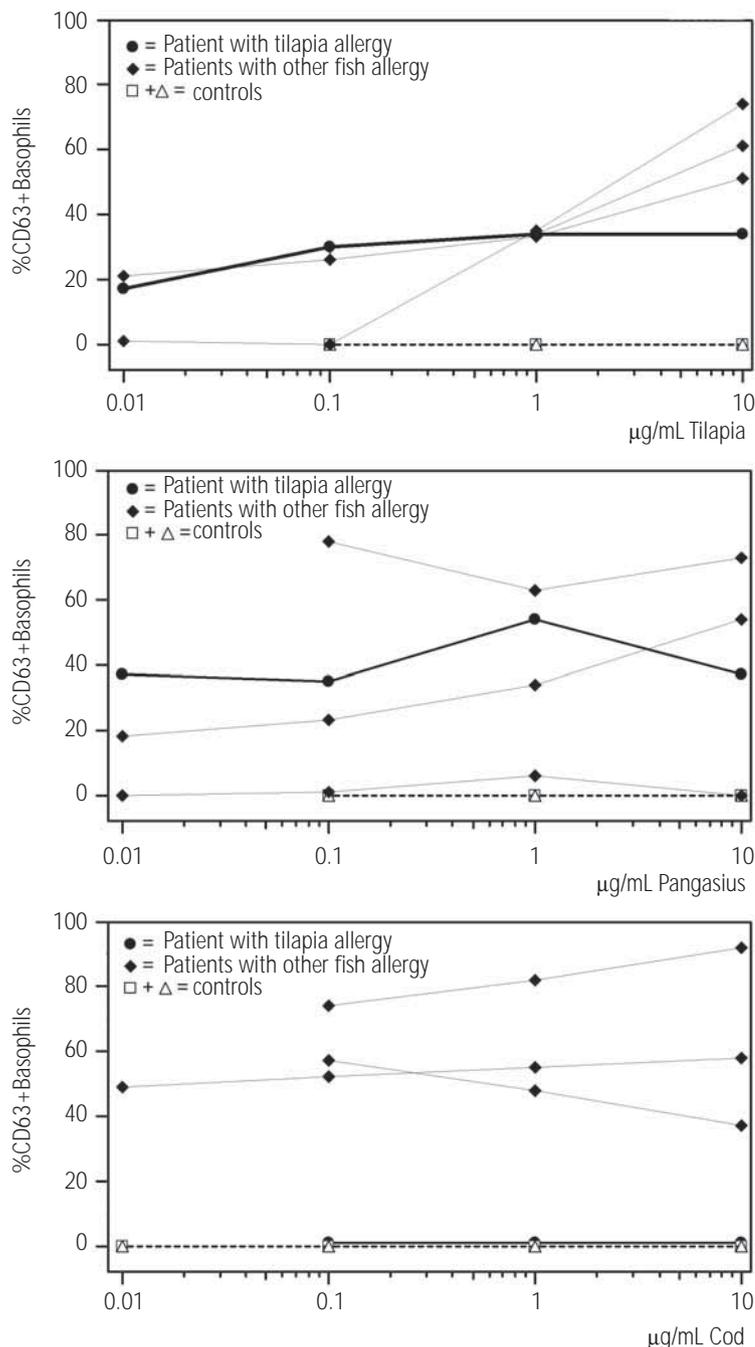


Figure 1. Pangasius and tilapia (Figures 1A and 1B) but not cod (Figure 1C) induced a consistent basophilic upregulation of CD63 above spontaneous expression in the patient (●). In contrast, fish-induced expression of CD63 from 2 healthy control individuals (□, △) was negative (Figures 1A-1C). In 3 patients with cod allergy (◆), the basophil activation test was positive for all 3 fish species in 2 of the patients and positive for cod and tilapia in the other one (Figures 1A-1C).

In the patient, pangasius and tilapia, but not cod, induced a consistent basophilic upregulation of CD63 above spontaneous expression. Fish-induced expression of CD63 from 2 healthy control individuals, however, remained entirely negative. In 3 patients with documented cod allergy, the BAT was positive for all 3 fish species in 2 of the patients and for cod and tilapia in the other.

Protein Extraction and Purification

Fish muscle extracts were prepared from pangasius (*Pangasius hypophthalmus*), Nile tilapia (*Oreochromis niloticus*), salmon (*Salmo salar*), cod (*Gadus morhua*), tuna (*Thunnus albacares*), mackerel (*Scomber scombrus*), and carp (*Cyprinus carpio*) as described elsewhere [15]. Native parvalbumins from pangasius, salmon, cod, and mackerel were purified by ion exchange and gel filtration chromatography. Recombinant β -1 parvalbumins from salmon and cod were obtained by expressing the complementary DNAs (Swiss-Prot Q91482, Q90YL0) in *Escherichia coli* M15 [3,4]. These parvalbumins were purified by affinity and gel filtration chromatography. Protein identity was confirmed by immunodetection using a commercial mouse monoclonal anti- β -parvalbumin antibody (Swant, Bellinzona, Switzerland).

IgE Quantification by Enzyme-Linked Immunosorbent Assay

Ninety-six-well plates (Maxisorb, Nunc, Wiesbaden, Germany) were coated overnight at 4°C with 5 μ g/mL of purified native and recombinant parvalbumins in phosphate buffered saline, pH 7.2. IgE were quantified versus a characterized patient serum with a known titer to cat serum albumin. The patient's serum was used in different dilutions (1:3, 1:5, and 1:10). An anti-parvalbumin antibody (Swant) and serum from a fish-allergic individual (5 kUa/L for cod, ImmunoCAP) were used as positive controls.

The enzyme-linked immunosorbent assay (ELISA) results for IgE reactivity to purified fish parvalbumins are shown in the Table. The patient's IgE antibodies did not bind to native isoform mixtures or to recombinant single isoforms of parvalbumins from 4 common fish species.

IgE Immunoblot and Immunoblot Inhibition

Fish protein extract was analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, Massachusetts, USA) [15,16]. The membranes were incubated overnight with diluted patient serum (1:3) and the anti-parvalbumin antibody (Swant). In Ca^{2+} -depletion experiments, 5 mM of ethylene glycol tetraacetic acid (EGTA) was added to the primary antibodies during incubation with blotted extract. To analyze cross-reactivity, the patient's serum was incubated with pangasius, tilapia, or cod protein extract at a final concentration of 1 mg/mL for 2 hours, prior to incubation with blotted pangasius or tilapia extract.

In the immunoblot analysis of total fish extracts, the patient's IgE detected strong protein bands of about 18 kDa and 45 kDa in pangasius and tilapia and weak bands

Table. Immunoglobulin (Ig) E antibody binding to purified fish parvalbumins in the patient was quantified by enzyme-linked immunosorbent assay. Specific IgE from a fish-allergic individual were titrated as a positive control

Coating Parvalbumin		Ua/L (Patient)	kUa/L (Positive Control)
Native	Pangasius	<0.1	0.5
	Cod	<0.1	4.5
	Salmon	<0.1	3.2
	Mackerel	<0.1	0.3
Recombinant	Cod, β -1	<0.1	5.2
	Salmon, β -1	<0.1	3.9

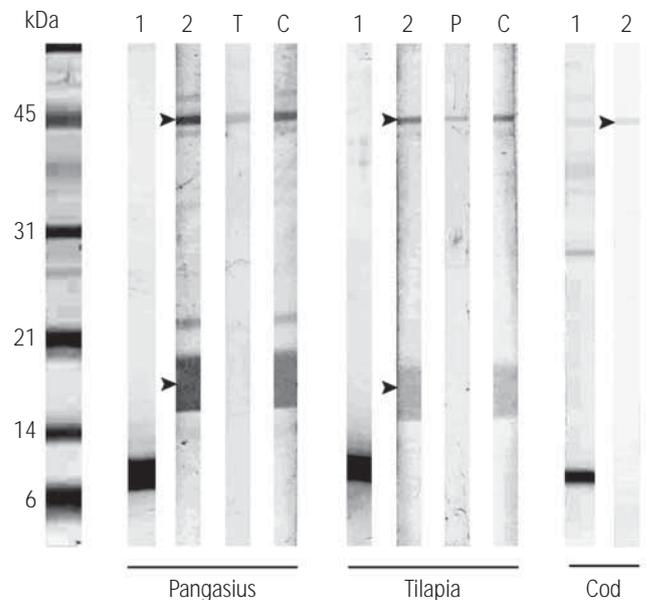


Figure 2. Total extracts from pangasius, tilapia, and cod were analyzed by immunoblotting. 1, anti- β -parvalbumin antibody detected parvalbumins in the fish extracts. 2, the patient's immunoglobulin (Ig) E detected putative new allergens of 18 kDa and 45 kDa (▶), but not parvalbumins. T, The patient's IgE reactivity to the 18-kDa and 45-kDa pangasius proteins was inhibited by tilapia extract. P, the patient's IgE reactivity to the 18-kDa and 45-kDa tilapia proteins was inhibited by the pangasius extract. C, serum incubation with cod extract did not affect IgE binding to either of the putative allergens from pangasius and tilapia.

in cod (Figure 2). No signals were obtained for protein extracts from salmon or carp (data not shown). Notably, no IgE reactivity was detected with extracts from mackerel or tuna, both close relatives of tilapia and belonging to the same order of perch-like fish (Perciformes). The patient's IgE did not recognize the parvalbumin-like bands detected by the monoclonal antibody in any of the fish extracts. IgE detection of the putative 18-kDa- and 45-kDa allergens was

not influenced by incubation with 5 mM EGTA (data not shown). Cross-reactivity between pangasius and tilapia was analyzed by immunoblot inhibition (Figure 2). IgE binding to putative 18-kDa allergens in pangasius and tilapia extracts was abolished upon serum cross-inhibition with the extracts and IgE detection of putative 45-kDa allergens in both extracts was markedly reduced. Serum incubated with cod extract did not affect IgE reactivity to either of the putative allergens in pangasius or tilapia.

Discussion

Parvalbumins are recognized as major fish allergens that cause broad IgE cross-reactivity, even in tropical fish species [1,2,17]. Recently, parvalbumin was cloned from Mozambique tilapia (*Oreochromis mossambicus*) [18], but homologous muscle proteins have not yet been characterized in pangasius or Nile tilapia.

We studied putative fish allergens in a patient with clinical symptoms exclusively due to pangasius and Nile tilapia. SPTs were positive only for pangasius and tilapia extracts, confirming the clinical history. Notably, immunoblot and ELISA analysis showed that fish parvalbumins were not the causative allergens.

Pangasius and tilapia are distantly related in taxonomy, but tilapia is a member of the same order as mackerel and tuna (*Perciformes*). No IgE reactivity was found to protein extracts from mackerel or tuna in our patient, indicating that cross-reactivity between fish species cannot necessarily be assumed on the basis of taxonomic classification.

Sensitization to single fish species caused by allergens other than parvalbumin has already been reported [8-10]. In our patient, the presence of 2 cross-reactive, homologous allergens of 18 kDa and 45 kDa in pangasius and tilapia can be assumed from cross-inhibition assays. The identity of both proteins remains to be determined. The higher molecular weight protein might even represent a dimer of the 18-kDa allergen with preserved IgE reactivity. Weak IgE binding to a 45-kDa cod protein may reflect clinically nonsignificant cross-reactivity under denaturing conditions, a hypothesis supported by the negative BAT results and cross-inhibition assays with cod extract. In the present case, native conditions were more representative of clinical relevance than testing of IgE binding to denaturated proteins.

Diagnosis of IgE-mediated fish allergy is based on a precise clinical history and corroborated by different *in vitro* and *in vivo* tests. ImmunoCAP analysis is helpful to establish cross-reactivity, but is less sensitive and not always available for single species. In our patient, *in vitro* diagnosis of pangasius and tilapia allergy was documented by flow-assisted analysis of *in vitro* activated basophils (BAT) [19]. Simulating native conditions, this technique correlated well with the patient's history of fish allergy and allowed discrimination between clinically relevant and irrelevant specific IgE.

In summary, we have reported the clinical case of a fish-allergic patient sensitized to pangasius and tilapia, but not to other types of fish. Cross-reactivity between pangasius and tilapia was not caused by the panallergen parvalbumin, but by

putative 18-kDa and 45-kDa fish allergens. Both fish allergens might be representatives of a new fish allergen family with homologous proteins in other species, which gives impact to the present report. For developing strategies in component-resolved diagnosis, the exact nature of these new fish allergens needs to be investigated.

Acknowledgments

The authors would like to thank Christel Mertens and Paul van Enderit for their technical input. This study was supported by the Instituut voor de Aanmoediging van Innovatie door Wetenschap en Technologie van Vlaanderen: basophil activation new applications in nutritional allergy (BANANA) project (IWT 060521) and by the National Research Found, Luxembourg: food safety project SECAL I and II (FNR/03/07/03).

References

1. Hansen TK, Bindslev-Jensen C, Skov PS, Poulsen LK. Codfish allergy in adults: IgE cross-reactivity among fish species. *Ann Allergy Asthma Immunol.* 1997;78:187-94.
2. Van Do T, Elsayed S, Florvaag E, Hordvik I, Endresen C. Allergy to fish parvalbumins: Studies on the cross-reactivity of allergens from 9 commonly consumed fish. *J Allergy Clin Immunol* 2005; 116 (6): 1314-20.
3. Van Do T, Hordvik I, Endresen C, Elsayed S. The major allergen (parvalbumin) of codfish is encoded by at least two isotypic genes: cDNA cloning, expression and antibody binding of the recombinant allergens. *Mol Immunol.* 2003; 39: 595-602.
4. Lindstrom C, van Do T, Hordvik I, Endresen C, Elsayed S. Cloning of two distinct cDNAs encoding parvalbumin, the major allergen of Atlantic salmon (*Salmo salar*). *Scand J Immunol.* 1996; 44: 335-44.
5. Bugajska-Schretter A, Pastore A, Vangelista L, Rumpold H, Valenta R, Spitzauer S. Molecular and Immunological Characterization of Carp Parvalbumin, a Major Fish Allergen. *Int Arch Allergy Immunol.* 1999; 118 :306-8.
6. Das Dores S, Chopin C, Villaume C, Fleurence J, Gueant JL. IgE-binding and cross-reactivity of a new 41 kDa allergen of codfish. *Allergy.* 2002; 57 (72): 79-83.
7. Kuehn A, Hilger C, Hentges F. Anaphylaxis provoked by ingestion of marshmallows containing fish gelatin. *J Allergy Clin Immunol.* 2009; 123 (3): 708-9.
8. Kondo Y, Komatsubara R, Nakajima Y, Yasuda T, Kakami M, Tsuge I, Urisu A. Parvalbumin is not responsible for cross-reactivity between tuna and marlin: A case report. *J Allergy Clin Immunol.* 2006; 118 (6): 1382-3.
9. Kelso JM, Jones RT, Yunginger JW. Monospecific allergy to swordfish. *Ann Allergy Asthma Immunol.* 1996; 77 (3): 227-8.
10. Asero R, Mistrello G, Roncarolo D, Casarini M, Falagiani P. True monosensitivity to a tropical sole. *Allergy.* 1999; 54 (11): 1228-9.
11. Sten E, Hansen TK, Stahl Skov P, Andersen SB, Torp A, Bindslev-Jensen U, Bindslev-Jensen C, Poulsen LK. Cross-reactivity to eel, eelpout and ocean pout in codfish-allergic patients. *Allergy.* 2004; 59 (11): 1173-80.

12. Rosmilah M, Shahnaz M, Masita A, Noormalin A, Jamaludin M. Identification of major allergens of two species of local snappers: *Lutjanus argentimaculatus* (merah/ red snapper) and *Lutjanus johnii* (jenahak/ golden snapper). *Trop Biomed*. 2005; 22 (2): 171-7.
13. Rehbein H: New Fish on the German Market. consumer protection against fraud by identification of species. *J Verbr Lebensm*. 2008; 3: 49-53.
14. Alenius H, Kalkkinen N, Lukka M, Reunala T, Turjanmaa K, Makinen-Kiljunen S, Yip E, Palosuo T. Prohevein from the rubber tree (*Hevea brasiliensis*) is a major latex allergen. *Clin Exp Allergy*. 1995;25:659-65.
15. Hilger C, Grigioni F, Thill L, Mertens L, Hentges F. Severe IgE-mediated anaphylaxis following consumption of fried frog legs: definition of α -parvalbumin as the allergen in cause. *Allergy*. 2002; 57: 1053-8.
16. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970; 227: 680-685.
17. Lim DL, Neo KH, Yi FC, Chua KY, Goh DL, Shek LP, Giam YC, Van Bever HP, Lee BW. Parvalbumin – the major tropical fish allergen. *Pediatr Allergy Immunol*. 2008; 19: 399-407.
18. Lee SJ, Ju CC, Chu SL, Chien MS, Chan TH, Liao WL. Molecular cloning and expression analysis of parvalbumin in tilapia, *Oreochromis mossambicus*. *J Exp Zool*. 2007; 307A: 51-61.
19. Ebo DG, Bridts CH, Hagendorens MM, Aerts NE, De Clerck LS, Stevens WJ. Basophil activation test by flow cytometry: present and future applications in allergology. *Cytometry B Clin Cytom* 2008; 74:201-10.

■ *Manuscript received February 6, 2009; accepted for publication, May 15, 2009.*

■ **WJ Stevens**

Department of Immunology, Universiteit Antwerpen
Campus Drie Eiken, Universiteitsplein 1
BE-2610 Antwerpen, Belgium
E-mail: immuno@ua.ac.be