

Original article

Allergenic components of a novel food, Micronesian nut Nangai (*Canarium indicum*), shows IgE cross-reactivity in pollen allergic patients

Background: New foods may present a risk for food hypersensitive patients. Several examples exist of allergic reactions caused by cross-reactive plant-derived foods, and new foods should be scrutinised before introducing them to the market. We have evaluated the clinical and serological relevance of cross-reactivity between Nangai and pollen allergens.

Methods: Cross-reactivity was examined with Maxisorp RAST (radioallergosorbent test), RAST inhibition and Western blot, using sera from patients allergic to grass, birch and mugwort pollen. None of the patients reported having seen or eaten Nangai previously. To determine the biological and clinical relevance of the cross-reactivity, histamine release (HR) test, skin prick test (SPT) and food challenge were used.

Results: There was prevalence for reactivity against Nangai in the group of pollen allergic patients. This cross-reactivity seems to be related – at least in part – to carbohydrate epitopes. Three out of 12 patients tested with Nangai were positive upon open challenge, but using double blind placebo controlled food challenge (DBPCFC) this could not be confirmed in two patients. The biological effects of Nangai on allergic patients were confirmed using HR and SPT.

Conclusion: The Nangai specific IgE found among pollen allergic patients addresses the need for control of new or changed foods before introduction to the market.

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Key words: carbohydrate specific IgE; food allergy; Nangai; novel food; pollen cross-reactivity.

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Accepted for publication 30 November 2001

The number of individuals developing immunoglobulin E (IgE) dependent allergic reactions against different substances is increasing at a high rate in the industrial countries. At the same time new foods, food additives and genetically modified (GM) foods are being produced and introduced to the consumers, in many cases with poor evaluation of the probability of inducing allergic reactions.

So far no one has been able to predict whether an antigen will be allergenic, or in what way. It also remains unexplained why some patients react to grass, for example, while others react to mugwort, and some to both (1). For this reason, both *in vivo* and *in vitro* methods must be used to determine the reactivity of a specific protein (2).

Nangai is an edible tree nut cultivated by people on

the islands in the Pacific. Its Latin name is *Canarium indicum* and it belongs to the *Burseraceae* family. There is some interest in the introduction of Nangai to the European market and, although the European Union (EU) has classified the nut as a novel food, no studies have been reported on its potential allergenicity. No other members of this family are frequently imported to Europe as food.

By using both serological and biological methods we have evaluated the risk of IgE-mediated food allergic reactions against Nangai, in a previously non-exposed population (3). We found that a group of pollen allergic patients had specific IgE against Nangai and that this IgE seemed to mediate biological reactivity. According to our results we conclude that Nangai contains epitopes that bind to cross-reactive IgE specificities and that these IgE molecules have biological relevance.

Material and methods

Patients

For the serological studies, patients (referred to by capital letters) screened for Nangai reactivity with Maxisorp RAST were selected as

Abbreviations: c.p.m.: counts per minute; DBPCFC: double blind placebo controlled food challenge; Ig: immunoglobulin; FCS: fetal calf serum; HR: histamine release; RAST: radioallergosorbent test; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SPT: skin prick test.

having specific IgE against at least one of three pollens (mugwort, grass and birch) determined by CAP (Pharmacia, Uppsala, Sweden). The inclusion criteria for patients (referred to by numbers) undergoing oral challenge and biological tests were pollen allergy documented by SPT. There was no overlap between the two groups of patients. This study was approved by the local ethical committee (Den videnskabetiske komité for Vejle og Fyns amter) and informed consent was obtained from the patients.

Extracts

The Nangai nuts were imported from West Java, Indonesia.

The Nangai extract was produced by homogenizing 5 g of Nangai in 20 ml Pipes buffer, pH 7.4 (10 mM piperazine-N,N (bois-ethane sulfonic acid), 140 mM sodium acetate, 5 mM potassium acetate, 0.6 mM CaCl₂ and 1.1 mM MgCl₂). The suspension was then centrifuged at 1850 g for 10 min to remove insoluble debris. The extract was aliquoted and stored at -20°C until use. A hazelnut extract was produced in the same way using 5 g hazelnut/10 ml buffer. Mugwort, grass, birch, cat and dog allergen extracts were obtained from Pharmacia. The extracts were biologically standardized according to the Nordic Guidelines using BU/ml (4). The carbohydrate epitopes were a kind gift from Dr Stefan Vieths (5). MXF are N-linked glycopeptides purified from pineapple stem bromelain that contain only two to four amino acid residues. They have the glycan structure Man α 1-6(Xyl β 1-2)Man β 1-4GlcNAc β 1-4 (Fuc α 1-3) GlcNAc. The MM control glycopeptides prepared from bovine fibrin have the structure Man α 1-6(Man α 1-3)Man β 1-4GlcNAc β 1-4 GlcNAc.

Protein content of extract

The protein content of the Nangai extract was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin (BSA) as standard; 10 μ l of standard or sample was placed in microtitre plate wells and 200 μ l of Bio-Rad dye was added to each well. The plate was incubated at room temperature for 5–15 min and analyzed in a spectrophotometer Tim-200 (Teknunc, Roskilde, Denmark) at 600 nm. Standard curves were plotted as absorbance vs BSA concentration and the protein concentration of the extract was calculated in μ g/ml.

Maxisorp RAST

The Maxisorp RAST was performed as previously described (6). Briefly, Maxisorp tubes (Nunc, Roskilde, Denmark) were coated with 250 μ l of allergen extract diluted in phosphate buffer saline (PBS) and incubated at 37°C overnight. The Nangai extract was diluted to a protein content of 2 μ g/ml before being used for coating the tubes. Tubes were washed and incubated for 1–2 h at 37°C with 250 μ l of cell culture medium (RPMI 1640) including 5% fetal calf serum (FCS). After a second wash the tubes containing 250 μ l sample (serum) diluted in washing buffer (10 mM PBS 0.1% Tween) were incubated at room temperature overnight. The next day 250 μ l of anti-IgE-I¹²⁵ (Pharmacia) diluted 12 times in washing buffer was added to the washed tubes, which were incubated overnight at room temperature. After washing the gamma radiation was measured on a 1471 Wizard counter (Wallac, Allerød, Denmark) for 2 min. All samples were tested in duplicate. All incubations were performed on a vibrator. Values three times higher than those obtained with a serum pool from nonallergic patients were considered positive.

RAST inhibition

The RAST inhibitions were performed in the same way as the Maxisorp RAST described above, except that the sera were preincubated overnight at 4°C together with allergen extracts (mugwort, grass, birch, cat and dog extract at 10 000 BU/ml – 10

BU/ml; hazelnut, Nangai, MXF and FF at 20 μ g/ml – 0.02 μ g/ml) and centrifuged at 1650 g for 20 min, before use. The inhibition was calculated in percent as the c.p.m. value for the inhibited sample divided by the value of the uninhibited. The background values determined with a pool of sera from nonallergic donors were at all times subtracted.

Histamine release (HR)

Direct and passive HR was performed by the glass fibre technique according to the manufacturer (RefLab, Copenhagen, Denmark) (7, 8). Each extract was tested in six 3.5-fold dilutions, C1-C6 (stock solution 10% weight/volume). Histamine release results were expressed as Class C1 to C6 corresponding to the lowest dilution inducing a release > 15 ng histamine/ml blood.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), protein transfer and immunoblotting.

SDS-PAGE and Western blots were performed in accordance with the protocol described by Laemmli, 1970 (9). Briefly, the extracts were run on a 1.5-mm 16% gel with a 4% stacking gel. Samples were diluted in a reducing buffer and cooked before use. 40 μ l of sample or 10 μ l Rainbow marker (Amersham Pharmacia Biotech) was added to each well. The proteins were blotted to a PVDF membrane (Millipore, Bedford, MA) for 1.5 h (I = 1.0 mA/cm²). The membranes were blocked with Tris buffer saline (TBS) with 0.5% Tween (TBST) and 5% bovine serum albumin (BSA) for 2 h at room temperature before incubating overnight at 4°C together with the sera. For inhibition studies the sera were preincubated with extract (mugwort, grass, birch, cat and dog extract at 2000 BU/ml; hazelnut at 50 μ g/ml; Nangai at 20 μ g/ml) overnight before use. After washing the membranes were incubated with anti-IgE-HRP (Dako AS, Glostrup, Denmark) for 2 h and then washed again. ECL (Amersham Pharmacia Biotech) was used as substrate.

Skin prick test (SPT)

The SPT was performed according to the Nordic Guidelines (10) as a prick-prick in duplicate. SPT with a wheal diameter > 3 mm (average of two SPT) was considered positive.

Challenge

For open challenge the patients were given four doses with increasing

Table 1. Summary of the results of sera from A–K. Columns 1–2 show the Nangai specificity determined by RAST and passive histamine release. Columns 3–5 demonstrate the pollen reactivity determined by CAP. Columns 6–10 show the results of RAST inhibition of the Nangai specific IgE

Sera	RAST		CAP			RAST inhibition				
	Nangai	HR	Grass	Birch	Mugwort	Grass	Birch	Mugwort	Cat	Dog
A	+	+	4	1	3	+	+	+	nd	–
B	+	–	4	3	3	+	+	+	–	nd
C	+	+	3	4	4	–	–	+	nd	nd
D	+	+	4	4	4	+	+	+	–	–
E	+	–	2	2	2	+	+	+	–	–
F	+	+	4	4	3	+	+	–	–	–
G	+	nd	3	4	3	+	+	+	nd	nd
H	+	nd	4	4	3	+	+	+	–	–
I	+	nd	1	0	1	+	+	+	nd	nd
J	+	nd	4	3	3	+	+	+	nd	nd
K	+	nd	0	4	0	+	–	–	–	–

nd = not determined.

amounts of Nangai, to a total amount of 3.6 g. The patients were observed for 15 min between each dose and after the final dose for a minimum of 2 h.

Double blind placebo controlled food challenge (DBPCFC) was performed according to the EAACI guidelines (11). The Nangai nut or placebo (oats) was hidden in chocolate and given in five doses with a total amount of 5 g.

Results

To evaluate possible allergenicity we screened 64 patients with known pollen allergy, for Nangai specific IgE using Maxisorp RAST. These patients had specific IgE against at least one of the three pollens, mugwort, grass and birch, determined by CAP. Eleven (17%) of these patients were found to have Nangai specific IgE (Table 1, patients A–K). The c.p.m. values varied between 250 and 1500 when the sera were diluted 10 times and the sera could be diluted 1000 times before losing reactivity. To evaluate the Maxisorp RAST we preincubated the sera with Nangai extract and calculated the inhibition of binding. Using 20 µg/ml of Nangai extract at least 50% of the specific binding was inhibited. The CAP results of the screened donors and their Nangai reactivity are compared in Fig. 1A. Each circle represents IgE binding to one pollen extract, and sera with the capacity to bind Nangai are shown in black, and the rest in grey. The Nangai positive samples were predominantly (nine out of 11) found in the group of patients with reactivity against all three pollens. Sera from 12 control patients, without any known allergy, were tested for Nangai specific binding using Maxisorp RAST. All 12 were found to be negative (data not shown).

Randomly chosen patients (*n*=18) referred for allergy evaluation were screened for Nangai reactivity

Table 2. Summary of the results for the 18 patients with reactivity against Nangai

Patient number	Nangai specific			Open challenge
	Maxisorp RAST	SPT	HR	
1	+	+	+	–
2	–	+	+	–
3	–	–	+	–
4	–	+	+	–
5	–	–	+	–
6	+	–	–	–
7	–	+	–	nd
8	–	–	+	nd
9	+	+	–	+
10	–	–	+	nd
11	–	–	+	+
12	–	–	+	nd
13	–	–	+	nd
14	–	–	+	–
15	–	–	+	–
16	–	–	+	+
17	+	–	–	nd
18	+	–	+	–

nd = not determined.

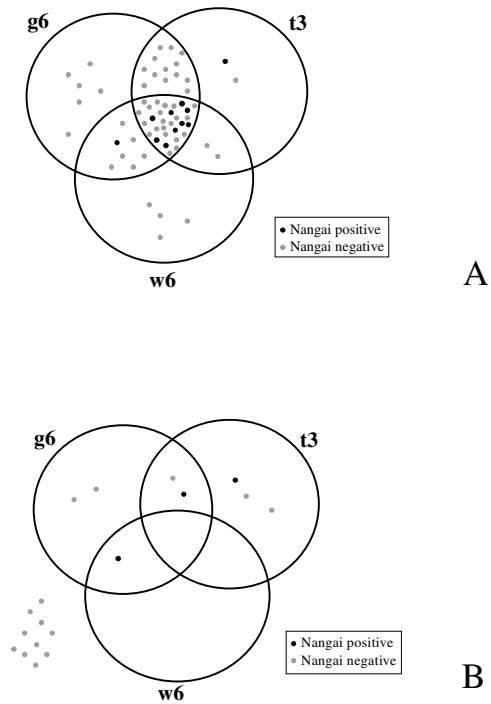


Figure 1. (A) Demonstration of the CAP results of the screened sera. Each dot demonstrates one serum and its reactivity against the three pollen extracts and Nangai determined by Maxisorp RAST. (B) Demonstration of the results of direct histamine release of randomly chosen patients with pollen and Nangai. g6 = grass, w6 = mugwort and t3 = birch.

using direct HR. These results were divided after pollen sensitivity, in the same way as the RAST results. The patients were not selected for pollen reactivity, and this sensitivity was only found in eight out of 18. However all three (out of 18; 17%) with Nangai specific reactivity (black dots) were also sensitive to at least one pollen (Fig. 1B).

Six of the sera with Nangai specific IgE (A–F) were tested using passive HR. Of these, four were able to mediate Nangai-induced histamine release (Table 1).

Thirty-six patients with documented allergy against one or more of the three pollens (grass, birch and mugwort) were chosen for biological tests. Eighteen (numbers 1–18) of these had reactivity against Nangai in at least one of three tests: SPT, HR or Maxisorp RAST (Table 2). Out of five RAST-positives, two were only positive using this test, and were negative in SPT and HR. Five patients had a positive SPT with Nangai and 14 were positive using HR. Three of these showed positive results using both SPT and HR of which one was positive in RAST as well. Similar differences in the results between RAST, SPT and HR were seen for pollen.

Twelve of the 18 patients with at least one positive Nangai specific test result were challenged openly with Nangai. Three of these had a positive reaction, two of

Table 3. Results of skin prick test, histamine release and DBPCFC of the patients with positive open challenge against Nangai. The sera were numbered according to Table 2

Patient number	SPT				HR				Challenge	
	Nangai	Mugwort	Grass	Birch	Nangai	Mugwort	Grass	Birch	Open	DBPC
9	+	+	+	+	-	+	+	+	+	-
11	-	+	-	+	+	+	+	+	+	nd
16	-	+	+	+	+	+	+	+	+	-

nd = not determined

which were further examined using DBPCFC. The reaction against Nangai could not, however, be confirmed with DBPCFC (Table 3).

Twelve control persons with no known allergy and no detectable Nangai specific IgE, according to the Maxisorp RAST results mentioned above, were also tested for Nangai reactivity with SPT and HR. All 12 were negative in SPT. Four out of 12 had positive HR but two of these were negative using anti-IgE, which was the positive control. One was positive in HR for a single concentration but showed no dose-dependency.

The sera (patients A–K in Table 1) determined to have Nangai specific IgE in the screening were used to study cross-reactivity. For this, RAST inhibition was performed using sera preincubated with pollen extracts. For eight out of 11 sera the binding to Nangai was inhibited by all three pollen extracts, grass, birch and mugwort. Inhibition curves for four representative samples are shown in Fig. 2A–2C. The amount of extract needed for 50% inhibition of the Nangai specific binding differed widely between the donors (150–9300 BU/ml). However, each individual patient demonstrated IC₅₀ values in the same range for grass, birch and mugwort. Fig. 2D shows a control inhibition using cat and dog extracts. These extracts could in no case significantly inhibit the IgE binding to Nangai.

Five samples were used for further cross-reactivity studies; numbers 1, 9, 17, and 18 (see Table 2) and patient D (see Table 1). The Nangai specific binding of four out of these five was inhibited by preincubation of

Table 4. Results from inhibition of the Nangai specific binding using RAST and immunoblot. The patients were numbered according to Tables 1 and 2

Patient	RAST								Western blot					
	w6	t3	g6	e1	e5	H	MXF	MM	w6	t3	g6	e1	e5	H
D	+	+	+	-	-	+	+	-	+	+	+	-	-	+
1	+	+	+	-	-	+	+	-	+	+	+	-	-	+
9	+	+	+	-	-	+	+	-	+	+	-	-	-	+
17	+	+	+	-	-	+	+	-	+	+	-	-	-	+
18	-	-	-	-	-	+	-	-	+	+	-	nd	nd	+

w6 = mugwort, t3 = birch, g6 = grass, e1 = cat, e5 = dog, H = hazelnut, MXF = carbohydrate epitope from pineapple stem bromelain, MM = carbohydrate epitope from bovine fibrin; nd = not determined.

*Only the 18 kDa band was inhibited.

the sera with mugwort, grass and birch determined by Maxisorp RAST (data not shown, patient D see Table 1). These sera were also preincubated with a hazelnut extract leading to inhibition of the binding, in all five cases, to the same extent as pollen (data not shown).

To determine if the cross-reactivity of Nangai could be due to carbohydrate specific IgE the sera were preincubated with carbohydrate epitopes from pineapple stem bromelain (MXF) and carbohydrate epitopes from bovine fibrin (MM), as a control (12). The Nangai specific IgE binding for four out of five sera was inhibited by MXF, whereas MM epitopes were in no cases able to inhibit the Nangai specific binding determined by RAST (Table 4).

For immunoblot, Nangai and pollen extracts were run on SDS-PAGE gel under reduced conditions and blotted to PVDF membranes. The complex pattern of IgE binding proteins were similar for all five patients tested, with the approximate size of the most common bands being 14, 18, 22, 28, 35, 40, 44, 46, 50 and 65 kDa, one of which is shown in Fig. 3, lane 4. The results of all five patients are summarized in Table 4. By preincubation of the sera with extracts of pollen we were able to inhibit the IgE binding to a large extent (results shown for one patient, Fig. 3, lanes 1–3). The

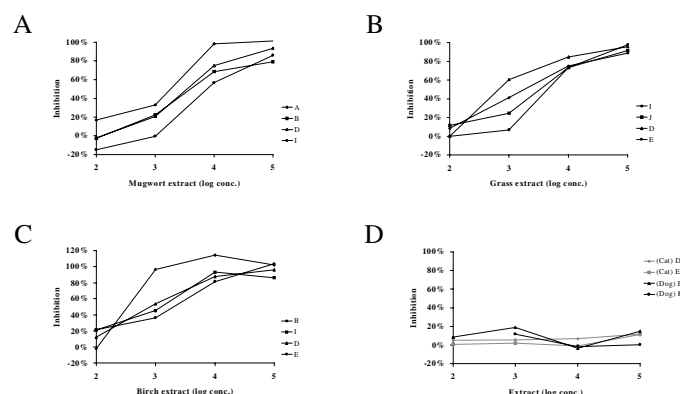


Figure 2. Inhibition of the Nangai specific binding by preincubation of the sera with different extracts (BU/ml). (A) mugwort extract, (B) grass extract, (C) birch extract, (D) cat or dog extracts.

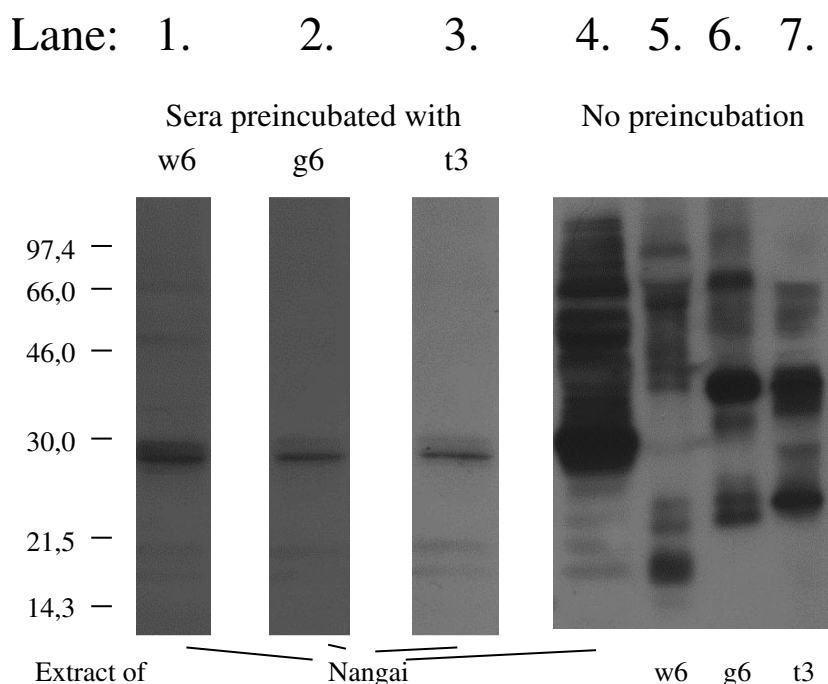


Figure 3. Demonstration of Western blot results with serum from patient D. Lanes 4–7 shows the specific IgE in the sera against the different extracts (Nangai, w6 = mugwort, g6 = grass and t3 = birch) while lanes 1–3 shows the binding to Nangai after preincubation of the sera overnight with pollen extracts 1) mugwort, 2) grass, and 3) birch.

IgE binding to Nangai was inhibited by birch extract in five, by mugwort extract in four, and by grass extract in two out of five cases (Table 4). In most cases the binding to all IgE specific proteins were reduced. In one case however (serum 9) only the binding to the 18 kDa band was inhibited when the sera were preincubated with birch or mugwort (data not shown). Hazelnut extract was also able to inhibit the binding to Nangai, while cat or dog extracts could not (Table 4).

Discussion

Food hypersensitive patients always face risk of reactions caused by unintentionally eaten non-tolerated food. In many cases this is due to insufficient labelling of processed foods, but there might also be a risk with new food items which are introduced to consumers without pre-screening for allergenicity. To avoid the risk of unwanted and unexpected cross-reactivities leading to allergic reactions, there is a definite and immediate need for tools to evaluate the allergenicity of new products. The Food and Agriculture Organization of the United Nations (FAO) has proposed new labelling procedures for foods of potentially allergenic nature. FAO states that foods and ingredients known to cause hypersensitivity should always be declared. Among the listed foods tree nuts are found. So far, no general control procedures have been established for this kind of evaluation of food allergenicity. We used a set-up whereby we screened patients with known allergies for specific IgE and used their sera for cross-

reactivity studies. Patients with the allergy pattern found to cross-react with Nangai were then further studied with both serological and biological methods.

Plant-derived food allergy is often correlated with hypersensitivity to pollen and in northern Europe most commonly to birch, grass and mugwort pollen (13). Among the pollen allergic patients up to 70% suffer from allergy against a variety of raw fruit, vegetables and nuts (14). Most of these allergic reactions are related to cross-reactive IgE caused by the sharing of common epitopes. The major birch pollen allergen Bet v 1 cross-reacts with similar proteins in a variety of foods like apple (Mal d 1), and celery (Api g) (15–17). The Bet v 1 related proteins are reported to be 16–20 kDa in size and might correlate with the 18 kDa band seen in Nangai. The protein found at 14 kDa could be related to profilin (Bet v 2), a pan-allergen frequently found to give rise to cross-reactivity within the botanic family. For grass pollen proteins of 41, 38, 24, 22, and 14 kDa have been reported to cross-react with kiwi (18–21). It is unlikely, however, that the Nangai extract should contain all of the above mentioned cross-reactive proteins. An alternative explanation could be IgE specific to carbohydrate determinants of glycoproteins (22). There are several reports regarding carbohydrate specific IgE in relation to cross-reactivity between vegetable foods, pollen and venom. Opinions differ about the biological and clinical relevance of such IgE (23, 24). Recently, clinical zucchini allergy was suggested to be related to carbohydrate epitopes comprising multiple bands

between 29 kDa and 96 kDa (12). Such multiple band patterns are also seen with Nangai. We hypothesize that Nangai contains carbohydrate determinants, which cross-react with pollen allergens. Preliminary experiments suggest that this cross-reactivity could also be extended to hazelnut. Patient variations in cross-reactivity patterns could be related to the primary sensitizing allergen or varying sensitivities of the methods towards different allergens.

Because of the common appearance of false positive results for food allergy using serological tests there is a need to perform DBPCFC to confirm clinical allergy (10). Both patients tested here for Nangai were negative. Two patients may be too small a sample to get a clear picture of the clinical relevance of Nangai. As we have only looked at the cross-reactivity between IgE

specific to Nangai and other allergens it is still unknown to what extent Nangai could act as primary sensitizing allergen.

Here we demonstrate the usefulness of a protocol to determine the risk of introducing new food to an earlier nonexposed market. By using both serological and biological methods we have demonstrated that Nangai cross-reacts with pollen and that this cross-reactivity, at least in part, is related to carbohydrate epitope specific IgE.

Acknowledgments

This study is part of the project "BioRisk" which is supported by The Danish Medical Research council. We thank Dr S. Vieths, Paul Ehrlich Institut, Langen, Germany for providing us with the carbohydrate epitopes.

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