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Cloning and expression of Ani s 9, a new *Anisakis simplex* allergen[☆]

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ABSTRACT

The larvae of the nematode *Anisakis simplex* parasitize seafood. When people eat raw or undercooked parasitized fish, they can suffer anisakiasis, an important immune human response to parasitic infection of the gastrointestinal tract. Even more, allergic manifestations like angioedema, urticaria or anaphylaxis can occur in sensitized patients. The aim of this work was to clone Ani s 9-cDNA and overproduce this recombinant allergen in *Escherichia coli*. The finding of this allergen was an unexpected result of a PCR using degenerate primers designed to amplify Ani s 5. The complete cDNA for Ani s 9 was obtained by RACE-PCR, cloned and sequenced. Expression of recombinant allergen was performed in *E. coli*. Immunodetection and immunoblot inhibition assays tests were carried out with sera from *Anisakis* allergic patients. The recombinant Ani s 9 (rAni s 9) is a protein of 147 amino acids. By immunoblot inhibition assay, it was located as a 14 kDa band present in a crude extract of the parasite. This new allergen is heat stable and is present in excretory/secretory products. Ani s 9 belongs to the SXP/RAL-2 family and shares amino acid sequence identity of 60% with As-14, an *Ascaris suum* allergen. Five of thirty-six *Anisakis* allergic patients (13.8%) were positive to rAni s 9 and natural Ani s 9 by immunodetection. In conclusion, Ani s 9 is a new allergen in *Anisakis* allergy and it has been cloned and successfully expressed in *E. coli*.

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1. Introduction

Anisakis simplex is a nematode parasite that can infect humans when live larvae are eaten in raw or undercooked seafood. The onset of infection may present acute epigastric pain, sometimes accompanied by nausea and vomiting [1]. The other common manifestation of human anisakiasis is an allergic reaction in sensitized individuals. These allergic symptoms can include urticaria, angioedema [2] and anaphylaxis appearing few hours after ingestion of infected fish [3].

Eight different proteins have been identified as allergens. Ani s 1 is a secretory protein of 21 kDa and the only major allergen described with no function nor homology described to date [4,5]. Ani s 2 (paramyosin, 100 kDa) [6] and Ani s 3 (tropomyosin, 41 kDa) [7] are muscular proteins. Ani s 6 (7 kDa) [8] and Ani s 4 (9 kDa) are protease inhibitors [9,10]. Ani s 5 (15 kDa) [8] and Ani s 8 (15 kDa) [11] are members of the SXP/RAL-2 family proteins. No information is available to date about Ani s 7 (139 kDa).

The SXP/RAL-2 proteins are a family of proteins specific to nematodes [12], including plant nematodes, like *Globodera rostochiensis* [13] and *Meloidogyne incognita* [14]. At our knowledge, nothing is known about the function of these proteins, but a great number of members of this family have been described as immunological active proteins. For example, Wb-SXP1 from *Wuchereria bancrofti* [12] and Bm-SXP1 from *Brugia malayi* [15], are useful for the diagnosis. Further, two *Ascaris* members of this family, As 14 and As 16 have been shown to confer protective immunity to *Ascaris suum* infections [16,17].

We are interested in identifying, characterizing and cloning of allergens from *A. simplex*. When we were cloning Ani s 5 by PCR with degenerated primers, an unexpected PCR product was obtained. This sequence had homology with As 14, from *A. suum*. Since this protein was described as allergen, we suspected that our sequence could be a new allergen and we decided to complete the sequence, clone and characterize it.

We report here the identification, cDNA cloning and expression in *Escherichia coli* of a new *Anisakis* allergen belonging to SXP/RAL-2 family protein like Ani s 5 and Ani s 8. This new allergen has been designated Ani s 9 by the WHO/IUIS Allergen Nomenclature Sub-Committee.

2. Materials and methods

2.1. *Anisakis* protein extract

Larvae were manually extracted from muscle tissue of *Micromesistius poutassou*. Approximately 150 larvae were ground in a

[☆] Note: The nucleotide sequence of Ani s 9 cDNA has been deposited in DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number EU074790. The new allergen has been submitted to the World Health Organization/International Union of Immunological Societies Allergen Nomenclature Sub-Committee.

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mortars with 5 ml of PBS. The mixture was centrifuged for 5 min at $10,000 \times g$ and the pellet was discarded to obtain the crude extract. This crude extract was heated in a boiling water bath for 30 min, centrifuged for 5 min at $10,000 \times g$ and the pellet was discarded. The supernatant was considered as a boiled extract.

To obtain an enriched preparation of Ani s 9, an ethanol fractionation was performed. The crude PBS extract obtained as above, was mixed with the same amount of absolute ethanol (50%, v/v) and left for 30 min at 4°C . Thereafter, the mixture was centrifuged at $4,000 \times g$ for 15 min, and the pellet was discarded. The ethanol content of the supernatant was increased to 66%. After 30 min at 4°C , the mixture was centrifuged at $4,000 \times g$ for 15 min, and the pellet obtained was discarded. The ethanol content of the last supernatant obtained was then increased to 90%, incubated for additional 30 min and centrifuged at $4,000 \times g$ for 15 min. The pellet obtained was resuspended in PBS and considered as 66–90% ethanol fraction [18].

The excretory/secretory products were obtained incubating 1.2 g of live larvae in 10 ml of 50 mM HCl for 18 h at 37°C . Then, the larvae were discarded and supernatant pH was adjusted to 7.2, dialysed against distilled water, freeze dried and resuspended in 300 μl of distilled water.

2.2. Sera and antibodies

Thirty-six sera of patients sensitized to *Anisakis* with a convincing history of *Anisakis* allergy and having specific IgE between class 2 and 6 determined by CAP FEIA (Phadia, Uppsala, Sweden) were tested with recombinant Ani s 9 by immunoblot. Five control sera with negative CAP to *Anisakis* were tested with recombinant Ani s 9 by immunoblot.

2.3. Isolation of cDNA encoding Ani s 9

Total RNA from larvae of *Anisakis* was extracted with Trizol (Gibco, Invitrogen, Carlsbad, CA, USA). The cDNA was synthesized using the Omniscript RT kit (Qiagen, Venlo, Netherlands). The cDNA obtained was amplified by PCR using two degenerated primers, a sense 5'-GCACCCCAAGATGTTGTSAAAGC-3' and antisense 5'-CYTSTYTGWCTGCTGGTGARAAT-3'. These primers were designed according to the alignment of As 16 (*A. suum*), Wb 14 (*W. bancrofti*), SXP-1 (*B. malayi*) DNA sequences (accession numbers AB089179, AF063940, M98813, respectively) and N-terminal protein sequence of Ani s 5 obtained in our laboratory. This N-terminal sequence was obtained after purification by ethanol fractionation and purification by reverse phase HPLC. The ethanol fractionation was done as described above and the 66–90% fraction was loaded on a Hypersil 300-C4 column (250 mm $\times$ 46 mm; particle size 5 μm ; Teknokroma, Barcelona, Spain) and eluted using a linear gradient of acetonitrile (10–90% in 1 h; 1 ml/min) in 0.1% trifluoroacetic acid. Peaks containing Ani s 5 in each chromatographic separation were identified by SDS-PAGE and immunodetection. N-terminal amino acid sequencing of Ani s 5 was performed automatically using a 477 gas-phase sequencer (Applied Biosystems, Foster City, CA) at the Protein Chemistry Department, Biological Investigation Centre, Madrid. The sequence obtained was: DDTPPPPFLAGAPQDVVKAFELL, this sequence was identical to the sequence described by Kobayashi et al. [8].

The PCR products were cloned into pGEM-T Easy (Promega) and sequenced. The sequence of one of these cloned PCR products showed that the antisense primer did not anneal and the PCR product obtained could only be attributed to the sense primer. But nevertheless, the sequence of this PCR product was similar to As 14, another allergen of *A. suum* [16].

To obtain the complete sequence of this protein 3'-RACE and 5'-RACE PCRs were performed using BD Smart RACE cDNA Ampli-

fication Kit (BD Biosciences, Erenbodeguen, Belgium), primer 5'-AATGTTGGAGCAAGGCAAAGCTCGA-3' for 3'-RACE and primer 5'-GTCGTTGTTTCTCTCCGACTTTGAGG-3' for 5'-RACE. The BLAST network server of the National Center for Biotechnology Information [19] was used to analyze the nucleotide sequence and deduced amino acid sequences in determining similarities with previously reported sequences in GenBank. Analysis of the signal sequence was performed using SignalP V 3.0 at the Center of Biological Sequence Analysis (<http://www.cbs.dtu.dk/services/SignalP/index.html>).

2.4. Expression of Ani s 9 fusion protein in E. coli

The coding region of Ani s 9, except the signal peptide was amplified by PCR using two primers, a sense primer 5'-GACGACGACAAGATGAAGCTCTGCATC-3' and antisense primer 5'-GAGGAGAAGCCCGTTAGCCTTGCAATTC-3'. The primers employed included the appropriate sequence (underlined) for directional cloning into the plasmid expression vector pET46 EK/LIC (Novagen, Merck KGaA, Darmstadt, Germany). This plasmid produces a protein with a histidine N-terminal tag. The resultant plasmid was transferred into *E. coli* BL21 Star (DE3) One Shot (Invitrogen, Carlsbad, CA, USA). Transformed bacteria were grown to an OD₆₀₀ of 1.0 at 37°C in SOB medium supplemented with ampicillin 50 $\mu\text{g/ml}$. Then, IPTG was added to a final concentration of 1 mM, and the culture was grown for additional 2 h at 37°C . The *E. coli* cells were pelleted and resuspended in lysis buffer (50 mM potassium phosphate, pH 7.8; 400 mM NaCl; 100 mM KCl; 10% glycerol; 0.5% Triton X-100; 10 mM imidazole). The suspension was disrupted with an ultrasonic cell disruptor (Misonix, New York, USA) on ice to obtain the lysate. Insoluble material was removed by centrifugation at $16,000 \times g$ for 10 min at room temperature.

2.5. Electrophoresis and immunological characterization

SDS-PAGE was performed in 16% polyacrylamide gels. After electrophoresis, proteins were stained with Coomassie Blue or were transferred by diffusion to two nitrocellulose sheets for 18 h and incubated with the sera (diluted 1:20). Specific IgE determination was performed with a monoclonal anti-IgE antiserum (Ingenasa, Madrid, Spain) and an alkaline phosphatase-labeled goat anti-mouse antiserum (Biosource Int., Camarillo, CA). Finally, the membranes were washed, and the signal was visualized with the alkaline phosphatase 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/4-nitroblue tetrazolium (NBT) system (Amresco, Solon, Ohio) for 30 min [4].

Alternatively, blocked membranes were immunodetected with mouse monoclonal antibodies to the histidine tag (1:1000 dilution, Novagen), then treated with anti-mouse IgG alkaline phosphatase conjugate (1:5000 dilution, Novagen) and finally developed as described above.

Immunoblot inhibition was performed as described above, except that the sera (500 μl) from *Anisakis* allergic patients were preincubated for 2 h at room temperature with 100 μl of bacterial extract containing recombinant Ani s 9.

3. Results

The cDNA encoding Ani s 9 comprised a 444 bp open reading frame coding for a protein of 147 amino acids. The first 17 amino acids corresponded to a signal peptide (Fig. 1A). Removal of the signal peptide resulted in a mature protein with 130 amino acids. The calculated molecular mass of mature protein was 13,781 Da and had an isoelectric point of 9.06. Four different clones were sequenced, but no differences at amino acid level were found.

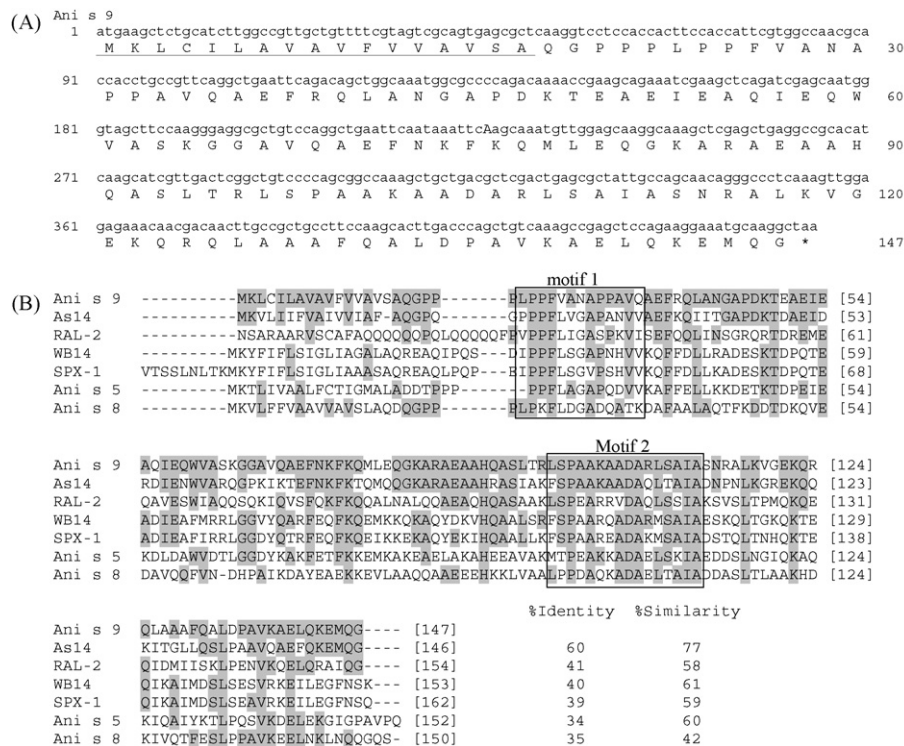


Fig. 1. Primary structure of Ani s 9. A. Ani s 9 nucleotide sequence. The deduced amino acid sequences are denoted below the nucleotide sequences. In frame stop codon is indicated by asterisk. The predicted peptide signal is underlined. B. Alignment of the deduced amino acid sequence with As 14 protein from *Ascaris suum* (AB057441), RAL-2 from *Acanthocheilonema viteae* (AF000609), WB-14 antigen from *Wuchereria bancrofti* (AF063940), SPX-1 antigen from *Brugia malayi* (M98813), Ani s 5 from *Anisakis simplex* (AB274998), Ani s 8 from *Anisakis simplex* (AB300647). Percentages of sequence identity and similarity with respect to Ani s 9 are included at the bottom right of the figure. The boxes show the motifs 1 and 2 of the SXP/RAL-2 family. Accession number of the Ani s 9 is EU074790*.

A homology search performed by BLAST [19] revealed that Ani s 9 shared the highest amino acid sequence identity with AS14 protein from *A. suum* (60%). Ani s 9 shared 41% identity with RAL-2 protein from *Acanthocheilonema viteae*, 40% with WB-14 from *W. bancrofti*, and 39% with SPX-1 from *B. malayi*. Ani s 9 shares a 34% of identity of sequence with Ani s 5 and a 35% of identity of sequence with Ani s 8 (Fig. 1B). All of these proteins with similarity with Ani s 9 belong to the SXP/RAL-2 protein family. For this reason, this new allergen should be included into this family of nematode proteins.

As a previous step to study the reactivity of Ani s 9 to IgE of sera from *Anisakis* allergic patients, the pET46 EK/LIC expression vector carrying the Ani s 9 cDNA coding sequence was used to transform *E. coli* BL21 cells. The recombinant Ani s 9 was expressed after induction with IPTG (2 h) and found to migrate as an 15–16 kDa fusion protein with a hexahistidine tag by SDS-PAGE (Fig. 2A). Most of rAni s 9 was recovered as soluble protein in the supernatant fraction (not shown). Then, we analyzed the *E. coli* lysate of the induced culture by immunodetection to confirm the correct expression of the protein. We used a monoclonal antibody directed against the hexahistidine amino-terminal fusion peptide of rAni s 9. As expected, a band with a molecular weight of 15–16 kDa corresponding to rAni s 9 was recognized in the lysate of *E. coli* induced by IPTG but not in the lysate of non-induced bacteria (Fig. 2B).

To localize the band protein corresponding to native Ani s 9, an immunoblot inhibition with recombinant Ani s 9 was performed (Fig. 3). The recombinant Ani s 9 inhibited completely the IgE binding to a 14 kDa band present in the crude extract of *Anisakis*. Besides, the same molecular weight band was inhibited in a boiled extract, in a 66–90% ethanol fractionated extract enriched in Ani s 9 (see Section 2) and in excretion/secretion products. This 14 kDa band was not inhibited when an immunoblot inhibition with an extract

of bacteria not expressing Ani s 9 was performed (data not shown). These results led us to conclude that the 14 kDa band that disappeared in all tested extracts is the natural Ani s 9. Furthermore Ani s 9 is present in excretion/secretion products, although it is more abundant in the crude extract (Fig. 3).

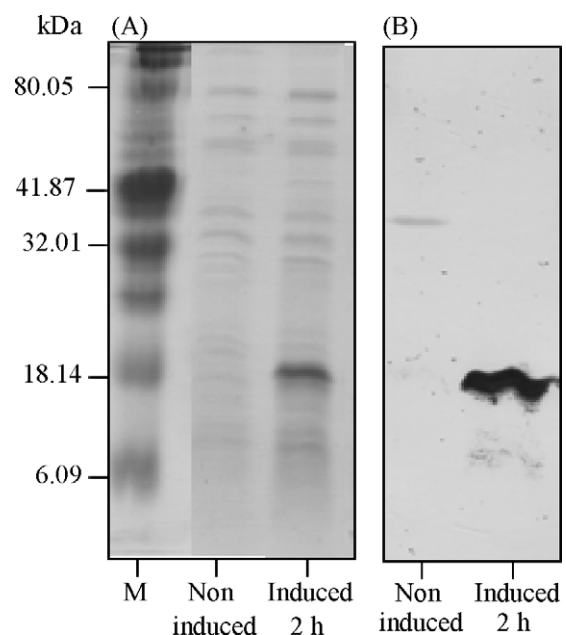


Fig. 2. Expression of rAni s 9. A. Time course of the recombinant production was analyzed by Coomassie Blue staining after SDS-PAGE at different times; not induced, before IPTG addition; induced 2 h, after IPTG addition. B. Immunodetection with mouse monoclonal antibodies directed against the hexahistidine amino terminal tag.

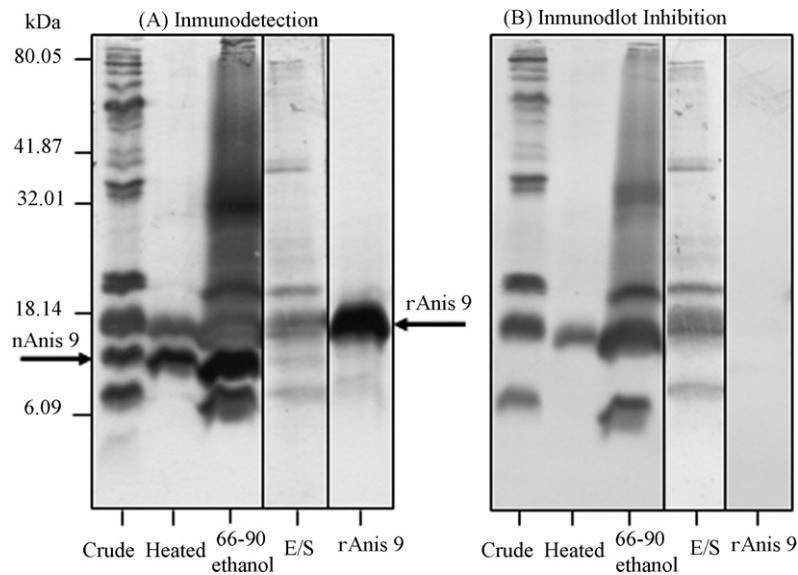


Fig. 3. Identification of Ani s 9 in different extracts. Crude extract, boiled extract, 66–90% ethanol enriched extract, E/S excretion/secretion products, rAni s 9 is *E. coli* lysate containing rAni s 9. A. IgE binding to nitrocellulose-blotted samples after SDS-PAGE with a serum from an Ani s 9 positive patient, the arrows show the bands corresponding to natural Ani s 9 (nAni s 9) and recombinant Ani s 9 (rAni s 9), respectively. B. The same immunodetection but inhibited with 100 μ l of *E. coli* lysate expressing rAni s 9.

To study the allergenicity after heat treatment of Ani s 9, we tested a heated rAni s 9 by immunoblotting (Fig. 4). The patient sera recognized both non-heated and heated protein. This fact means that Ani s 9 is a heat-stable allergen.

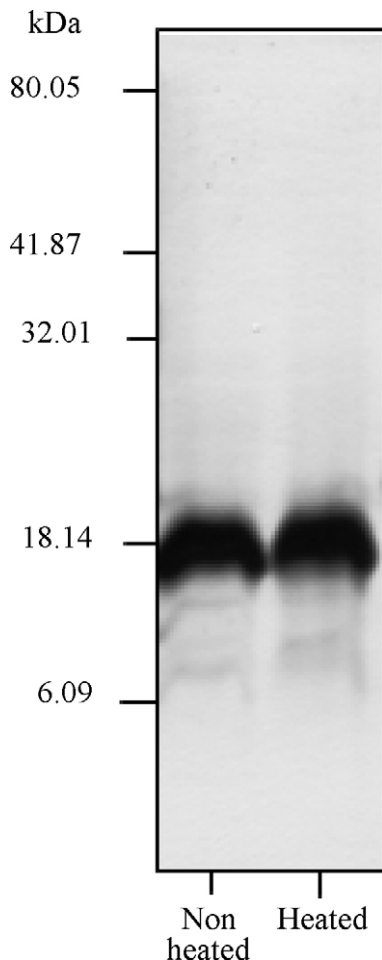


Fig. 4. Analysis of heat stability of rAni s 9 by immunoblot. Heated: rAni s 9 boiled (95 °C) for 30 min.

A total of thirty-six patients with positive CAP to *Anisakis* and convincing history of *Anisakis* allergy (Table 1) were tested using recombinant Ani s 9 and natural Ani s 9 by immunoblot. Of those patients, five recognized Ani s 9 (13% of the patients), and all of them recognized both, the natural and the recombinant form (Fig. 5). Besides, we tested five negative controls with recombinant Ani s 9 by immunoblot. None of them recognized recombinant Ani s 9 (data not shown).

4. Discussion

Anisakis infection and *Anisakis* allergy are prevalent particularly in countries where seafood is an important part of the diet such as Japan and Spain [20]. People of these countries usually eat undercooked or marinated fish, specially served with acid sauces, habits that increase the risk of *Anisakis* infection and/or allergic symptoms [21].

Ani s 9 belongs to SXP/RAL-2 family proteins, an intriguing family with unknown function. This family is only present in nematodes, including plant nematodes [13,14]. There are two more *Anisakis* allergen members of this family, Ani s 5 and Ani s 8. Ani s 9 shares a 34% sequence identity with Ani s 5 and a human sera monosensitized against Ani s 5 did not recognize Ani s 9. This preliminary fact suggest there are not cross-reaction between Ani s 5 and Ani s 9. The sequence identity with Ani s 8 is 35%, but we do not know if there is any cross-reactivity between Ani s 8 and Ani s 9.

The SXP/RAL-2 proteins seem to be important for parasitism in nematodes. In this way, nasal immunization with rAs 14 protects against *A. suum* infection in mice [16], and gerbils immunized with recombinant *B. malayi* SXP-1 had significantly reduced worm burdens after challenge [22]. The SXP/RAL-2 protein family has two characteristic motifs, the first motif is ([I/V]PPFLXGAPXXVV) and the second motif is ([F/L]SP[E/A]A[R/K]XADAX[M/L][S/T]AIA) [12]. With regard to the first motif, Ani s 9 has Valine instead of Leucine at position 5, which is a conservative change. Besides, Ani s 9 has two other non-conservative changes, at position 7 Asparagine instead of Glycine, and position 13 Glutamine instead of Valine. Since we do not know the function of these proteins, we cannot know if these changes are related with some function. The second motif is well conserved in our allergen.

Table 1Clinical data, specific IgE levels and sensitization to Ani s 9 of the thirty-six selected patients with allergy to *Anisakis simplex*

Patient	Age (years)/sex	Symptoms	Specific IgE (kU/l)	Ani s 9
1	68/M	Urticaria	67.4	
2	67/M	Urticaria/epigastralgia/diarrhea	26.5	
3	40/F	Urticaria/diarrhea/abdominal pain/vomit	>100	
4	29/F	Urticaria/epigastralgia	36.7	
5	58/M	Anaphylaxis/urticaria	41.7	
6	62/F	Epigastralgia	89.4	
7	28/F	Urticaria	22	
8	39/M	Urticaria	8.3	+
9	60/F	Urticaria/pruritus/dyspnea	>100	
10	42/F	Contact urticaria/epigastralgia	>100	+
11	31/M	Urticaria	1.2	
12	53/F	Urticaria	>100	+
13	29/F	Urticaria/epigastralgia/contact pruritus	26	
14	62/F	Urticaria/ dyspnea/diarrhea	72.6	+
15	40/F	Vomit/nausea/diarrhea	20.7	
16	39/F	Urticaria/contact dermatitis/epigastralgia	3.5	
17	51/F	Urticaria/vomit/dyspnea	1.3	
18	59/M	Pruritus	4.9	
19	25/M	Epigastralgia	16.9	
20	50/F	Urticaria	9	
21	43/M	Vomit/diarrhea/urticaria	6.9	
22	67/M	Urticaria/epigastralgia	25.1	
23	32/F	Urticaria/epigastralgia/dyspnea	18.7	+
24	30/F	Epigastralgia	7.1	
25	55/F	Urticaria/epigastralgia	2.2	
26	32/M	Epigastralgia/nausea/angioedema	2.1	
27	18/F	Abdominal pain/urticaria/angioedema	16	
28	62/F	Epigastralgia	50.3	
29	58/M	Nausea/vomit/urticaria	18.7	
30	80/F	Urticaria	91.8	
31	56/M	Urticaria	1.1	
32	68/F	Pruritus	40.4	
33	30/M	Epigastralgia	2.7	
34	43/M	Anaphylaxis/urticaria	>100	
35	42/M	Urticaria	6.3	
36	65/F	Urticaria	63.3	

M, male; F, female; negative result for IgE < 0.35 kU/l.

It has been described that excretory/secretory products from the *Anisakis* larvae are more allergenic than somatic products and more useful for diagnosis due to their higher specificity [21]. Since Ani s 9 has 77% of similarity with As 14 from *A. suum* and this allergen is present in the excretory/secretory products [16], we wanted to test the presence of Ani s 9 in excretory/secretory products of third-stage larvae of *A. simplex*. According to our results, Ani s 9

is expressed in excretory/secretory products, but at lower level than in the crude extract. The protein As 14 is as abundant in the excretory/secretory products as in the crude extract [16]. It is possible that the different way employed to obtain these products can influence the expression of Ani s 9. Another possibility is *Anisakis* and *Ascaris* are different organisms and the composition of excretory/secretory products is therefore different, so it is possible that

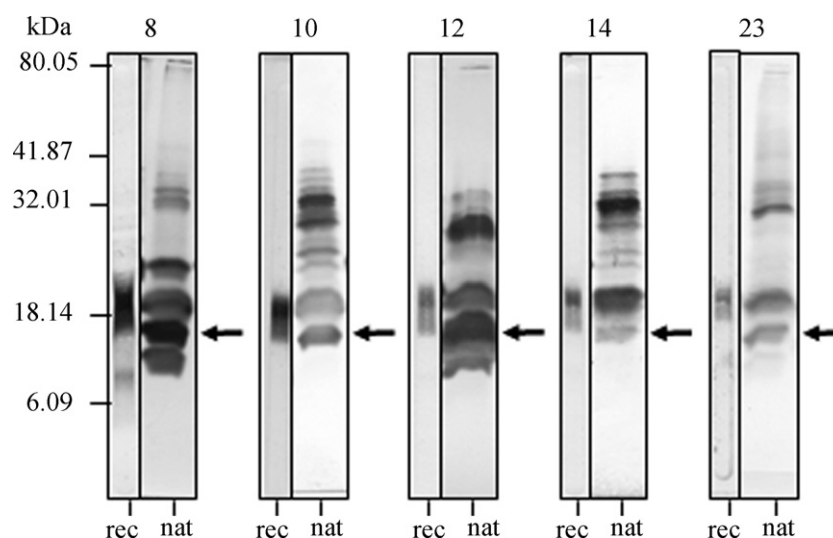


Fig. 5. Immunoglobulin E-immunodetection with five individual sera from *Anisakis* allergic patients. Rec: supernatant fraction from *Escherichia coli* lysate expressing rAni s 9, Nat: 66–90% ethanol extract enriched in natural Ani s 9. The arrows show the band corresponding to natural Ani s 9.

As 14 has high levels of expression in *Ascaris* and Ani s 9 has low levels of expression in *Anisakis*.

The heat-stable allergens are related with allergic episodes with well cooked or canned fish [9]. For this reason, these allergens are highly relevant, although they are minor allergens attending to their frequency of recognition. However, frequency of recognition is not always equivalent to clinical relevance [23]. The other allergens of *Anisakis* having heat stability and low frequency are Ani s 4 [9] and Ani s 8 [8].

Due to clinical interest of the allergens of *Anisakis*, even minor allergens such as Ani s 9 can be useful for the diagnosis of some patients and may be useful in a future therapy of *Anisakis* infection or allergy. In conclusion, a new heat-stable 15 kDa allergen (Ani s 9) was identified, cloned and successfully expressed in bacteria.

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