

Original article

Characterization of allergens of *Anisakis simplex*

Background: *Anisakis simplex* is an intestinal parasite of sea mammals. The larvae infect crustaceans, cephalopods and fish. Humans may consume *A. simplex* third stage larvae (L3) when eating infected raw or under-cooked fish. Consumed larvae cause an inflammatory reaction when they penetrate the digestive mucosa. The larvae or their secretory/excretory products can sensitize humans and induce an immunoglobulin E (IgE)-mediated allergic reaction. This parasite is now being implicated in numerous cases of allergic reactions after eating fish. The purpose of this study was to evaluate the allergenicity of proteins present in an extract of the third stage larva.

Methods: Rabbit antiserum raised to *A. simplex* somatic extract (L3) was reacted by crossed immunoelectrophoresis (CIE) with the same somatic extract. Crossed radioimmuno-electrophoresis (CRIE) was also performed by incubating CIE gels first in the sera of 13 individuals with positive immunoCAP to *A. simplex* and then in radiolabeled anti-human IgE.

Results: Twelve to 16 antigen–antibody precipitin peaks were visualized on Coomassie blue stained CIE gels in which somatic extract was reacted with somatic-antiserum. Autoradiography of CRIE gels showed that 18 different proteins bound IgE in patient sera. Individual patients had serum IgE directed at two to 10 different allergens. Five of these allergens were recognized by ≥50% of the patients. No allergen was recognized by every patient and no patient had serum IgE directed at all 18 allergens.

Conclusion: Somatic extracts of *A. simplex* L3 larva contain a large number of allergenic molecules and there is significant variability between patients in their sensitivity and reactivity to these allergens.

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The nematode, *Anisakis simplex*, is an intestinal parasite of sea mammals. Eggs from the female parasite are passed in the feces of these definitive hosts and then are eaten by marine crustaceans where the first stage larvae (L1) emerge from the eggs. Fish and cephalopods consume the infested crustaceans. In these intermediate hosts, the L1 larvae develop to the second stage larvae (L2) and then to the third stage larvae (L3). Whales, dolphins, sea lions and other marine mammals eat the infected fish and cephalopods containing the L3 larvae. The L3 develop to the fourth stage larvae (L4) and then into adults in the intestine of these hosts.

Humans may consume *A. simplex* L3 larvae when eating infected raw, cooked or under-cooked fish or cephalopods. Fish known to contain L3 larvae include herring, red fish, hake, sockeye salmon, cod, sardine, pollack, mackerel, sole and anchovies (1–6). The L3 larvae may invade the human gastric or intestinal mucosa where they generally eventually die (7). However, consumed live L3 larvae can cause an inflammatory/immune reaction when they penetrate the digestive mucosa and can migrate to tissues of the lungs, pancreas and liver (8). This condition is known as anisakidosis. The L3 larva can

also develop to the L4 stage in the human intestine or other tissues (9).

This parasite is now being implicated in numerous cases of immunoglobulin E (IgE)-mediated food allergy (10) after persons eat cooked or raw fish. The presence of serum IgE to *A. simplex* is high in some populations and sensitivity may range from 6 to 56% in adult patients (11–14). It has been suggested that both dead and live larva or larval secretory products can sensitize humans and induce an IgE-mediated food allergy but this is unclear. A study by Sastre et al. (15) suggested that only ingestion of live larvae induces an allergic reaction because ingestion of an extract of lyophilized larvae did not. Regardless, extracts made from larva do induce a positive skin test in many patients (14).

The allergens of *A. simplex* have not yet been fully characterized. Western blotting studies have identified many IgE binding peptides in extracts made from homogenized L3 larval bodies or excretory/secretory products collected from L3 larva maintained *in vitro* (9, 16, 17). Monoclonal antibodies that recognize epitopes on somatic and excretory/secretory allergens have been developed and some of these may be candidates for serodiagnosis of anisakidosis (9). Some of the allergens of

A. simplex are heat stable, maintain their allergenicity in cooked fish and may be responsible for inducing symptoms (18–20). Others have been reported to be susceptible to thermal degradation with a 5-min incubation at 100°C (conditions typically used in the preparation of samples for electrophoresis) destroying the ability of these allergens to bind human IgE (21).

The purpose of this study was to characterize the allergenicity of proteins present in an extract of the third stage larvae of *A. simplex* using crossed-immunoelectrophoresis. This technique was employed because it does not subject samples to the elevated temperatures that have been reported to alter IgE binding to the allergens of this parasite.

Materials and methods

Antigen extracts

Aqueous extracts were prepared from homogenates of *A. simplex* third stage larval (L3) somatic material by C.B.F. Leti, Tres Cantos, Spain. Live larvae of *A. simplex* were collected from *Micromesistius poutassou* (Bacaladilla, Couch's Whiting) and *Gadus merluccius* (Merluza, Hake) with forceps and identified by expert parasitologists. Extracts were prepared by methods previously described (6). Twenty grams of larvae were frozen at –80°C for 5 days and lyophilized for 3 days. The lyophilized larvae were crushed and extracted 1 : 50 (W/V) in ammonium bicarbonate, 0.125 M, pH 8.2, for 16 h at 4°C, and centrifuged. The pellets was discarded and the remaining solution was filtered through 0.22 µm, dialyzed in 3.5 kD membranes and lyophilized. Lyophilized material was reconstituted in glass distilled water.

Rabbit antiserum

Antiserum was raised in two rabbits (#2091 and 2098) immunized with the *A. simplex* somatic extract. Antiserum was supplied by C.B.F. Leti, Tres Cantos, Spain.

Human serum

Serum from 13 individuals (mean age 46 years, range 32–72 years) who had experienced allergic reactions (urticaria/angioedema, abdominal pain or anaphylaxis) after eating undercooked or marinated fish and who showed a positive immunoCAP (>0.35 kU/ml) to *A. simplex* was obtained from Fundación Jiménez Díaz, Spain (Table 1). A serum pool was prepared using equal volumes of the 13 positive sera. Negative control serum (US1 and US2) was from two subjects with no history of exposure to any of the materials tested in this study. All subjects provided informed consent.

Crossed-immunoelectrophoresis

Crossed immunoelectrophoresis (CIE) was performed as previously described (22). Briefly, 10 µl (42 µg) of *A. simplex* somatic extract was electrophoresed for 30 min at 200 V in the first dimension 1% agarose gel. Lanes containing separated proteins were excised and placed transversely onto a piece of GelBond film (FMC Bioproducts, Rockland, ME). An upper gel (anodic) containing 75 µl of rabbit antiserum in 3 ml of 1% agarose and a lower gel (cathodic)

Table 1. ImmunoCAP scores for the 13 *Anisakis simplex* sera used in this study. CAP values >0.35 kU/ml were considered to be positive

Serum code	CAP value kU/ml
ANS N94	10.9
ANS L927	>100
ANS 757	>100
ANS N188	49
ANS L815	93.4
ANS L811	>100
ANS N0965	>100
ANS N315	>100
ANS 2065	40.6
ANS 1851	7.68
ANS N177	>100
ANS 2081	21
ANS N341	92

with 25 µl of serum in 1 ml of agarose were poured and electrophoresed overnight at 50 V. Gel plates were washed, pressed, dried and stained with Coomassie Brilliant Blue R-250 (Sigma Chemicals Co., St Louis, MO).

Crossed-radioimmunoelectrophoresis

Crossed radioimmunoelectrophoresis (CRIE) was performed on homologous gels prepared using *A. simplex* somatic extract precipitated with rabbit antiserum raised against this antigen. Unstained gels (two per tray) were incubated overnight at room temperature in 0.5 ml of serum diluted in 20 ml of 0.1 M phosphate buffer, pH 7.5 with 2 mg/ml of bovine serum albumin, 1% Tween 20 and 0.05% sodium azide (RAST buffer). Immunoglobulin E-binding allergens were visualized as previously described (22) by incubating gels with 0.5 µCi ¹²⁵I-labeled anti-human-IgE (¹²⁵I-labeled goat anti-human-IgE tracer; Diamed Inc., Windham, ME) diluted in 20 ml of buffer. Gels were washed, dried and placed in cassettes with tungsten-enhancement screens and Kodak X-OMAT LS film (Eastman Kodak Co., Rochester, NY) at –80°C. Film was exposed for 2, 3, 7, or 19 days. To standardize and to make comparisons, all gels were processed identically in one batch with one lot of reagents.

Results

Reaction of the *A. simplex* somatic extract with the serum from the two rabbits immunized with this material resulted in the precipitation of 16 Coomassie blue stained antigens (Figs 1 and 2). When homologous CIE gels were incubated in the serum from the immunoCAP-positive human patients, antigen/antibody complexes bound IgE. Three antigens (1X, 2X, and 3X) were not visualized by Coomassie Blue staining. Because the two antisera gave different peak heights for the various antigens (making some antigens easier to see with one antiserum than with the other), both were used in the CRIE experiment.

All of the immunoCAP-positive test sera showed IgE binding to the CRIE gels while no IgE binding was observed from either of the control sera (Tables 2 and 3). The individual patients showed IgE binding to from two to 10 different allergens. Antigens 4, 5, 9, 11, and 16 were the

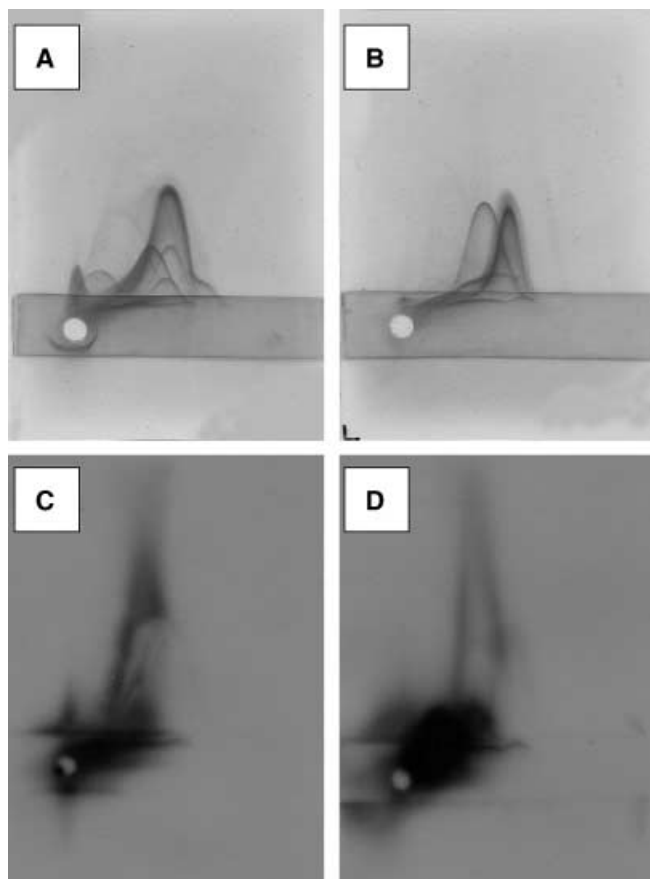


Figure 1. Homologous crossed immunoelectrophoresis and crossed radioimmuno-electrophoresis of *Anisakis simplex* somatic extract. (A, C) Gels using antiserum from rabbit #2091. (B, D) Gels using antiserum #2098. (A, B) Coomassie blue stained gels. (C, D) Gels probed with the serum from patient N0965 followed by ^{125}I -anti-IgE. Autoradiography films exposed for 3 days.

major allergens and showed IgE binding on both sets of CRIE gels by $\geq 50\%$ of the test subjects. In most cases, these allergens were also visible after only 2 or 3 days of autoradiography. Allergens 6, 10, 1X, and 2X were also important because they showed IgE binding to one or the other set of CRIE gels by $\geq 50\%$ of patients. One antigen-antibody complex did not bind IgE from any of the reference sera. No allergen was recognized by every patient and no patient had serum IgE directed at all 18 allergens.

Discussion

In this study, we used CRIE in order to identify allergenic molecules in a somatic extract of the L3 larva of *A. simplex*. The CRIE was used because this technique allows identification of intact allergenic molecules. Each peak on a CRIE gel represents an intact molecule that has been precipitated by polyclonal antibodies in antisera built in rabbits immunized with the extract in question. Although

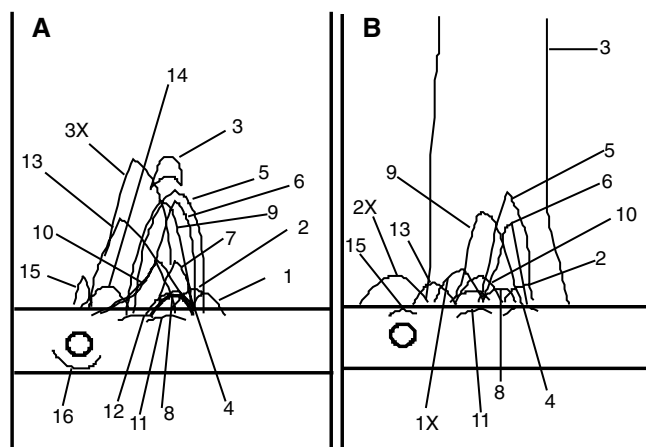


Figure 2. Schematic representations of homologous crossed immunoelectrophoresis of *Anisakis simplex* somatic extract. Individual antigens/allergens are identified by number based on migration rate in the first dimension. Allergens numbered with X were visible on autoradiograms but not on the Coomassie blue stained gel. (A) Schematic of gel using serum from rabbit #2091. (B) Schematic of gel using serum from rabbit #2098.

sodium dodecyl sulphate-polyacrylamide gel electrophoresis coupled with Western blotting identifies allergenic proteins/peptides and provides their approximate molecular weights, this technique typically includes a heating step during sample preparation. A recent study demonstrated that some of the allergens of *A. simplex* are very thermolabile while others are quite heat stable (21). We chose to use CIE/CRIE in this study as these techniques do not include any heating during sample preparation and would therefore preserve the IgE-binding properties of all the allergen molecules present in the *A. simplex* extract.

With the CRIE technique, it is possible that the rabbit antiserum may completely bind to allergenic epitopes on these molecules in the antigen-antibody complex and thus these allergenic epitopes may not be available for binding by IgE in the serum of a sensitized patient. However, extensive research (including our own using house dust and storage mites) shows this is rarely, if ever, the case and most allergenic molecules from a source can be identified. Rather, the rabbit antibody-antigen complex that forms leaves many allergenic epitopes available for IgE in human serum to bind. It is also possible that a protein that is allergenic for humans may not be precipitated because it is not an immunogen for rabbits that therefore produce no antibody. This is also rarely the case.

Our studies showed that immunized rabbit antisera contained antibody that recognized at least 19 different immunogenic proteins in an extract made from the bodies of L3 larva of *A. simplex*. Eighteen of these proteins bound IgE from our reference sera that were obtained from *A. simplex* sensitized (allergic) individuals (patients). Five of the allergens were major allergens as $\geq 50\%$ of the patient sera contained IgE that recognized epitopes on these molecules. These were visualized after 2–3 days of

Table 2. Antigens visualized on crossed radioimmuno-electrophoresis gels of *Anisakis simplex* somatic extract precipitated with serum from rabbit 2091

Subject	Antigen number																			Total
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	1X	2X	3X	
N94																				0
L927	+			+++	+++	+++			+++	+++	++++	++++				++				9
757			++	+++	+++	+++	+++		+++		+++	+++			++	+				10
N188			++	+		++				+	++	++	+			+				8
L815				++	+				+	++	++					+				7
L811				+++	+++	+++				+++	+++		+		+++	++				8
N0965			++	+++	++++				+++	++++	+++	+++	+	+		+				10
N315																				
2065				++	+						++					+				4
1851					++++	++++			++		+++		+		++					6
N177			++++			++++					++++				++					4
2081							++		++	++										3
N341				+++	++				++	++	++++		++		++++				++	8
# +	1	0	4	8	8	7	2	0	7	7	10	4	5	1	5	7	0	0	1	
% +	8%	0	31%	62%	62%	54%	15%	0	54%	54%	77%	31%	38%	8%	38%	54%	0	0	8%	
Pool			+	++	++	+++				++	+++				+					7
US1																				0
US2																				0

++++, First seen at 2 days; +++, first seen at 3 days; ++, first seen at 7 days; +, first seen at 19 days.

Shaded row for N315 indicates that the CIE incubated in this serum did not run well.

Sera N94 to N341 were from patients who had documented IgE-mediated food allergy. The serum pool was prepared by mixing equal volumes of the 13 positive sera. Sera US1 and US2 were negative control sera from two subjects with no history or exposure to *A. simplex*.

Table 3. Antigens visualized on crossed radioimmuno-electrophoresis gels of *Anisakis simplex* somatic extract precipitated with serum from rabbit 2098

Subject	Antigen number																			Total
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	1X	2X	3X	
N94				+												++		+++		3
L927				+++	+++				+++							+				4
757																+	+++	+++		3
N188				+	+				+	+						+	++	++		7
L815				++					+		+++					+	+			5
L811				++	+++	++		+	+++		++					+	+			8
N0965			++	+++	+++				+++	++++	+++	++				+	++++	+++		10
N315				+++	++				++	+++	+++					+	+++	+++		8
2065				++							++									2
1851			+		++				++		+++						+++	+		6
N177			+++			++++		++			+++						+			5
2081				+		++			++	+							++			6
N341			+	++++	+++			++	+++		++++							++		7
# +	0	0	4	10	7	3	0	3	9	4	9	1	0	0	0	8	9	7	0	
% +	0	0	29%	71%	50%	21%	0	21%	64%	29%	64%	7%	0	0	0	57%	64%	50%	0	
Pool			+	++	++	++			++		++						+	+		8
US1																				0
US2																				0

++++, First seen at 2 days; +++, first seen at 3 days; ++, first seen at 7 days; +, first seen at 19 days.

Shaded columns indicate that these peaks (#1, 7, 13, 14) were not visible on the stained CIE using serum 2098.

Sera N94 to N341 were from patients who had documented IgE-mediated food allergy. The serum pool was prepared by mixing equal volumes of the 13 positive sera. Sera US1 and US2 were negative control sera from two subjects with no history or exposure to *A. simplex*.

autoradiography. This would suggest that the patients had high titers of IgE to a few allergenic epitopes or moderate levels of serum IgE to many epitopes on these molecules. This could not be determined because the allergenic

epitopes on *A. simplex* molecules have not been identified or sequenced. A second set of four other allergens were also visualized by >50% of the sera but these took longer to appear on the CRIE autoradiograms.

It is unclear if the allergens of the L3 larvae are derived from some component of the whole body or if the allergens are secretory/excretory products released from live larva into the intestine as the larva migrate through (invade) tissue. Sastre et al. (15) report that none of 12 patients who experienced allergic reactions after eating marinated fish experienced positive reactions after the ingestion of a lyophilized larva or its equivalent in *A. simplex* liquid extracts. However, the same material gave positive skin prick tests and conjunctival challenges in these patients. A similar study of patients with a previous history of gastroallergic anisakidosis found that none reacted after injection of dead frozen larva (23).

Our results clearly showed that *A. simplex* somatic extract (made from lyophilized L3 larvae) contained allergenic proteins. These results confirm studies that show positive skin prick tests and conjunctival challenges to similar extracts. However, the question remains: 'What is the source of the sensitizing allergens?' L3 may invade

tissue and die and then the antigenic material is released from the body as it breaks down as suggested by Kennedy (20). It is also possible that, as they invade tissues, L3 larvae secrete materials that may be allergenic and sensitize the patient before the larvae die. This issue remains to be resolved. The results of studies that show no reaction after ingestion of the dead larva support either mechanism for sensitization. Regardless, it appears that these larva induce allergic sensitization similar to that of other helminths that must invade tissue and they are not like sensitization to food allergens (20).

Conclusion

Somatic extracts of *A. simplex* L3 larva contain a large number of allergenic molecules and there is significant variability between patients in their sensitivity and reactivity to these allergens.

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