

Cross-sectional study of serum reactivity to *Anisakis simplex* in healthy adults in Niterói, Brazil

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Abstract

Although the incidence of anisakiasis is rising worldwide, its frequency is still unknown in Brazil. The aim of this study was to verify immunoreactivity to *Anisakis simplex* antigens in healthy adults and determine its possible relationship with frequency of fish consumption and allergy symptoms. A prospective cross-sectional study was carried out with 67 volunteers recruited from a military facility in Niterói, Brazil. The subjects completed a structured questionnaire and serum titers of specific anti-*Anisakis* IgE and IgG antibodies were measured. The association between frequency of fish intake and IgE reactivity was evaluated by Fisher's exact test. Almost all subjects (97.0%, 65/67) that consumed seafood; 64.6% (42/65) ate fish at least once weekly. Of all seafood consumers, 56.9% (37/65) reported allergy symptoms, being gut allergies most often cited (35.5%). IgE seroreactivity to *Anisakis simplex* was found in 20.9% of subjects (14/67), with 13.4% (9/67) reacting exclusively to somatic antigen, 3.0% (2/67) exclusively to excretory/secretory antigens and 4.5% (3/67) to both antigens. There was a significant association between frequency of fish consumption and positive serology ($p = 0.019$). An immunoblot assay for *Anisakis* antigens showed different positive bands for IgG. The direct relationship between ELISA reactivity and frequency of fish intake and absence of association with allergy symptoms suggests previous contact with *Anisakis simplex* antigens.

Keywords

Anisakis simplex, fish, excretory/secretory antigen, ELISA, immunoblotting

Introduction

Although recent data have shown that adverse reactions to foods are increasing, there are countless unknown agents that may cause allergy, and an estimated 27.2% of all cases of anaphylaxis do not have a defined diagnosis (Moro Moro *et al.* 2011).

Anisakis simplex is a white, round nematode approximately 3 cm in length. It is a parasite of sea mammals and has fish, cephalopods and planktonic crustaceans as intermediary hosts. Humans are accidental hosts due to the ingestion of raw or undercooked fish (Pinkus *et al.* 1975) contaminated with the infecting larvae (L3). Infection, known as anisakiasis, has a wide range of clinical presentations, varying from complete lack of signs and symptoms (Polimeno *et al.* 2010) to reactions such as urticaria or anaphylaxis (Daschner *et al.* 2012).

The association between allergy symptoms and anisakiasis is not straightforward in most cases, due to the long latency between fish intake and symptom onset (Daschner *et al.* 1998).

Ingestion of live larvae often leads to mechanical lesions of the gut mucosa, and is probably responsible for priming of the immune system. This leads to the development of various forms of immune response, such as the induction of allergic responses (Daschner *et al.* 2012). Although several *Anisakis simplex* allergens (Park *et al.* 2012) have been identified, many unknown allergens may still exist (Kobayashi *et al.* 2011). As there is no serological diagnostic technique for all forms of anisakiasis, excretory/secretory antibodies are currently recognized as the most specific and sensitive method for the diagnosis of human infection (Anadon *et al.* 2010).

Although the number of cases of anisakiasis diagnosed each year is increasing throughout the world and the presence

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of *Anisakis* nematodes has been confirmed in several species of fish in the Brazilian coast (Pereira *et al.* 2000, Knoff *et al.* 2001, Melo *et al.* 2006, Knoff *et al.* 2007, Motta *et al.* 2008), the infectious status of the human population along the coast of Brazil is unclear. To the best of our knowledge, there are no proven reported clinical cases or serological analyses of the Brazilian population. To confirm the diagnosis of anisakiosis the presence of anisakid larvae is imperative (Kim *et al.* 2011). Although Cruz *et al.* (2010) have reported the first case of an anisakidosis infection in the Brazilian population these authors are not certain of their diagnosis once they only presume that the infection was acquired by eating raw marine fish and even though they did remove the larva it was not fully identified (Cruz *et al.* 2010). Thus, the aim of this study was to determine immunoreactivity to *Anisakis simplex* antigens in healthy adults and ascertain whether a relationship exists with the frequency of fish intake and allergy symptoms.

Materials and Methods

Subjects, blood samples and interviews

During the second semester of 2010, a cross-sectional study was carried out after three lectures presented to approximately 150 people affiliated with a military facility in the municipality of Niterói, State of Rio de Janeiro, Brazil. All individuals undergo regular medical checkups and are the target of several disease prevention protocols, including for prevention of intestinal parasitic conditions, as ordered by the force. After applying the exclusion criteria, which removed from the cohort any individuals with health problems, those who did not provide informed consent and those who had not taken part in the explanatory lectures for any reason, the final sample consisted of 67 healthy adult participants. All provided written consent, had 5 ml of peripheral blood drawn for serum collection, and answered a structured interview.

During the interview, participants completed a structured questionnaire on aspects such as age, professional or occasional fishing activity, and pattern of seafood ingestion, which included frequency and form of preparation (well done, undercooked or raw). Participants were also asked to report any history of respiratory, gastrointestinal, or cutaneous allergy symptoms, associated or not with seafood ingestion. This study was entered into the Brazilian National System of Ethics in Research registry (SISNEP protocol 0167.0.258.000-08).

Anisakis larvae and specific antigens

Anisakis simplex larvae were obtained from the gut of fresh fish of a variety of species, mainly from the pink cusk-eel, *Genypterus brasiliensis*, Regan, 1903, cutlassfish, *Trichiurus lepturus*, Linnaeus, 1758, and red porgy, *Pagrus pagrus* Linnaeus, 1758. These were acquired at a local fish market in the municipality of Niterói, Brazil, and transported to the fish re-

search laboratory at the Universidade Federal Fluminense School of Veterinary Medicine for removal and taxonomical identification of parasites.

Once classified, the parasites were disintegrated in a Potter-Elvehjem glass homogenizer for collection of total body antigens (Wilson 1966). The resulting homogenate was centrifuged and the supernatant was collected and stored. In order to obtain excretory/secretory antigens, live larvae were incubated in physiological saline containing 50 mM of HCl at 37°C for 18 hours (Caballero *et al.* 2008). The culture medium was then collected. Protein concentrations were determined (Lowry *et al.* 1951) and all antigens were stored at -70°C until use.

Imunoblotting

All antigens were analyzed by means of electrophoresis using 12% SDS-PAGE gels (Laemmli 1970), followed by electrophoretic transfer to nitrocellulose membranes (Towbin *et al.* 1979). After blocking the nitrocellulose membranes overnight at 4°C with blocking buffer (PBS containing 5% skim milk), membranes were incubated with the sera from each volunteer, diluted 1:50 in blocking buffer for 2 hours at room temperature under constant agitation, followed by a wash with phosphate buffered saline + Tween solution (PBS-T). The next step consisted of the addition of HRP-goat anti human IgG (H+L) or HRP-mouse anti-human IgE (ϵ chain-specific) antibodies, diluted as recommended by the manufacturer (Invitrogen, Camarillo, CA, USA), followed by 2 hours of incubation at room temperature with constant agitation. Reactions were carried out by immersing the strips in a solution containing 5 mg of 3,3'-diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, MO) and 0.03% hydrogen peroxide. After gel staining, the protein profile was analyzed.

ELISA

The presence of specific anti-*Anisakis* IgE antibodies in the blood of the volunteers was determined by enzyme-linked immunosorbent assay (ELISA). Briefly, 50 μ l of the *Anisakis* excretory/secretory or total body antigens solution, containing 20 μ g protein/ml, was added to each well of a 96-well microplate (Nunc® MaxiSorp™, Thermo Fisher Scientific) and incubated overnight at 4°C. After a wash with PBS-T, the plates were blocked with PBS - 1% gelatin (PBS-G) for 2 hours at room temperature. Then, 50 μ l duplicates of each serum, diluted 1:50 v/v in PBS-G, were added to each well. After 2 hours of incubation at 37°C, plates were once again washed with PBS-T and 50 μ l of HRP-mouse anti-human IgE (ϵ chain-specific) (Invitrogen, Camarillo, CA, USA) was added to each well according to manufacturer instructions, followed by another hour of incubation at 37°C. Finally, after a last wash, the reaction was revealed by adding *o*-phenylene-diamine and H₂O₂ diluted in citrate-phosphate buffer (pH 5.0). The reaction was stopped after 20 minutes with 2N sulfuric

acid. The individual optical density of each well was read with an automatic plate reader (Anthos 2010) at 490 nm. The results are expressed as the mean of each duplicate. The cutoff level for positive reactivity was established by calculating three times the average optical densities of 20 wells of the ELISA reaction described above, substituting human sera with PBS-T.

Statistics

Student's t-tests were used to compare quantitative variables, after confirmation of sample normality using the Shapiro-Wilk test. For between group comparisons the chi-square test was performed and odds ratios (OR) were calculated. The association between frequency of fish intake and IgE reactivity was evaluated by Fisher's exact test. For the purposes of calculation, consumption of fish more than once a week was considered high intake (Corres *et al.* 2001). Frequency of multiple responses was used to analyze self-reported allergy complaints. A binary logistic model was constructed including variables associated to *Anisakis simplex* sensitization and with IgE sensitization (0/1) as the dependent variable. The analysis was also performed using self-report allergy complaints (0/1) as the independent variable. The significance level for statistical analyses was set to $p < 0.05$. Tests were performed using Excel® and SPSS® for Windows.

Results

Among the 67 volunteers, age ranged between 21 and 59 years (median, 40 ± 8.4 years). According to interviews, participants bought fish and other seafood predominantly from local supermarkets (53.7%, 36/67) and/or the Niterói fish market (28.3%, 19/67). Direct handling of fish was reported by 49.2% (33/67) of participants, mostly in domestic circumstances (84.8%, 28/33). The most common frequency of fish handling was once monthly (51.5%, 17/33), followed by weekly (18.2%), bimonthly (15.2%, 5/33), occasional (9.1%, 3/33), and daily (6.1%, 2/33). All but two participants (97.0%, 65/67) consumed seafood.

Of the seafood consumers, all ate fish (65/65), 73.8% (48/65) ate shrimp and 24.6% (16/65) ate mollusks (namely, octopus). The most frequently consumed fish species were

guttled whitemouth croaker (*Micropogonias furnieri*) (56.72%), sardine (*Sardinella brasiliensis*) (35.82%), cutlassfish (*Trichiurus lepturus*) (23.88%), hake (*Merluccius merluccius*) (20.90%), salmon (*Salmo salar*) (19.40%), and bluefish (*Pomatomus saltatrix*) (19.40%). All seafood consumers eat cooked fish (boiled, roasted, or fried), while only 17.9% (12/65) also eat raw seafood in the form of Japanese cuisine (sushi and sashimi). The frequency of fish consumption followed a normal distribution curve, which varied from sporadic to daily eating. On the basis of these data, the sample can be stratified into three groups: those that eat fish less than once a week (35.3%, 23/65), once a week (40.0%, 26/65) or at least once a week (24.6%, 16/65).

Of all seafood consumers, 56.9% (37/65) reported allergy symptoms with the following distribution, from frequency analysis by multiple responses: 35.5% reported gut allergy, 30.6% respiratory allergy, 16.1% skin allergies and 17.7% others complaints. Mean age was significantly lower ($p < 0.05$) among participants with a history of allergic complaints (36.6 years) than in those with no such complaints (40.8 years).

Among all volunteers, 20.9% (14/67) had a positive anti-*Anisakis simplex* IgE response when tested by ELISA, 13.4% (9/67) reacted to somatic antigen, 3.0% (2/67) to excretory/secretory antigens and 4.5% (3/67) to both antigens. Among those who reported allergies, only 29.7% (11/37) had self-described allergic reactions to fish intake. Of these, only 18.1% (2/11) had positive serology: one participant reacted to both antigens, and the other, to somatic antigen.

Further characterization of the 14 volunteers with IgE reactivity on ELISA showed that 71.4% (10/14) ate fish more than once a week; of these, three include raw fish in their diet. Only 42.9% (6/14) handle fish, mostly at home. As in the sample as a whole, 50.0% of anti-*Anisakis* IgE-positive subjects (7/14) purchased their seafood at local supermarkets, and all claimed to eat fish. The order of preferred seafood differed slightly in this subgroup: IgE-positive volunteers predominantly ate croaker, and cutlassfish. Allergy-related complaints were reported by 42.9% (6/14) of reactive subjects; only two of these associated symptoms with fish intake, and both reported the presence of gastrointestinal symptoms, with abdominal pain and vomiting. Both reacted to the somatic antigen, and one also exhibited a reaction to excretory/secretory antigen. Eight *Anisakis* reactors did not report any allergy symptoms.

Table I. Risk Estimate of frequency of fish consumption (at least once a week) and IgE immunoreactivity by healthy volunteers, Niterói, Brazil

	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for ELISA IgE (reactive)	4.667	1.309	16.637
For cohort frequency = weekly or less	1.647	0.961	2.824
For cohort frequency = 2 x week or more	0.353	0.160	0.779
N of Valid Cases	65		

Table II. Binary Logistic Regression: self-reported allergy symptoms (complaints), related or not to frequency fish consumption, among healthy volunteers, Niterói, Brazil

		Variables in the Equation				
		B	S.E	Wald	df	Sig
Step 1 ^a	Frequency of fish intake (1)	1.580	0.663	5.689	1	0.017
	Complaints (1)	0.793	0.648	1.501	1	0.221
	Constant	2.187	0.553	15.665	1	0.000

a. Variable(s) entered on step 1: Reactive ELISA-IgE^a.

A logistic model was constructed including the two co-variables, frequency of fish intake and self-reported allergy symptoms (complaints) associated to *Anisakis simplex* sensitization (0/1) as dependent variable.

Short title: Serum reactivity to *Anisakis* in adults

One quarter of subjects who ate raw seafood (12/65) was serologically positive. Furthermore, subjects who reported raw fish consumption accounted for 43.7% of those who eat fish twice a week or more often.

The Fisher's exact test ($p = 0.019$) and odds ratios (ORs) calculations (Table I) showed a significant association between frequency of fish consumption and positive *Anisakis* serology. On the other hand, no significant association was observed between clinical symptoms and positive serology (Table II).

Immunoblotting showed that the serum of the 14 ELISA positive subjects recognized total *Anisakis* antigens. All reactive sera show at least two positive bands between 45 and 95 kDa when anti-human IgG, is used, while no positive reactions were observed for anti-human IgE. There was no predominance in the reactivity to a specific band.

Discussion

Anisakiasis is a public health issue (Dias *et al.* 2011) of particular relevance in areas where the population are habitual fish eaters (Daschner *et al.* 2010). Most cases are reported in Japan, where consumption of raw fish is traditional. However, the number of new cases worldwide has risen steadily over the last decades (Audicana and Kennedy 2008).

Studies within the Spanish population, which has a high rate of raw seafood consumption, have demonstrated a high prevalence of sensitization to *Anisakis simplex* (Puente *et al.* 2008), despite strict hygiene measures for seafood handling that have been established by the Spanish government (Corres *et al.* 2001). According to the Rio de Janeiro State Department of Agriculture and Fisheries (Ceasa 2007), this State has the highest rate of fish consumption in Brazil, with approximately 23 kilograms eaten per capita per year. Although the State of Rio de Janeiro accounts for only 0.5% of the Brazilian territory, it is the third largest fish producer in Brazil, outpaced only by the States of Pará and Santa Catarina (Ceasa 2007). So far, there have been no studies of human *Anisakis simplex* infection and its consequences in Brazil, other than a brief report

which described three suspected cases of anisakiasis that were not confirmed serologically (Neto *et al.* 2007). Considering not only that 17.9% (12/65) of subjects in our cohort reported consumption of raw seafood, but also that Niterói has a population of approximately 500,000, we can estimate that 85,000 people are at risk of infection with *Anisakis simplex*. One limitation of the results of this study was the absence of prior knowledge on whether consumed fish were fresh or frozen. As the data show, 64.6% of subjects in the sample (42/65) ate seafood at least once weekly.

Among the various fish species of the Brazilian coast, the whitemouth croaker, *Micropogonias furnieri*, has been overfished and is appreciated by many. In this cohort, it was the most frequently consumed fish. A recent study on this species along the Brazilian coast revealed that 93.97% of specimens are infected with at least one parasite species, including *Anisakis* sp., *Terranova* sp. and *Contracaecum* sp. (Luque *et al.* 2010). Apparently, *Sardinella brasiliensis*, the second most consumed fish by this cohort, is not parasitized by *Anisakis* (Luque and Poulin 2004), nor are sardines, *Sardina pilchardus* form the Northeast coast of Spain (Gutiérrez-Galindo *et al.* 2010); on the other hand, 28.1% of sardines caught off the West coast of Portugal were parasitized with *Anisakis* sp. in one study (Silva and Eiras 2003).

This evidence is of major public health importance (Del Rey Moreno *et al.* 2006), as subclinical sensitization is common in healthy populations with high fish and seafood intake levels (Audicana *et al.* 2002).

Our results are within the upper range of seropositivity incidence as compared with the work performed by Spanish researchers using ELISA. These studies found an incidence of specific anti-*Anisakis simplex* IgE antibody reactivity ranging from 6.6% to 27.5% (Del Rey Moreno *et al.* 2006, Audicana and Kennedy 2008, Puente *et al.* 2008, Cuellar *et al.* 2012). In our study, as expected, a direct association between the frequency of fish intake and anti-*Anisakis* IgE positivity on ELISA could be established (Table II). However, no association was found between positivity and self-reported allergy symptoms. It is important to say that the most anisakiasis episodes are subclinical and up to now no allergen or specific

factor has been associated with overt allergic symptoms and self-reported symptoms could also be due to fish-allergy and others causes.

Although the pattern of IgE bands in immunoblot testing of our volunteers differed from that reported by Del Rey, who found a strong presence of anti-*Anisakis* IgE, our data are similar to those of Del Pozo, who demonstrated the presence of anti-*Anisakis* IgG and absence of anti-*Anisakis* IgE antibodies in the sera of their subjects (Del Pozo *et al.* 1996).

Although somatic antigens are used more often and are easier to obtain, they may cross-react with antigens derived from other nematodes, such as *Ascaris lumbricoides*, *Ascaris suum* and *Toxocara canis* (Fernandez-Caldas *et al.* 1998, Weiler 2007). Despite being less abundant, excretory/secretory antigens are more specific and should be preferred in diagnostic kits. Excretory/secretory antigens are secreted by the parasite to help penetrate the gut mucosa and are probably the antigens involved in stimulation of the host immune system during active infection (Valls *et al.* 2005). In an experimental study, Anadón *et al.* demonstrated that anti-Ani s 7 IgE antibodies induced by live *Anisakis* larvae peaked on day 30 post-infection, decreasing over the course of two months (Anadón *et al.* 2009), and that their presence could constitute a possible recent infection (Anadón *et al.* 2010). In the present study, 7.46% (5/67) of volunteers were seroreactive to excretory/secretory antigens. This finding is suggestive of recent infection with *Anisakis* sp., particularly in one volunteer, who reported abdominal pain associated with fish consumption.

Although our results are consistent with international data, further research is still required to establish better preventive measures against this infection and guide food hygiene policies in the country.

In conclusion, our results indicate an association between frequency of fish intake and systemic sensitization and no direct association with allergies, which suggests previous contact with *Anisakis simplex*.

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