

Short communication

Prevalence of and risk factors for IgE sensitization to *Anisakis simplex* in a Spanish population

Background: The number of allergic reactions to *A. simplex* reported in Spain has increased dramatically in the last decade. Nevertheless, there have been no studies of the prevalence of and possible risk factors for IgE sensitization to this parasite, possibly because suitably specific diagnostic methods have only recently become available. The objective was to investigate the prevalence of and risk factors for IgE sensitization to *A. simplex* in Galicia, a region of northwestern Spain with a population of about 3 million and high average fish consumption (78.5 g/person per day).

Methods: The study was performed with a random sample of 2801 healthy blood donors distributed in 53 geographic areas, proportional to the density of donors. IgE sensitization to *A. simplex* was tested by a capture ELISA method that has proved to be the most specific method currently available.

Results: The results showed a total of only 12 positive subjects, of whom five also showed IgG1 sensitization. All positive subjects and 101 randomly selected seronegative subjects were then included in a case-control study of risk factors for sensitization to *A. simplex*, based on a telephone interview about fish consumption (especially raw and undercooked fish). All seropositive subjects (but only 25% of seronegative subjects) reported consumption of undercooked fish or homemade raw-fish products.

Conclusions: Our results strongly suggest that sensitization to *A. simplex* is caused only by live larvae, and not by allergens contained in fish tissues, and that ingestion of homemade *boquerones* (anchovies [*Engraulis encrasicolus*] in vinegar), and to a much lesser extent of undercooked fish, are the main risk factors for IgE sensitization to *Anisakis* in this region.

B. Valiñas¹, S. Lorenzo¹, A. Eiras²,
A. Figueiras³, M. L. Sanmartín¹,
F. M. Ubeira¹

¹Laboratorio de Parasitología, Departamento de Microbiología y Parasitología, Universidad de Santiago de Compostela; ²Centro de Transfusión de Galicia, Santiago de Compostela; ³Departamento de Medicina Preventiva y Salud Pública, Universidad de Santiago de Compostela, Spain

Key words: allergy; anchovies; *Anisakis simplex*; IgE; marine fish; monoclonal antibodies; nematode; seroprevalence.

Dr Florencio M. Ubeira
Laboratorio de Parasitología
Facultad de Farmacia
Universidad de Santiago de Compostela
15706-Santiago de Compostela
Spain

Accepted for publication 22 February 2001

Man can be infected by numerous species of helminths after having eaten raw or undercooked fish (mostly freshwater fish). These species include the cestode genus *Diphyllobothrium*; the trematode digenids *Clonorchis*, *Opisthorchis*, *Heterophyes*, *Metagonimus*, and *Nanophyetus*; and the nematode genera *Gnathostoma*, *Capillaria*, *Pseudoterranova*, and *Anisakis* (1). Of these, only *Anisakis simplex* and, to a lesser extent, *Pseudoterranova decipiens* have been reported to be of importance for human health in Europe, and especially Spain (2, 3).

Human infection by *A. simplex* larvae was first reported by van Thiel et al. (4) in 1960. Since then, thousands of cases have been reported around the world, mainly in Japan, where the consumption of raw fish dishes (e.g., sushi or sashimi) is very high. However, it is only in the last decade that *A. simplex* has attracted the attention of allergologists as an important cause of food-related allergy (5–7). In Spain, several hundred cases of allergy to *A. simplex* have been reported in the last few years (8–10). More recently, however, two independent

studies have shown that sensitized patients (with a history of severe allergic disease to this parasite) did not experience any adverse reaction after an oral provocation test with dead *A. simplex* larvae (11, 12). These studies suggest that *Anisakis*-induced allergic reactions are a consequence of active infection, and are not due to parasite antigens ingested with fish. If so, knowledge of the prevalence and the source(s) of human sensitization to *A. simplex* is of crucial importance for public health in affected countries.

In light of this, and following the recent development in our laboratory of the first specific method (UA3-ELISA) for determination of anti-*Anisakis* IgG and IgE levels (13), we here report a study of the seroprevalence of and risk factors for IgE sensitization to *A. simplex* in Galicia (northwestern Spain), a region of nearly 3 million inhabitants with the highest per capita fish consumption in Spain. Specifically, we determined levels of anti-*Anisakis* IgE/IgG1 antibodies in serum samples from 2801 healthy blood donors. Subsequently, all seropositive

subjects and a subset of 101 seronegative subjects were interviewed about fish-eating habits, with the aim of identifying risk factors for IgE sensitization to *Anisakis*.

Material and methods

Design, population, samples, and data collection

A cross-sectional study was performed with 2801 subjects from a population of normal unpaid-volunteer blood donors ($n=151\,950$, 1999 census) from Galicia (2 750 000 inhabitants). The sample was stratified in proportion to the population of each of 53 geographic subareas. A signed consent form was obtained from each individual participant in the study, for extraction of 5 ml of blood and for subsequent telephone interview if necessary. After 12 subjects tested positive for sensitization to *A. simplex* in antigen-capture ELISA (see below), we randomly selected 105 of the 2789 subjects that tested negative, as controls. Four of these 105 were excluded from the study because they had changed address and could not be located. The remaining subjects (12 cases, 101 controls) were interviewed by telephone about fish-eating habits, and particularly raw or undercooked fish consumption. The questionnaire included items related to:

- 1) personal and occupational details
- 2) fish consumption preferences (fresh, frozen, salted, etc.)
- 3) consumption of raw fish including source, method of preparation (home-made, industrial), and preventive measures for anisakiosis (e.g., freezing)
- 4) frequency and place of consumption (home, restaurant, etc.)
- 5) preferences as regards undercooked fish consumption.

Capture ELISA

IgE and IgG1 sensitization to *A. simplex* were tested by an antigen-capture ELISA procedure (13, 14) in which the serum to be tested is added to wells containing *O*-deglycosylated *A. simplex* antigen bound by the immobilized mAb UA3 (14).

Optical densities (OD) at 492 nm were calculated by subtracting the OD values given by the same serum in the absence of antigen. All isotype determinations were done in duplicate. For data analysis, sera were considered positive if the OD was more than $\bar{x}+4$ SD, where $\bar{x}$ and SD are the corresponding mean OD and standard deviation for 25 CAP-negative control sera from normal blood donors aged 18–24 years, as described previously (13). IgE values are expressed as kU/l, as calculated from the calibration curve obtained for IgE (13).

Statistical analysis

The effects of the different dietary habits on risk of sensitization to *A. simplex* IgE were estimated as odds ratios (OR) with 95% confidence limits (CI 95%) estimated by Woolf's approximation. If the contingency table contained cells with zero frequencies, 0.5 was added to all frequencies before analysis. OR values were considered statistically significant if the 95% confidence interval did not include 1. All analyses were performed with the program GraphPad Instat® (GraphPad Software, San Diego, CA, USA).

Results

Sensitization to *A. simplex*

Of the 2801 serum samples analyzed for anti-UA3R IgE by antigen-capture ELISA, only 12 tested positive (prevalence: 0.43%) (Fig. 1). These subjects were nine

men and three women aged 22–49 and 24–33 years, respectively. IgE levels were high in one subject (20 kU/l), intermediate in five subjects (2–10 kU/l), and low in six subjects (0.5–2 kU/l). Five (41.6%) of these 12 sera also presented anti-*Anisakis* IgG1 antibodies (Fig. 1).

Fish-eating habits (seronegative population)

Of the 101 seronegative subjects used as controls, 51 were male (50.5%) and 50 female (49.5%), with mean ages of 34.4 and 32.3 years, respectively. The data obtained in the interview revealed that 40/101 (39.6%) of these subjects habitually ate one or more class of raw fish, with *boquerones* (anchovies [*Engraulis encrasicolus*] in vinegar) being the most frequent (27/101; 26.7% of individuals). About half of these *boqueron* consumers (13/101, 12.8%) prepared them at home from fresh fish, without any previous measure (e.g., freezing) to ensure killing *Anisakis* larvae if present. The basic procedure for the preparation of *boquerones* is maceration of the fish fillets in vinegar with a small amount of salt for 12–24 h, and then dressing with olive oil, garlic, and parsley before consumption. The remaining *boqueron* consumers (14/101, 13.8%) ate this product in restaurants or bars, where they are often (although not always) of industrial origin (i.e., prepared from frozen anchovies) (Table 1). *Boquerones* are mostly eaten during the summer, and most subjects eat them only a few times a year (mean 7.5 ± 8.6 times per year; CI 95% = 2.3–12.7; Table 2). The consumption of other raw-fish products (e.g., sushi, sashimi, etc.) is very infrequent in the study region. However, a total of 16/101 (15.8%) of these subjects reported consumption of fish prepared in ways favoring undercooking (notably charcoal-grilled ungutted sardines).

Fish-eating habits (seropositive population)

Of the 12 seropositive subjects, 10 (83.3%) reported consumption of homemade *boquerones*, without previous freezing (Table 1). Most of these subjects reported eating homemade *boquerones* at most 15 times a year (Table 2), generally during the peak-availability period in summer. Of the two remaining positive subjects, one ate *boquerones* in restaurants (not more than twice per year), and also reported a preference for undercooked fish; the other did not eat *boquerones* but did eat sardines (*Sardina pilchardus*) prepared in the traditional manner (ungutted and charcoal-grilled) during street festivals. It is noteworthy that none of the 12 seropositive subjects remembered suffering abdominal pain or any other potential symptom of *A. simplex* infection.

Risk factor analysis

Our data (Table 1) strongly suggest that ingestion of homemade *boquerones* dramatically increased the risk

of IgE sensitization to *A. simplex* (OR: 33.8; CI 95%=6.6–172.1). Furthermore, all the seropositive subjects reported consumption of homemade *boquerones* and/or undercooked fish: even after applying a correction for zero values, the presence of one or both of these factors increased the risk of IgE sensitization to *A. simplex* 75 times (OR: 75; CI 95%=4.3–1313.2). None of the other variables considered were statistically significant risk factors.

Discussion

This work provides data on the seroprevalence of and risk factors for anti-*Anisakis* IgE sensitization in Galicia. By comparison with previous studies in other Spanish populations (15), the seroprevalence value obtained (0.46%) is very low. A possible explanation for this discrepancy is that, before the introduction of UA3-ELISA (13), the methods employed to detect anti-*Anisakis* IgE antibodies were not specific enough to detect true sensitization (15, 16), leading to overestimation of seroprevalence.

With respect to the risk factors involved in anti-*Anisakis* IgE sensitization, it was surprising that all subjects with anti-*Anisakis* IgE in serum reported consumption of raw or undercooked fish (i.e., sources of live *Anisakis* larvae). Indeed, more than 80% of the seropositive subjects (but less than 15% of seronegative subjects) showed a single risk factor, consumption of homemade *boquerones*. One of the subjects (interestingly, the subject with the highest anti-*Anisakis* serum IgE value) reported that homemade *boquerones* were the only fish product eaten by him. *Boquerones* are widely eaten in Spain, particularly in the Basque Country, Andalucia, Madrid, and Galicia. Only two of the seropositive subjects reported consumption of

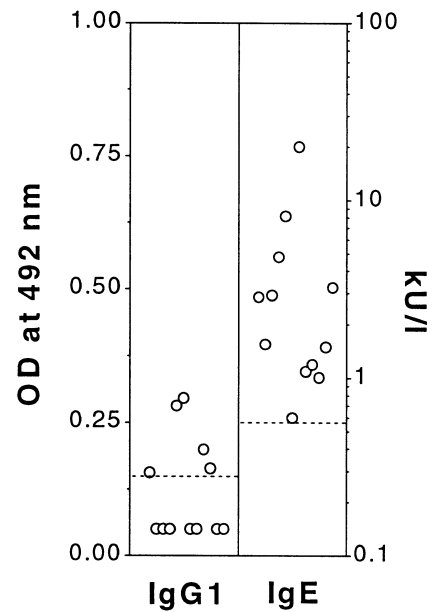


Figure 1. Serum IgG1 and IgE responses to *Anisakis* UA3R antigens in seropositive population ($n=12$). Each point represents individual serum. IgG1 levels are expressed as OD and IgE levels as kU/l (as obtained from calibration curve for IgE; see Material and methods). Horizontal bars represent cutoff values (mean OD + 4 SD) for each isotype (IgG1: 0.135; IgE: 0.105), as obtained on basis of results for 25 control sera.

undercooked fish. The close association with *boqueron* consumption may explain why most reported cases of allergy to *A. simplex* are from Spain.

The prevalence of L3 larvae of *A. simplex* in *E. encrasicholus* is not well known. A recent study in Spain (17) reported it to be highly variable (3–34%), depending on area where caught. In our laboratory, however, we did not detect any larvae in 650 specimens of *E. encrasicholus* caught in the Ria de Arosa, the largest estuary in Galicia. This may explain the marked variation in *A. simplex* infection

Table 1. Number of subjects in each group (cases and controls) reporting consumption of raw or undercooked fish. For each category of fish product, odds ratio for corresponding two-by-two contingency table was calculated: only statistically significant values (95% confidence interval does not include 1) are shown here

	Homemade raw or undercooked				Commercial raw				Any ⁹
	<i>Boqueron</i> ¹	Sardine ²	U/cook ³	Any raw or u/cook ⁴	<i>Boqueron</i> ⁵	Smoked herring ⁶	Smoked salmon ⁷	Other ⁸	
Cases	10/12 (83.3%)	0/12	2/12 (16.6%)	12/12 (100%)	1/12 (8.3%)	0/12	0/12	0/12	12/12 (100%)
Controls	13/101 (12.8%)	1/101 (0.9%)	16/101 (15.8%)	25/101 (24.8%)	14/101 (13.8%)	20/101 (19.8%)	16/101 (15.8%)	2/101* (1.9%)	40/101 (39.6%)
Odds ratio	33.8	–	–	75	–	–	–	–	37.9
CI 95%	6.6–172.1	(NS)	(NS)	4.3–1313.2	(NS)	(NS)	(NS)	(NS)	2.2–659.6

¹Homemade *boqueron*, i.e., *Engraulis encrasicholus* in vinegar, homemade without prior freezing.

²*Sardina pilchardus* in vinegar, homemade.

³U/cook: undercooked fish (notably charcoal-grilled ungutted sardines).

⁴Any of categories 1–3.

⁵Restaurant-purchased *boqueron*, i.e., *E. encrasicholus* in vinegar, often (although not always) industrial product made with frozen fish.

⁶Smoked and salted *Clupea harengus* (herring).

⁷Smoked *Salmo salar*.

⁸Other raw fish products (sushi, sashimi, carpaccio, etc.).

⁹Any of categories 1–8.

*One of these subjects ate sushi prepared at home.

Table 2. Frequency of consumption of homemade *boquerones* in seropositive ($n=12$) and seronegative ($n=101$) populations

	1–5 times/year	6–15 times/year	16–50 times/year	Never
Cases	2/12 (16.6%)	5/12 (41.6%)	3/12 (25.0%)	2/12 (16.6%)
Controls	9/101 (8.9%)	2/101 (2.0%)	2/101 (2.0%)	88/101 (87.1%)

risk among the different Spanish regions. The fact that 13% of our control subjects were seronegative despite reported consumption of homemade *boquerones* may be attributable to the following factors:

- 1) variation in the prevalence and/or intensity of fish infection
- 2) variation in the effects of product preparation and subsequent mastication on parasite survival and/or infection capacity
- 3) variation in individual susceptibility to infection.

In agreement with previous studies using oral provocation tests (11, 12), our results also indicate that allergy to *A. simplex* is not in fact a classical food-related allergy, but rather the clinical manifestation of an infection in which the allergenic component is particularly relevant (notably, those cases classified as “gastro-allergic anisakiosis” [18, 19]). If allergy to *A. simplex* were a classical food-related allergy, we would expect to find IgE sensitization not only in subjects who eat undercooked fish and homemade raw-fish products, but also in some subject who eat only cooked fish and/or industrial raw-fish products (i.e., about 75% of the Galician population). This conclusion is of interest since it seems to indicate that allergy to *A. simplex*, like classical anisakiosis, is probably preventable if fish is eaten well-cooked or previously frozen. This has significant implications for public health, since extrapolation of our data on seroprevalence to the Spanish population (37 million inhabitants) suggests that more than 150 000 individuals may have IgE sensitization to *Anisakis*.

It might be argued that our results cannot be meaningfully extrapolated to the general population, since our serum samples were from blood donors. In fact, all individuals in this subpopulation are aged less than 65 years, and the 18–35-year age group is proportionally larger than in the general population. Our seroprevalence estimate is thus almost certainly an underestimate, since it has been reported that IgE sensitization to *Anisakis* antigens increases with age (5,

8), and since subjects who have recently suffered nonsubclinical episodes of *Anisakis*-related disorder will probably have been hospitalized and thus excluded from subsequent blood donation. Nevertheless, our data reveal a strong association between ingestion of raw fish and seropositivity (see odds ratio in Table 1) despite the probable exclusion of many subjects who have recently suffered *Anisakis*-related disorder, thus reinforcing our conclusions.

Finally, and although we have not observed any cases of IgE sensitization to *Anisakis* in the no-risk subpopulation (i.e., subjects who do not consume homemade *boquerones* or undercooked fish), this and previous studies do not completely rule out the possibility that, as with other oral allergens, a small number of previously sensitized patients may suffer an allergic reaction after ingestion of dead larvae of *Anisakis*, or even of other as-yet-unidentified cross-reacting allergens present in other foods. This possibility is most likely to arise if allergens reach the systemic circulation, as favored by situations in which the gastric and/or intestinal mucosa is altered (as in cases of gastrointestinal hemorrhage [20], or during treatment with nonsteroid anti-inflammatory drugs (NSAIDs) [21]). These and other possibilities need to be further investigated by diagnostic methods with high specificity for *Anisakis*, such as the capture ELISA used in the present study.

Acknowledgments

We thank Dr R. Villaescusa (Director) and the staff of the Centro de Transfusión de Galicia (Santiago de Compostela, Spain), for their help in obtaining serum samples, and Rosana Rego (Centro de Transfusión de Galicia), for her help with the telephone interviews. This work was supported in part by grant 1028/77 from the Fondo de Investigación Sanitaria (Ministerio de Sanidad y Consumo, Spain), and grant XUGA20305B98 from the Secretaría Xeral de Investigación e Desenvolvemento (Xunta de Galicia, Spain), and by the Dirección Xeral de Saúde Pública (Xunta de Galicia, Spain).

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