

High Prevalence of Seropositivity to a Major Allergen of *Anisakis simplex*, Ani s 1, in Dyspeptic Patients

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Finding evidence of anisakidosis requires invasive methods. We have developed a serological assay based on the detection of an immunoglobulin E (IgE) specifically directed against Ani s 1 protein, a major parasite allergen of *Anisakis simplex*, which has shown a high level of accuracy in the diagnosis of anisakidosis. We used this tool to determine the prevalence of anti-Ani s 1 IgE in dyspeptic patients and to investigate if its seropositivity could be related to epidemiological factors other than raw fish consumption. A total of 174 dyspeptic patients who submitted to upper digestive tract endoscopy were studied. Specific IgE against Ani s 1 was determined by immunoblotting. Quantitative information on smoking, alcohol consumption, and fish consumption as well as a history of gastric surgery was recorded. Twenty-four (13.8%) patients were seropositive for Ani s 1 protein. The seroprevalence of anti-Ani s 1 IgE increased with age in patients who were less than 62 years old ($P = 0.047$). Seropositivity to Ani s 1 was associated with the consumption of fish in vinegar ($P < 0.001$), raw fish ($P = 0.001$), and smoked fish ($P = 0.007$). There was no relationship between anti-Ani s 1 IgE seropositivity and the number of cigarettes smoked ($P = 0.098$) or alcohol intake ($P = 0.179$). Five patients had undergone previous gastric surgery, and three of those patients were seropositive for Ani s 1 ($P = 0.019$). In multivariate analysis, the consumption of fish in vinegar ($P = 0.006$), raw fish ($P = 0.017$), and smoked fish ($P = 0.002$) and a history of gastric surgery ($P = 0.005$) were independent factors associated with anti-Ani s 1 IgE detection. In conclusion, at present, anisakidosis might frequently be underdiagnosed, and it might have a clinical role in patients with upper dyspeptic symptoms. Uncooked-fish ingestion and previous gastric surgery were associated with seropositivity for Ani s 1 protein.

Anisakis simplex is a nematode parasite that belongs in the *Anisakidae* family. Its life cycle involves larval stages with several intermediary hosts and the adult stage, during which the worm parasitizes the stomachs of marine mammals. Humans can be accidental hosts by eating raw or undercooked fish or seafood that contains the third-stage larvae of *A. simplex*. After ingestion, larvae can be invasive, penetrating the digestive tract and producing a disease, known as anisakidosis or anisakiasis, that can mimic other gastrointestinal disturbances (12).

The finding of evidence of anisakidosis is based on visualization of larvae by upper digestive tract endoscopy within the first 24 h after fish ingestion or by surgical findings (12, 18). For these reasons, it is probable that only the most severe cases that require invasive techniques are diagnosed and that many of the mild cases remain underdiagnosed (21). Although serologic techniques have been developed for the detection of *A. simplex* infection, cross-reactivities with other helminths have been a serious limitation (8, 10, 11, 17). In an attempt to improve the diagnosis, we purified and characterized a major immunoglobulin E (IgE)-binding protein from the parasite. This major parasite allergen, named Ani s 1, is a protein from the excretory gland that shows no homology to a previously described protein (14). Recently, we have demonstrated that

the detection of IgE specifically directed against Ani s 1 has a high accuracy in the diagnosis of anisakidosis (sensitivity, 86%; specificity, 90%) (6).

Up to now, the use of invasive methods for anisakidosis diagnosis has restricted the study of this disease, and, therefore, the true prevalence of the disease in dyspeptic patients is unknown. On the other hand, although risk factors, such as the consumption of raw or undercooked fish, are well known, other studies have suggested that host factors could be involved. However, those series published about anisakidosis are retrospective studies with a limited epidemiological value (13, 16, 19). The aims of this study were to determine the prevalence of *A. simplex* infection in dyspeptic patients and to investigate whether anisakidosis is related to epidemiological factors other than the consumption of raw fish in this population. We carried out a cross-sectional study of a group of dyspeptic patients who were investigated to determine the prevalence of primary drug resistance to *Helicobacter pylori*.

MATERIALS AND METHODS

Patients. From February to December 1998, 174 patients (91 men and 83 women; mean age, 49.3 ± 15.1 years; age range, 21 to 80 years) who underwent a programmed upper gastric endoscopy because of dyspeptic symptoms were studied. Dyspepsia was defined as intermittent or persistent pain or discomfort in the upper abdominal region or the lower part of the chest, heartburn, nausea, a feeling of postprandial fullness, or any other symptoms thought to be related to the upper gastrointestinal tract that lasted at least 3 weeks (4). Patients who had previously been treated for eradication of *H. pylori*, had received antimicrobial or antacid therapies during the previous 3 months, and/or had taken nonsteroidal

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anti-inflammatory drugs (including low-dose aspirin) within a month of endoscopy were not admitted. The present study was approved by the Ethics Committee of Hospital Carlos III, and informed consent to participate was obtained from each patient.

Endoscopy. In all patients, upper endoscopy was performed by using a conventional videoendoscope (FG-100FP; Fujinon, Tokyo, Japan). All endoscopic devices were disinfected with 2% glutaraldehyde. During the endoscopic procedure, the presence of macroscopic lesions in the stomach was carefully evaluated and at least five biopsies, three from the antrum and two from the corpus, were obtained.

Specific IgE determination for *A. simplex* extract and Ani s 1 protein. (i) **Sera.** At the time of the endoscopy, a serum sample was obtained from the patients and stored at -80°C until the time of use. All patients were tested for the presence of IgE against *A. simplex* extract. IgE specifically directed against *A. simplex* major allergen Ani s 1 was studied in subjects who were reactive to the *A. simplex* extract.

(ii) **Antigens.** The *A. simplex* extract was prepared with larvae obtained from muscle tissue of hake (*Merluccius merluccius*) and *Micromesistius poutassou*. Approximately 200 larvae were ground in a mortar with 5 ml of phosphate-buffered saline. The mixture was centrifuged for 10 min at $4,500 \times g$, and the pellet was discarded. The protein content of the supernatant was 2 mg/ml, as measured by the method of Bradford (5) with bovine serum albumin as a standard. *A. simplex* major allergen Ani s 1 was isolated from larvae and purified from the crude parasite extract by ethanol precipitation and reversed-phase high-performance liquid chromatography according to the method described in a previous work (14).

(iii) **Immunoblotting.** Ani s 1 (0.1 ml of a 100- $\mu\text{g}/\text{ml}$ dilution) or crude *A. simplex* extract (0.1 ml of a 1-mg/ml dilution) was mixed with 0.1 ml of a sample buffer. The mixture was subjected to electrophoresis in a 16% acrylamide minigel (150 V) under standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis conditions and transferred by diffusion to two nitrocellulose sheets for 18 h. The next day, after being blocked in 3% Nonidet P-40 for 30 min, the membranes were placed on a Mini-Protein II multiscreen (Bio-Rad Laboratories, Hercules, Calif.). For specific IgE determination, the different sera were diluted 1:6 in incubation buffer and incubated overnight in each independent chamber. After incubation and washing, the membranes were incubated for 3 h with 10 ml of a 1:1,000 dilution of a monoclonal human anti-IgE antiserum (Ingenasa, Madrid, Spain). After new washes, the membranes were incubated with 10 ml of a 1:4,000 dilution of an alkaline phosphatase-labeled goat anti-mouse antiserum (Bio-source, Camarillo, Calif.). Finally, the membranes were washed, and the signal was visualized with 5-bromo-4-chloro-3-indolyl phosphate-4-nitroblue tetrazolium (Amresco, Solon, Ohio) for 30 min.

***H. pylori* infection.** During endoscopy, biopsies were taken for histologic examination (two antrum and two corpus biopsies) and culture (one antrum biopsy). Diagnosis of *H. pylori* infection was established if culture and/or histologic examination was positive.

Data collection. All patients were interviewed prior to endoscopic examination. During the interview, information on cigarette smoking habits, alcohol intake, dietary practices, and a history of gastric operation was collected by questionnaire. With respect to smoking, the questionnaire asked if the patient smoked currently, in the past, or never. Current smokers were asked to indicate how many cigarettes per day they smoked. For alcohol intake, the questionnaire was conducted in a similar way, and current drinkers were asked to indicate the volume consumed per day and the frequency of drinking. The percentages of subjects who consumed alcohol of various types were estimated to be 3.7% for beer, 10% for wine, and 40% for spirits. The current alcohol intake was defined as the total volume of pure alcohol consumed in milliliters per week. Finally, the subjects were interviewed about fish-eating habits during the last year, including the type and form of consumption (fish in vinegar sauce, other raw fish dishes, and smoked and cooked fish). With regard to the frequency of consumption, the subjects were classified as nonconsumers (never eating fish), sporadic consumers (one or less than one time per month), or habitual consumers (more than one time per month).

Statistics. Categorical variables were analyzed with the chi-square test, chi-square test for linear trend, or Fisher exact test when appropriate. Multiple logistic regression analysis was performed to identify factors related to reactivity to anti-Ani s 1 IgE. The magnitude of the association was evaluated by determining odds ratios together with their 95% confidence intervals. A *P* value of <0.05 was considered to be significant. The Statistical Package for Social Sciences software version 10 (SPSS Inc., Chicago, Ill.) was used for all analyses.

RESULTS

No cases of active parasitism were diagnosed during endoscopic procedures. However, 24 (13.8%) of 174 patients were reactive for Ani s 1 protein. Table 1 shows epidemiological results for the 174 patients. There was no association between gender distribution and seroreactivity to Ani s 1 protein. Overall, we did not observe any association of anti-Ani s 1 IgE detection with age. However, when we examined the patients who were less than 62 years of age, the seroprevalence of anti-Ani s 1 IgE increased with age ($P = 0.047$). Seropositivity to Ani s 1 protein decreased in the group of patients over 61 years, but the difference did not achieve statistical significance ($P = 0.358$).

Sixty-six (37.9%) patients were smokers and 103 (59.2%) reported alcohol consumption. We found no evidence of a dose-dependent association between Anti-Ani s 1 IgE seropositivity and the number of cigarettes smoked ($P = 0.098$) or alcohol intake ($P = 0.179$).

With regard to fish consumption, nonconsumers and subjects with sporadic ingestion were regrouped into one category. Data obtained in the interviews revealed that the habitual consumption of fish in vinegar sauce, namely, anchovies (*Engraulis encrasicolus*), was frequent in our population (32 subjects; 18.4%) and was associated with seroreactivity to Ani s 1 protein ($P < 0.001$). Only five subjects (2.9%) usually ate other raw fish dishes (sardines [*Sardina pilchardus*] and hake [*Merluccius merluccius*]). All but one of these subjects were seropositive to Ani s 1 protein ($P = 0.001$). With regard to the consumption of smoked fish, seven (4%) patients were habitual consumers and four of those patients were anti-Ani s 1 IgE positive ($P = 0.007$). As expected, we did not find a relationship between the consumption of cooked fish and reactivity to Ani s 1 protein.

One hundred thirty-eight subjects (79.3%) were infected by *H. pylori*. No relationship was established between *H. pylori* infection and the detection of anti-Ani s 1 IgE. Five subjects had past histories of nonresective gastric surgery (pyloroplasty and vagotomy in all cases). Three of the patients were seropositive for Ani s 1 ($P = 0.019$).

To enable multivariate analysis, subjects were regrouped as under 62 years of age or 62 years of age and older, and the following variables were considered independent variables: tobacco consumption (smokers or nonsmokers); alcohol intake (drinkers or nondrinkers); consumption of raw fish, fish in vinegar sauce, and smoked fish (habitual consumers or sporadic consumers and nonconsumers); and previous gastric surgery (yes or no). Table 2 shows the results for multivariate analysis. Ingestion of fish in vinegar, raw fish, and smoked fish and a history of gastric operation were associated independently with anti-Ani s 1 IgE positivity.

DISCUSSION

In the present study, we found that 24 patients (13.8%) were seropositive to Ani s 1 protein. Therefore, these patients probably suffered previous episodes of *A. simplex* infection that had not been diagnosed. It is noteworthy that this work was carried out with dyspeptic patients in whom anisakidosis was not suspected. This finding highlights the observation that *A. simplex*

TABLE 1. Epidemiological data of 174 dyspeptic patients

Characteristic	No. of patients (%)			P
	Total	Positive for IgE against Ani s 1	Negative for IgE against Ani s 1	
Sex				
Male	91 (52.3)	10 (11)	81 (89)	0.366
Female	83 (47.7)	14 (16.9)	69 (83.1)	
Age (yr) ^a				
21–37	43 (24.7)	3 (7)	40 (93)	0.535 ^c
38–49	44 (25.3)	7 (15.9)	37 (84.1)	
50–61	45 (25.9)	10 (22.2)	35 (77.8)	
62–80	42 (24.1)	4 (9.5)	38 (90.5)	
Tobacco consumption				
Nonsmokers	108 (62.1)	12 (11.1)	96 (88.9)	0.098 ^c
Smokers (no. of cigarettes/day) ^b	66 (37.9)	12 (18.2)	54 (81.8)	
1–14	31 (17.8)	4 (12.9)	27 (87.1)	
15–50	35 (20.1)	8 (22.9)	27 (77.1)	
Alcohol consumption				
Nondrinkers	71 (40.8)	8 (11.3)	63 (88.7)	0.179 ^c
Drinkers (g of ethanol/wk) ^b	103 (59.2)	16 (15.5)	87 (84.5)	
1–40	54 (31)	6 (11.1)	48 (88.9)	
44–402	49 (28.2)	10 (20.4)	39 (79.6)	
Consumption of fish				
In vinegar				
Sporadic or nonconsumers	142 (81.6)	13 (9.2)	129 (90.8)	<0.001
Habitual consumers	32 (18.4)	11 (34.4)	21 (65.6)	
Raw				
Sporadic or nonconsumers	169 (97.1)	20 (11.8)	149 (88.2)	0.001
Habitual consumers	5 (2.9)	4 (80)	1 (20)	
Smoked				
Sporadic or nonconsumers	167 (96)	20 (12)	147 (88)	0.007
Habitual consumers	7 (4)	4 (57.1)	3 (42.9)	
<i>H. pylori</i> infection	138 (79.3)	20 (14.5)	118 (85.5)	0.788
History of gastric operation	5 (2.9)	3 (60)	2 (40)	0.019

^a Percentile, 25th.^b Percentile, 50th.^c Value was subjected to chi-square analysis to determine the linear trend.

infection might be more frequent than expected, since only the most severe cases with alarming gastric or allergic symptoms that require urgent upper endoscopy examination are being diagnosed at present. Moreover, we suggest that many cases of anisakidosis may be misdiagnosed and confounded with other conditions. In this context, studies about the clinical significance of seropositivity to Ani s 1 in dyspeptic patients are in progress.

This relevant seroprevalence of Ani s 1 IgE in our study can be due to several factors. First, Spain has a very elevated level of consumption of fish (85 g per person per day) (3). Second, a high level of fish parasitism of up to 45% has been detected in the market, depending on the species examined (1). Last, in our population, the habitual consumption of uncooked fish was very frequent, especially for anchovies in vinegar sauce

(18.4%). In contrast, other studies have reported a very low seroprevalence of anti-*Anisakis* IgE in the north of Spain (20). This low seroprevalence can be explained, aside from the methodology used, by the culinary habits among different regions of Spain. Our study was performed in the central area of the country (Madrid), where the consumption of uncooked fish is more frequent than in the north (9).

In our work, the seroprevalence of Ani s 1 IgE increased with age in patients younger than 62 years. However, in older patients, the rates decreased slightly. Previous studies have reported that cases of anisakidosis are uncommon in elderly patients. This finding has been related to a decrease in gastric acid secretion with age (15). The possible influence of gastric pH on anisakidosis may be related to an increase of larval activity (12). In fact, nematode parasites of warm-blooded hosts use chemical and thermal signs in their hosts in the subsequent resumption of development after a metabolic arrest (2).

Our data revealed a strong association between anti-Ani s 1 IgE seroreactivity and the consumption of uncooked fish. Therefore, the disease is completely preventable by the appropriate preparation of fish or by general prophylactic measures, such as freezing before marketing. In this sense, current European Community regulations require that species intended for marinating or salting at temperatures of <60°C must be stored at –20°C for 24 h. The U.S. Food and Drug Adminis-

TABLE 2. Multiple logistic regression analysis of factors associated with the detection of anti-Ani s 1 IgE

Variable	Odds ratio	95% confidence interval	P
Consumption of fish in vinegar sauce	4.4	1.5–12.8	0.006
Consumption of raw fish	3.9	1.3–12.1	0.017
Consumption of smoked fish	15.6	2.7–89.4	0.002
History of gastric operation	16.7	2.3–121.9	0.005

tration demands that all fish products not intended for cooking or processing at temperatures of $>60^{\circ}\text{C}$ should be deep-frozen at -35°C for 15 h or at -23°C for a minimum period of 7 days (7). These measures should reduce infection rates.

Anisakidosis has rarely been reported to occur in patients following gastrectomy. The decrease of gastric acid and/or the shortened length of a resected stomach might be the factors responsible (2). In contrast, we found an association between patients with previous nonresective gastric surgery and seropositivity to Ani s 1 protein. Although the number of subjects was small and this finding could be casual, we cannot rule out the possibility that the persistence of dyspeptic symptoms in these patients is due to misdiagnosed episodes of *A. simplex* infection.

In conclusion, anisakidosis is at present frequently underdiagnosed. It may play a clinical role in patients with dyspeptic symptoms. Further studies are necessary to determine the clinical significance of seropositivity to Ani s 1 in this clinical setting. Consumption of different preparations of uncooked fish is a risk factor for *A. simplex* seropositivity.

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