

Digestive haemorrhage in patients with anti-*Anisakis* antibodies

R. Gutiérrez-Ramos^a, R. Guillén-Bueno^c, R. Madero-Jarabo^b and C. Cuéllar del Hoyo^c

Objective *Anisakis simplex* parasite causes anisakidosis, a disease that often mimics other gastrointestinal diseases such as peritonitis, appendicitis, Crohn's disease, ulcer, etc. Patients with digestive haemorrhage caused by ulcers, varices or Mallory syndrome were analysed for antibodies to the worm *A. simplex*.

Methods Antibody detection was carried out by enzyme-linked immunosorbent assay (ELISA) and immunoblot using crude extracts of antigen and excretory/secretory products. Total immunoglobulin (Ig), IgG, IgM, IgA and IgE were studied.

Results Eighty-seven patients were studied. The following prevalence rates were found with crude antigen: total Ig 30% (95% confidence interval 21–40), with values for IgG, IgM, IgA and IgE of 22 (CI 14–31), 17 (CI 10–26), 37 (CI 27–47) and 12% (CI 6–20), respectively. Twenty-four positive sera for total Ig response and crude products were selected for determination of specific antibodies with excretory/secretory antigens. We obtained 8, 13, 3 and 16 positive cases for total Ig, IgG, IgM and IgA respectively. The percentages of positivity within the varices and Mallory groups of patients were higher although differences were not significant (35 and 50% respectively). In a healthy population, the prevalence for total Ig is much lower (6%). Twenty-five positive sera for total Ig response were tested by means of immunoblot analysis against crude larval antigen. Concerning total Ig antibody response, 12 of the

sera showed an immuno-recognition pattern concordant with the human anisakidosis reference serum (E17). Specific IgG bands were visualized in 30 sera; specific IgM and IgA in 6 and 12, respectively. Different clinical variables of these patients were studied: leucocytes, eosinophils, haemoglobin, prothrombin activity, thromboplastin time, fibrinogen, platelets and erythrocyte sedimentation rate. There were a few significant differences: for total Ig in prothrombin activity and platelets, and for IgM in eosinophils.

Conclusions The prevalence of detectable antibodies against *A. simplex* is higher in patients with digestive haemorrhage than in the healthy population. *Eur J Gastroenterol Hepatol* 12:337–343 © 2000 Lippincott Williams & Wilkins

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Introduction

Anisakidosis is a human disease caused by the accidental ingestion of larval nematodes belonging to the family Anisakidae. People acquire the infection by eating raw seafood dishes such as sushi, sashimi, ceviche and lomi-lomi, or undercooked fish and squid dishes [1]. Based on the lesion location, anisakidosis is divided into gastric, intestinal and heterologous anisakidosis (ectopic anisakidosis); the latter may be found in various human body locations, although it is not frequent. Generally, the clinical symptoms of anisakidosis include nausea, vomiting, abdominal and epigastric pain, abdominal dilatation by ascites or intestinal gas filling, movable soft induration and diarrhoea followed by normal stools or constipation [2]. In anisakidosis patients, occult blood is often present in the gastric

juice or faeces [3]. According to Oshima [4] and Van Thiel *et al.* [5], occult blood may also be present in intestinal anisakidosis. The anisakidosis pathology involves the development of a granuloma surrounding the worm, characterized by infiltration and proliferation of neutrophils, associated with a few eosinophils and giant cells, and the occurrence of haemorrhage [1,3,6,7].

These facts and our own observations on experimental animals led us to examine the anti-coagulant action of *Anisakis simplex* larvae on human blood *in vitro* [8]. With the larval crude extracts, a slight prolongation of the prothrombin time (PT) was observed. The excretory/secretory (ES) products of *A. simplex*, produced during invasion of the gastric mucosa, are recognized as antigens by the host [9]. Therefore, the anti-coagulant

activity of ES products collected from *in vitro* cultures of third-stage larvae was also investigated. Larval ES products were able to prolong coagulation times (PT and partial thromboplastin time), indicating that they contain an inhibitory or anti-coagulant property, suggesting a probable alteration of one of the coagulation proteins, namely factors Xa, IIa or Va.

However, anisakidosis is often misdiagnosed as a different gastrointestinal disease due to the vagueness of its symptoms [2]. Keeping the above facts in mind, the present study was undertaken to evaluate specific antibody levels against *A. simplex* larval antigens in sera from patients with gastrointestinal bleeding.

Materials and methods

Patients and serum samples

Eighty-seven patients with digestive haemorrhage were selected for this study between 1996 and 1997. They were hospitalized in the gastroenterology service of the Hospital La Paz. This is a 1500-bed university hospital that serves part of the Madrid metropolitan area (Spain).

For all patients, the following laboratory factors were analysed: leucocytes, eosinophils, haemoglobin, prothrombin activity, thromboplastin time, fibrinogen, platelets and erythrocyte sedimentation rate.

Human serum E17 (anisakidosis reference serum) was supplied by the Contrôle National de Qualité en Parasitologie (Département de Biologie Médicale 'E. Brumpt', Centre Hospitalier Gonesse, France). The serum of reference E17 was obtained two months post-infection and tested by Western blot, immunoelectrophoresis and the Ouchterlony test [10].

A. simplex antigen preparation

Third-stage larvae (L3) of *A. simplex* were extracted manually from the viscera, flesh and body cavity of naturally infected blue whiting (*Micromesistius poutassou*) and exhaustively washed in water. For the preparation of the crude extract (CE), *A. simplex* L3 were placed at 4°C in a PBS buffer. This material was homogenized in a hand-operated glass tissue grinder at 4°C, following sonication for 6 min (10 s/pulse) with a Virsonic 5 (Virtis Co. Inc, Gardiner, NY, USA) set at 70% output power. The homogenate was extracted in PBS at 4°C overnight and subsequently delipidized with *n*-hexane and then centrifuged at 8497 *g* for 30 min at 4°C (Biofuge 17RS, Heraeus Sepatech). The supernatant was dialysed overnight at 4°C in PBS and its protein content was estimated by the Bradford method [11]. The extract was frozen at -20°C until use [12].

To obtain ES products, viable *A. simplex* L3 were

carefully selected, and immersed for 1 h in NaCl (0.85%) containing gentamicin (4 g/l). One larva per ml was placed in each well of 24-well plates (Costar, Cambridge, MA, USA) containing 1 ml of minimum essential Eagle's medium with Earle's salts (ICN Biomedicals Inc., Costa Mesa, California, USA) supplemented with sodium bicarbonate (2.2 g/l), glutamine (0.292 g/l) and gentamicin (4 g/l) and maintained at 28°C. Supernatants were collected weekly. After dialysing against PBS (0.01 mol/l sodium phosphate; 0.15 mol/l NaCl) and concentrating in an Amicon YM-10, the protein content in the supernatant was estimated by the Bradford method [11] and finally frozen at -20°C until use [8].

Enzyme-linked immunosorbent assay (ELISA)

The 96-well microtitre plates (Nunc-Immuno Plate PoliSorp™) were sensitized overnight at 4°C by the addition of 1 µg/ml of larval CE antigen diluted in a carbonate buffer to 0.1 mol/l at pH 9.6 (100 µl per well). After washing three times with 0.05% PBS-Tween-20 (PBS-Tween), wells were blocked by the addition of 200 µl per well of 0.1% BSA in PBS, for 1 h at 37°C. After washing, 100 µl of duplicate dilutions of sera at 1/100 in PBS-Tween, containing 0.1% BSA, were added and incubated at 37°C for 2 h. After washing, 100 µl per well of affinity-isolated, peroxidase-conjugated, goat anti-human immunoglobulins (gamma, alpha, mu and light chains), IgG (gamma), IgM (mu) and IgA (alpha) (Caltag Laboratories, Burlingame, California, USA) at the appropriate dilution in PBS-Tween, 0.1% BSA, were incubated for 1 h at 37°C. After washing in PBS-Tween, 100 µl per well of substrate (*o*-phenylenediamine; Sigma) were added at 0.04% in phosphate citrate buffer (pH 5.0) with 0.04% hydrogen peroxide. The reaction was stopped with 3 N sulfuric acid and the plates were read at 492 nm. Simultaneously, an ELISA without antigen was performed as control [12].

To evaluate the results, we used the diagnostic index (DI): the ratio between the optical density resulting from the test serum and the optical density of the negative control once its corresponding non-specific reaction with the BSA used in the post-coating was subtracted [12]. For total immunoglobulin, IgG, IgM and IgA DI values <1.5 were considered negative, those between 1.5 and 2 as doubtful, and those ≥ 2 as positive.

ELISA for IgE determination

The plates were coated overnight by the addition of 1 µg per well of CE antigen. A post-coating was carried out as previously described. Test serum, at the dilution 1 in 2, was added in duplicate and incubated at 37°C for 2 h. After washing in PBS-Tween, 100 µl per well of a murine monoclonal antibody against epsilon human IgE chain (IgG1κ, E21A11; Ingenasa, Madrid, Spain)

was added and incubated for 2 h at 37°C. After washing, 100 µl per well of a goat anti-mouse IgG1 (gamma) horseradish peroxidase conjugate (Caltag Laboratories) was added and incubated for 1 h at 37°C. The following steps were performed as described in the previous ELISA method. DI values ≥ 2.5 were considered positive.

Immunoblotting analysis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out as described by Laemmli [13] and revised by Hames [14] using a Mini Protean® II cell (BioRad). The gels consisted of a 4% stacking gel and a 5–20% linear gradient separating gel. Larval CE antigen was dissolved in a sample buffer (50 mmol/l Tris–HCl buffer, pH 8.6, containing 2% SDS, 20% glycerol and 0.02% bromophenol blue) diluted 1:1 in electrode buffer (25 mmol/l Tris, 192 mmol/l glycine, pH 8.3), containing 1% SDS. Electrophoresis was performed for 2 h at a constant 100 V in Tris–glycine electrode buffer (see above). Broad-range molecular weight markers (6500–205 000 Da, BioRad) were incorporated into each electrophoretic run.

Following SDS–PAGE of the larval CE antigen, the protein bands were transferred onto a 0.22 µm pore size nitrocellulose membrane (Pharmacia) in a Multiphor II Nova Blot semi-dry transfer cell (Pharmacia) with 48 mmol/l Tris, 39 mmol/l glycine, 0.037% SDS and 20% methanol, pH 9.2. The transblot was carried out at 45 mA constant current for 12 h. The membrane was cut into strips and each strip was blocked for 3 h at room temperature with PBS containing 5% non-fat dry milk, prior to immunorecognition by incubation for 3 h with the sera diluted at 1/50 in PBS–Tween containing 1% non-fat dry milk. Each strip was then washed with PBS–Tween (3 × 5 min) and incubated for 3 h with affinity-isolated, peroxidase-conjugated, goat anti-human immunoglobulins (gamma, alpha, mu and light chains), IgG (gamma), IgM (mu) and IgA (alpha) at the appropriate dilution in PBS–Tween, containing 1% non-fat dry milk. To visualize bands, the strips were washed with PBS–Tween (3 × 5 min) and reacted with the substrate (PBS, containing 0.006% H₂O₂:methanol, containing 0.03% 4-chloro-1-naphthol). The reaction was stopped by exhaustive washing with distilled water, and the stained bands were photographed [12].

Statistical analysis

Data were analysed with SPSS/PC software. Statistical comparisons were done with the ANOVA one-way test. Multiple comparisons *post hoc* were performed with the Bonferroni method. When the case number in each group was less than 30, the Kruskal–Wallis one-way ANOVA was used. Student's *t* test or the Mann–Whitney *U* test was used for comparing quantitative data between two groups. Qualitative data were ana-

lysed with the Pearson χ^2 test. *P* values < 0.05 were considered to be statistically significant.

Results

We examined a total of 87 patients with digestive bleeding, 23 females (26%) and 64 males (74%). The mean age ($\pm$ SD) was 61.41 ± 14.89 years, range 23–88. Gastrointestinal haemorrhage was apparently caused by ulcer in 38 cases (22 gastric ulcers and 12 duodenal ulcers), oesophageal and gastric varices in 34 cases (10 of these patients also had an ulcer), haemorrhagic gastritis in five cases and Mallory–Weiss syndrome in ten cases. There was no previous diagnosis or evidence of a current *Anisakis* infection.

The mean DI value in each immunoglobulin group is shown in Table 1. The antibody prevalences are presented in Table 2. DI values ≥ 1.5 were found for 30% of the sera for specific total immunoglobulin (G+M+A) against *A. simplex*. For IgG, IgM, IgA and IgE, the percentages were 22%, 17%, 37% and 12% respectively. Greater positivity was observed within the varices and

Table 1 Mean diagnostic index values and standard deviation of the patients' sera

Diagnostic index	< 1.5	1.5–2	≥ 2
Total immunoglobulins (C)	0.90 $\pm$ 0.31	1.75 $\pm$ 0.12	2.59 $\pm$ 0.47
IgG(C)	0.80 $\pm$ 0.37	1.70 $\pm$ 0.13	2.23 $\pm$ 0.19
IgM(C)	0.66 $\pm$ 0.43	1.69 $\pm$ 0.15	2.65 $\pm$ 0.22
IgA(C)	0.81 $\pm$ 0.34	1.67 $\pm$ 0.14	2.63 $\pm$ 0.49
Total immunoglobulins (ES)	1.05 $\pm$ 0.25	1.67 $\pm$ 0.11	2.18 $\pm$ 0.09
IgG(ES)	1.17 $\pm$ 0.18	1.81 $\pm$ 0.18	2.41 $\pm$ 0.32
IgM(ES)	0.72 $\pm$ 0.36	1.73 $\pm$ 0.09	2.11
IgA(ES)	1.16 $\pm$ 0.33	1.87 $\pm$ 0.17	3.95 $\pm$ 1.86

Diagnostic index = ratio between the optical density resulting from the test serum and the optical density of the negative control. DI < 1.5 is negative; DI 1.5–2 is doubtful; DI ≥ 2 is positive). Total specific immunoglobulins (G + M + A) against *A. simplex*. IgG, IgM, IgA, IgE, specific immunoglobulins against *A. simplex*. C, crude antigen; ES, excretory/secretory antigen.

Table 2 Prevalence of detectable antibodies against *Anisakis simplex* in patients with digestive haemorrhage using crude antigen

Immunoglobulin	<i>n</i>	%	CI
Total immunoglobulins	26	30	21–40
IgG	19	22	14–31
IgM	15	17	10–26
IgA	32	37	27–47
IgE	9	12	6–20

n, number of patients with detectable antibodies: diagnostic index ≥ 1.5 for total immunoglobulins, IgG, IgM and IgA; DI ≥ 2.5 for IgE). %, percentages of patients (total number of patients 87, except IgE with 78). CI, 95% confidence intervals. IgG, IgM, IgA, IgE, specific immunoglobulins against *A. simplex*.

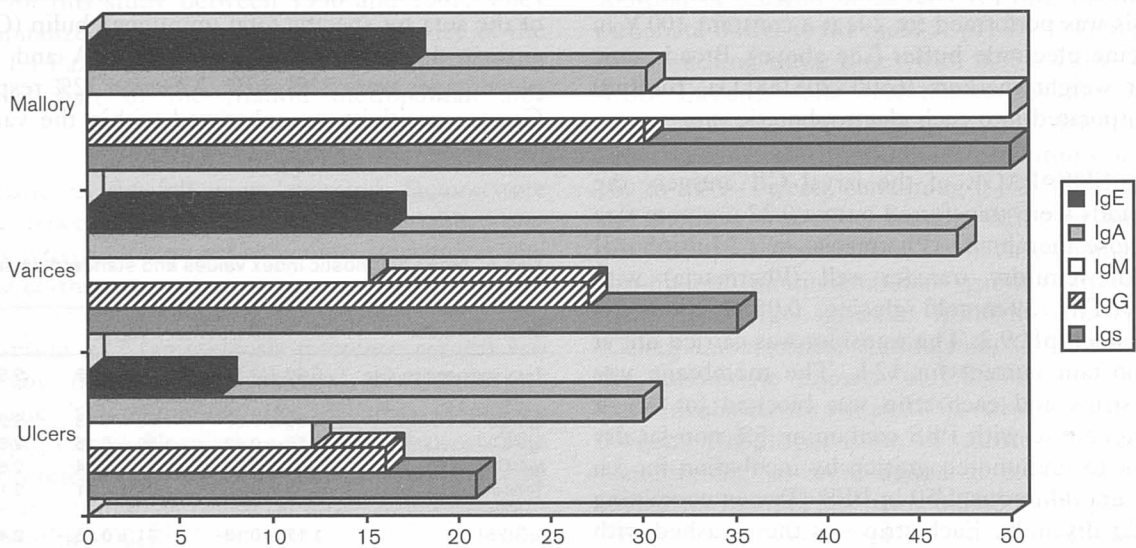
Mallory groups of patients (Fig. 1), although the difference was not statistically significant.

Twenty-four of the studied sera with $DI \geq 1.5$ for total immunoglobulin response were tested by ELISA against the excretory/secretory products of *A. simplex* larvae (eight patients with varices, three with varices and ulcer, eight with ulcers and five with Mallory–Weiss syndrome). Eight of these sera showed $DI \geq 1.5$ for specific total immunoglobulins. For IgG, IgM and IgA, the numbers were 13/24, 3/24 and 16/24, respectively. The greatest rate of positivity was observed for IgA (Fig. 5).

Twenty-five of the studied sera, with DI values ≥ 1.5 for total immunoglobulins against crude extract were

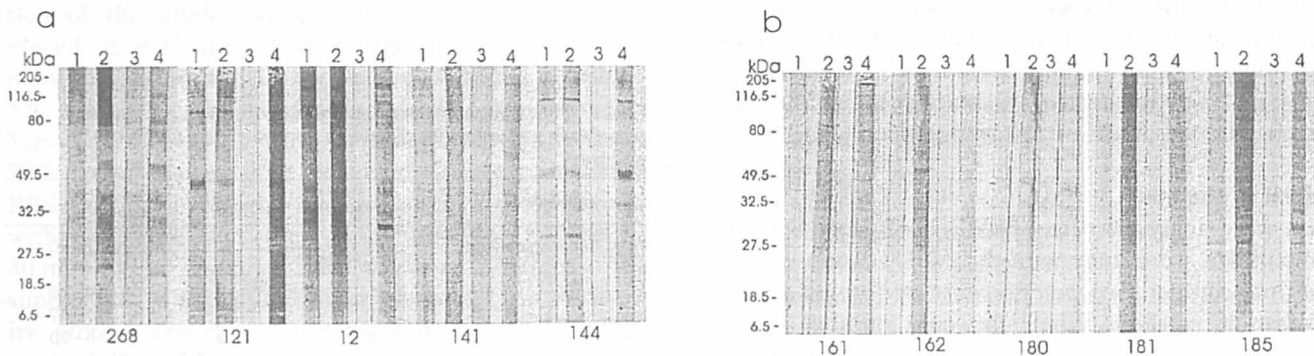
selected. These sera were tested by means of immunoblot analysis against crude larval antigen, under non-reducing conditions. For the total immunoglobulin antibody response, 12 of the sera showed an immunorecognition pattern markedly concordant with the human anisakidosis reference serum (E17). In 13 sera we visualized bands of specific IgG, in six sera specific IgM and in 12 sera specific IgA. Likewise, six sera with specific total immunoglobulin bands, seven with IgG, four with IgM and nine sera with specific IgA reacted with antigenic proteins situated approximately between 50 and 250 kDa, with a band of about 70 kDa which was observed for all cases. On the other hand, two of the sera which had shown total immunoglobulin bands and one with an IgG band only showed a reaction with antigenic proteins of high molecular weight (Figs 2–4).

Fig. 1



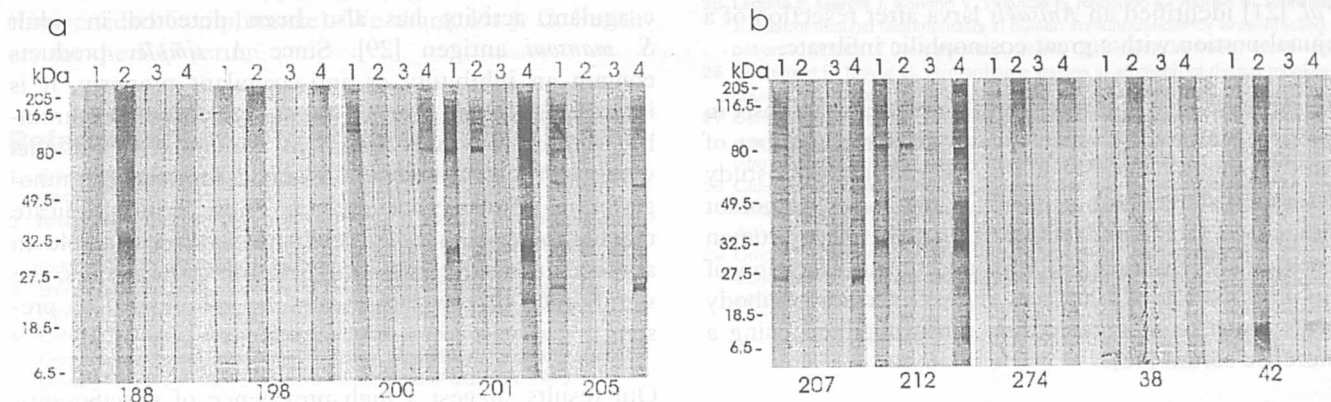
Percentages of patients with positive sera against *A. simplex* in ulcers, varices and Mallory groups of patients. Igs: total specific immunoglobulin against *A. simplex* (IgG + IgM + IgA). IgG, IgM, IgA, IgE: specific immunoglobulins G, M, A and E against *A. simplex*.

Fig. 2



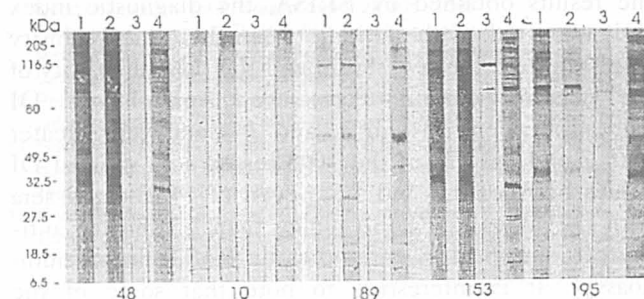
Immunorecognition patterns of the selected sera against the crude extract of *A. simplex*. (a) Patient numbers: 268, 121, 12, 141, 144. (b) Patient numbers: 161, 162, 180, 181, 185. Lane 1: total immunoglobulins; lane 2: IgG; lane 3: IgM; lane 4: IgA.

Fig. 3



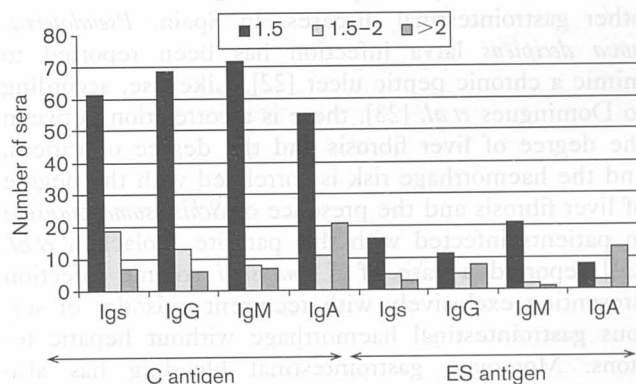
Immunorecognition patterns of the selected sera against the crude extract of *A. simplex*. (a) Patient numbers: 188, 198, 200, 201, 205. (b) Patient numbers: 207, 212, 274, 38, 42. Lane 1: total immunoglobulins; lane 2: IgG; lane 3: IgM; lane 4: IgA.

Fig. 4



Immunorecognition patterns of the selected sera against the crude extract of *A. simplex*. Patient numbers: 48, 10, 189, 153, 195. Lane 1: total immunoglobulins; lane 2: IgG; lane 3: IgM; lane 4: IgA.

Fig. 5



Number of sera with a diagnostic index that was negative (< 1.5), doubtful ($1.5-2$) or positive (≥ 2), against crude (C) and excretory/secretory (ES) *A. simplex* antigens.

Leucocytes, eosinophils, haemoglobin, prothrombin activity (PA), thromboplastin time, fibrinogen, platelets and erythrocyte sedimentation rate were analysed for each immunoglobulin group according to the DI values. For total immunoglobulin, prothrombin activity and the number of platelets were significantly lower in the doubtful groups (PA 58%, platelets $112 \times 10^3/\text{mm}^3$). For IgM, the number of eosinophils was significantly higher ($385 \times 10^3/\text{mm}^3$) in the doubtful groups (DI values between 1.5 and 2). We did not find any other significant differences.

Discussion

The first known human anisakidosis case in Spain was reported by Arenal *et al.* [15]. It was an acute abdomen case due to appendicular lumen occlusion caused by *Anisakis* larvae. Later, López-Vélez *et al.* [16] reported three cases of non-imported anisakidosis in Spanish

patients. In two of them, presented clinically as a bowel obstruction, a larva of *A. simplex* was found by intestinal biopsy in the abdominal wall. In the third case, which presented as a peptic ulcer with a longer evolution time, a *Pseudoterranova decipiens* larva was found during an upper gastrointestinal endoscopic procedure. Likewise, Valero *et al.* [17] identified an *A. simplex* larva after surgical intervention in a patient with a clinical suspicion of peritonitis. A case of anisakidosis was reported by Clavel *et al.* [18] in which a living nematode larva was found in the abdominal cavity of a woman. The worm was identified as an *Anisakis physeteris* larva. Martín *et al.* [19] described two new cases of non-imported anisakidosis after ingestion of raw anchovies. Further, Acebes *et al.* [20] related two additional cases, in which two and one larva, respectively, were

extracted by fibrogastroscopy, showing an oedematous gastric wall with erosive lesions. Moreover, Rodríguez *et al.* [21] identified an *Anisakis* larva after resection of a jejunal portion with a great eosinophilic infiltrate.

On the basis that the true prevalence of anisakidosis in Spain is presumably much greater than the number of cases previously described, we have performed a study of seroprevalence by means of ELISA and immunoblot with crude larval extracts of *A. simplex* using human sera from Spanish people with no clinical suspicion of anisakidosis: 6% of 1008 sera showed specific antibody levels equal to or greater than those obtained using a reference serum [12].

In this paper, we have investigated the relationship between gastrointestinal tract bleeding episodes and specific antibodies against *A. simplex*. We found a higher prevalence of specific detectable antibodies in patients with digestive haemorrhage than in healthy controls. This could be explained by the fact that the lesions caused by the parasite can mimic ulcer and other gastrointestinal diseases. In Spain, *Pseudoterranova decipiens* larva infection has been reported to mimic a chronic peptic ulcer [22]. Likewise, according to Domingues *et al.* [23], there is a correlation between the degree of liver fibrosis and the degree of varices, and the haemorrhage risk is correlated with the degree of liver fibrosis and the presence of *Schistosoma mansoni* in patients infected with this parasite. Rolachon *et al.* [24] reported a case of *S. mansoni* colonic infection presenting exclusively with recurrent episodes of serious gastrointestinal haemorrhage without hepatic lesions. Moreover, gastrointestinal bleeding has also been reported in human immunodeficiency virus-infected patients with visceral leishmaniasis [25].

On the other hand, *A. simplex* products prolonged the prothrombin time (PT) and the partial thromboplastin time (PTT) of human plasma [8]. Overstreet and Meyer [26] observed haemorrhagic lesions in the stomach and proximal duodenum of monkeys after *Hysterothylacium* worm administration. These authors explained the lack of any worms protruding into the stomach lumen or migrating within mucosae plus the presence of extensive haemorrhage as due to the fact that the anisakid larvae traverse the stomach wall rapidly.

Hookworms have been reported to possess a substance with anti-coagulant activity. Carroll *et al.* [27], through observations of the anti-coagulant effects of *Ancylostoma ceylanicum* in human and dog blood *in vitro* and infected dogs *in vivo*, have shown that the anti-coagulant properties of hookworms may have biological significance in infected animals. Cappello *et al.* [28] reported that the predominant anti-coagulant activity from the adult

hookworm *Ancylostoma caninum* is an inhibitor of the activated form of clotting factor X. Likewise, anti-coagulant activity has also been detected in adult *S. mansoni* antigen [29]. Since *A. simplex* products contain an inhibitory or anti-coagulant property, it is interesting to note that, in our study, a lower prothrombin activity was only found in the group of patients with DI values between 1.5 and 2 for total immunoglobulins against crude extract. These results indicate that although *A. simplex* products may have a role in anti-coagulant effects during invasion at local sites, the significance of this fact cannot be established at present.

Our results suggest a high prevalence of specific antibodies against *A. simplex* in patients with digestive haemorrhage when comparing with healthy patients. In a previous paper [12], the results obtained in a study of seroprevalence by means of ELISA and immunoblot with crude larval extracts of *A. simplex* using 1008 human sera from Spanish people showing no clinical suspicion of anisakidosis were given. For evaluation of the results obtained by ELISA, the diagnostic index (DI) was used, as the ratio between the optical density resulting from the test serum and the optical density of the negative control. Forty-seven sera showed DI values between 1.5 and 2, and 14 sera were greater than 2 and only 75 of the 1008 tested sera showed DI values between 1.3 and 1.5. Seven of 14 selected sera with DI values by ELISA higher than 1.3 showed anti-*Anisakis* specific IgE antibodies by RAST fluoroimmunoassay. It is interesting to note that some of the positive sera from haemorrhage patients when tested by means of immunoblot analysis showed an immunorecognition pattern markedly concordant with the human anisakidosis reference serum (E17). Total immunoglobulin and IgA antibody responses to the crude antigen were higher. The prevalence of an IgE-positive response was low. The cause of this is still unknown. One possibility is that total immunoglobulin masks the presence of IgE. Patients with haemorrhage caused by varices or Mallory-Weiss tears had a higher percentage of positive sera than the rest, though there were no significant differences. Whether an abnormal response to parasite antigens or parasite initiated this process must be investigated. In patients with a positive *Anisakis* history, oedema, congestion, bleeding and erosion of the gastric mucosa were observed by gastroscopy, as well as acute ulcerative changes by laparoscopy [2]. These facts suggest that the response to gastric damage could be aggravated in these patients. Several syndromes may result, such as the acute gastric mucosal lesion which includes erosion, ulceration, and a haemorrhagic lesion on the gastric mucosa. The aetiology of this symptom complex is not completely understood, but anisakidosis could be one of the causative factors [2].

Acknowledgements

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