

ELISA antibody determination in patients with anisakiosis or toxocariosis using affinity chromatography purified antigen

Marta Rodero, Ph.D.,* Carmen Cuéllar, Ph.D.,* Soledad Fenoy, Ph.D.,# Carmen del Aguila, Ph.D.,# Tomás Chivato, M.D.,§ José M. Mateos, M.M.,§ and Rafael Laguna, M.D.§ (Spain)

ABSTRACT

One of the fundamental aspects of a parasitic infection diagnosis is the use of adequate antigens to develop specific and sensitive immunoassays. This fact is especially complicated in nematode infection cases because of the high cross-reactivity among different parasites in this group. We performed an evaluation of *Anisakis simplex* antigens purified by affinity chromatography. We used sera from 38 patients diagnosed with *Anisakis* sensitization and sera from 35 patients with clinical suspicion of visceral larva migrans (VLM). These sera were assayed by the ELISA method against the crude extracts (CEs) and the purified antigens. When the sera from patients diagnosed with *Anisakis* sensitization were tested against the *A. simplex* CE, the IgG was the most abundant immunoglobulin. When the *A. simplex* larval antigens were purified using a column of IgG anti-*A. simplex* (PAK) or a column of IgG anti-*Ascaris suum* (PAS) were tested, we observed a higher diminution in the IgG levels, which coincides with the augmentation of the mean values against the "eluted of *Ascaris*" (EAS antigen). When the IgE was detected, only 18.4% of the sera reacted with the PAS antigen. We have observed that in the purification process of *A. simplex* antigen by affinity chromatography, the majority of the proteins that produced cross-reactivity against *A. suum* and *Toxocara canis* were eliminated.

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A possible and definitive diagnosis of human anisakiosis could be based on the morphologic characteristics of *Anisakis*, when the larva is expectorated by the patient or removed from the gut by endoscopy. However, a great number of cases exist in which the larva is not located in the gut because of extragastric migrations or, on the contrary, is degraded, making its identification impossible.¹

Although the immunologic techniques permit the determination of the presence of specific antibodies against different parasites, the introduction of extremely sensitive techniques, such as ELISA, does not avoid the presence of antibodies that provoke numerous cross-reactions, when the *Anisakis simplex* larval crude extract (CE) is used as a reactive for the determination of specific IgE levels in patients with clinical suspicion of anisakiosis.^{2,3} Because of these facts, we performed the evaluation by ELISA of previously de-

scribed *A. simplex* antigen purified by affinity chromatography^{4,5} using sera from patients previously diagnosed with *Anisakis* sensitization or toxocariosis.

MATERIALS AND METHODS

Parasites

A. simplex third stage larvae (L3) were collected manually from the viscera, flesh, and body cavities of naturally infected blue whiting (*Micromesistius poutassou*) and exhaustively washed in water.⁶ *Ascaris suum* was obtained from natural infections of swine.

Antigens

For preparing the CE of *A. simplex* crude extract (AK antigen), L3 were placed at 4°C in PBS (phosphate buffer saline). This material was homogenized in a hand-operated glass tissue grinder at 4°C, followed by sonication for 10 seconds with a Virsonic (Virtis, Gardiner, NY) 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 minutes at 4°C (Biofuge 17RS; Heraeus Sepatech, Gmb, Osterode, Denmark). The supernatant was dialyzed overnight at 4°C in PBS. The CE antigens from *A. suum* adults (AS antigen) were obtained using a modification of the Welch method⁷ by homogenization and extraction in PBS at 4°C overnight (instead of ultrasonic burst). Their protein contents

From the *Departamento de Parasitología, Facultad de Farmacia, Universidad Complutense, Madrid, Spain, #Facultad de Ciencias Experimentales y de la Salud, Urbanización Montepríncipe, Crta. Boadilla del Monte KM 5,600, Madrid, Spain, and §Servicio de Alergia e Inmunología, Hospital del Aire, Madrid, Spain
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Address correspondence and reprint requests to Carmen Cuéllar del Hoyo, Ph.D., Departamento de Parasitología, Facultad de Farmacia, Universidad Complutense, 28040 Madrid, Spain

E-mail address: cuellarh@farm.ucm.es

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were estimated by the Bradford method,⁸ and the antigen was frozen at -20°C until use.^{9,10}

Hyperimmune Sera

New Zealand rabbits that had ~ 3 kg of body weight were immunized with 1 mg of larval *A. simplex* or adult *A. suum* CE antigens by the intramuscular route. The animals were bled weekly postimmunization after the first inoculation (week 0). The experiments were carried out according to the European Council on applied animal experiments, published in the Guidelines 86/609ED, and controlled in Spain by Royal Decree 223/1988 of 14 March, on the protection of animals used for research and other scientific ends.^{10,11}

Human Sera

Thirty-eight human anti-*Anisakis* sera were obtained from the Servicio de Alergia del Hospital del Aire de Madrid. All of the sera showed a positive result when assayed with the CAP system (Pharmacia AB, Uppsala, Sweden) by fluoro-enzymo-immunoassay, which varied from values of CAP = 1 (3%), CAP = 2 (26%), CAP = 3 (47%), CAP = 4 (10%), CAP = 5 (8%), and CAP = 6 (5%). These patients ate fish regularly. They were diagnosed as having idiopathic acute recidivous urticaria. Patients diagnosed with any other allergic disease were excluded, as were patients taking corticosteroids or antihistamines. All patients underwent the usual protocol related to idiopathic acute recidivous urticaria (to exclude other trigger factors): complete blood count, biochemistry (urea, glucose, cholesterol, glutamic-oxaloacetic transaminase (GOT), gamma glutamyl transferase (GGT), total proteins, seric Fe, albumin, alkaline phosphatase, lactate dehydrogenase (LDH), and amylase), urine, C3, C4, C1q, total hemolytic complement, faecal parasites, hepatitis B and C, and skin-prick test (inhaled and food-derived allergens). Likewise, we selected 35 human sera from the Hospital Juan Canalejo de la Coruña from patients with clinical suspicion of visceral larva migrans (VLM) who were diagnosed as positive by ELISA using the excretory-secretory (ES) antigen of *Toxocara canis*, with diagnostic indexes ≥ 3 , according to Fenoy *et al.*¹²

Purification of the *A. simplex* CE Antigen by Affinity Chromatography

Protein A-Sepharose CL-4B bead (Pharmacia Biotech, Piscataway, NJ) columns were prepared according to the manufacturer's instructions. Rabbit anti-*A. simplex* or anti-*A. suum* antibodies, in sample buffer ([0.05 M of Tris and 0.5 M of NaCl, pH 8.0) were loaded into the columns. Fractions of 1 mL were then collected. The unbound immunoglobulins were washed with washing buffer (0.05 M of Tris and 0.5 M of NaCl). Then, the bound immunoglobu-

lins were eluted with glycine buffer (0.2 M of glycine and 0.5 M of NaCl, pH 2.8). Fractions were collected onto 100 μL of collection buffer (Tris-base, 1 M; pH 8.5) and read using a spectrophotometer at A280 to calculate the IgG concentration. A column was prepared with protein A affinity isolated IgG anti-*A. simplex*, at a concentration of 5 mg/mL in NaHCO_3 , 0.1 M, with NaCl, 0.5 M (pH 8.5), coupled to CNBr-activated Sepharose 4B, according to the manufacturer's instructions (Pharmacia Biotech). The *A. simplex* CE antigen in sample buffer was loaded into the column and incubated for 3 hours at room temperature. Fractions of 1 mL were then collected. The unbound antigens were washed with washing buffer and the bound antigens were eluted with glycine buffer followed by 50 mM of diethylamine in saline, pH 11.5, collecting them into glycine to neutralize the eluted fractions. The fractions were read at 280. This antigen was called PAK antigen. The same procedure was performed using columns prepared with rabbit IgG anti-*A. suum* to obtain the PAS antigen and the EAS antigen.^{4,5}

Determination of Specific Antibody Levels

Ninety-six-well microtiter plates (Nunc-Immuno Plate PolySorp, Nunc, Roskilde, Denmark) were sensitized overnight at 4°C by the addition of 100 μL /well of antigen diluted at 1 $\mu\text{g}/\text{mL}$ in a 0.1 M of carbonate buffer, pH 9.6. After washing three times with 0.05% PBS-Tween 20 (PBS-Tween), the wells were blocked by the addition of 200 μL per well of 0.1% bovine serum albumin (BSA) in PBS for 1 hour at 37°C . After washing, 100 μL of duplicate dilutions of the human sera at 1/400 in PBS-Tween containing 0.1% BSA were added and incubated at 37°C for 2 hours. Once the plates were washed, 100 μL per well of goat anti-human IgG or IgM that was peroxidase conjugated (Biosource International, Camarillo, CA) at the appropriate dilution in PBS-Tween, 0.1% BSA, was incubated for 1 hour at 37°C . After adding the substrate (phosphate-citrate buffer, containing 0.04% H_2O_2 and 0.04% *o*-phenylenediamine) the reaction was stopped with 3N-sulfuric acid and the plates were read at 490 nm.¹³ The results were expressed as problem serum (O.D.p)–control serum (O.D.c.) (optical density–optical density) indexes by subtracting the mean O.D. of the control from the mean O.D. of the test sera once the nonspecific reaction with the BSA used in the blocking was subtracted. For the determination of the specific IgE, the plates were coated with 1 μg of antigen (CE of *A. simplex*, and purified antigen PAK, PAS, and EAS) per well. The test sera, at the dilution $\frac{1}{2}$ were added in duplicate. A murine monoclonal antibody against an ϵ -human IgE chain (IgG1 κ , E21A11; INGENASA, Madrid, Spain) was added and incubated, followed by a goat anti-

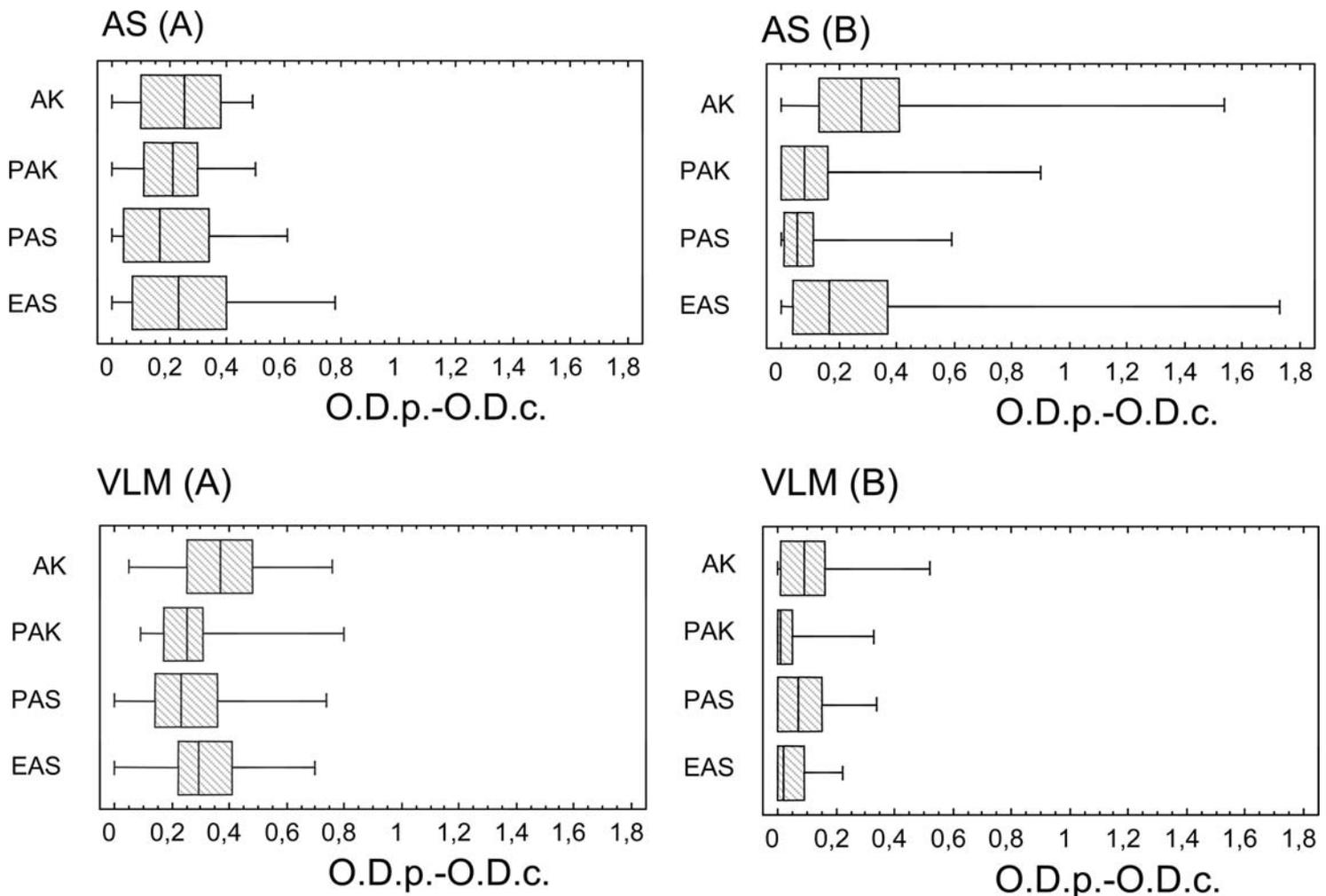


Figure 1. (A) IgM and (B) IgG levels in sera from patients diagnosed with *Anisakis* sensitization (AS) and VLM against AK, PAK, PAS, and EAS antigens. Mean and SD are shown.

mouse IgG1 (γ) horseradish peroxidase conjugate (CALTAG Laboratories, Burlingame, CA). The following steps were performed as described in the previous ELISA method.

Statistic Treatment

To determine the actual levels of agreement among the CE of *A. simplex* (AK antigen) and purified antigens (PAK, PAS, and EAS), the concordance determination was performed by the κ -coefficient. For the determination of the sensitivity and specificity of the different antigens, 2×2 tables were performed. In these tables, the number of sera from patients diagnosed with *Anisakis* sensitization and CAP values ≥ 3 and ≤ 2 against the different antigens were represented for each immunoglobulin. In addition, different O.D. cutting points were selected in function of positive and negative predictive value as well as the sensitivity and specificity, in the case of each immunoglobulins and assayed antigens (AK, PAK, and PAS).

RESULTS

ELISA Antibody Determination in Sera from Patients Diagnosed with *Anisakis* Sensitization or VLM Using the *A. simplex* Antigen Purified by Affinity Chromatography

When the sera from patients diagnosed with *Anisakis* sensitization were tested against the AK antigen, we observed that the IgG was the immunoglobulin with higher levels, followed by the IgM (Fig. 1; Table 1). When the IgGs were tested, the amount of sera from patients diagnosed with VLM, which cross-reacted with the AK antigen, was lower (20%). However, with the AK antigen higher IgM levels were obtained; 88.5% of the sera from VLM patients showed O.D.p.-O.D.c values ≥ 0.2 (Fig. 1, Table 1).

The PAK and the PAS antigens were tested using the sera from patients diagnosed with *Anisakis* sensitization. In both cases, the highest O.D. corresponded to the IgM followed by the IgG (Fig. 1; Table 1). Against the EAS antigen, the mean values of IgG increased

Table 1 Percentage of patients diagnosed of Anisakis sensitization (AS) or VLM with IgG, IgM, and IgE O.D.p-O.D.c indexes ≥ 0.2 against crude extract of *A. simplex* (AK) and against the purified antigens PAK, PAS, and EAS

	AK AS/VLM	PAK AS/VLM	PAS AS/VLM	EAS AS/VLM
IgG	57.89/20	18.42/2.85	7.89/5.71	44.73/5.71
IgM	60.52/88.57	50/65.71	44.73/62.85	55.56/80
IgE	15.7/0	10.5/0	18.4/0	21/0

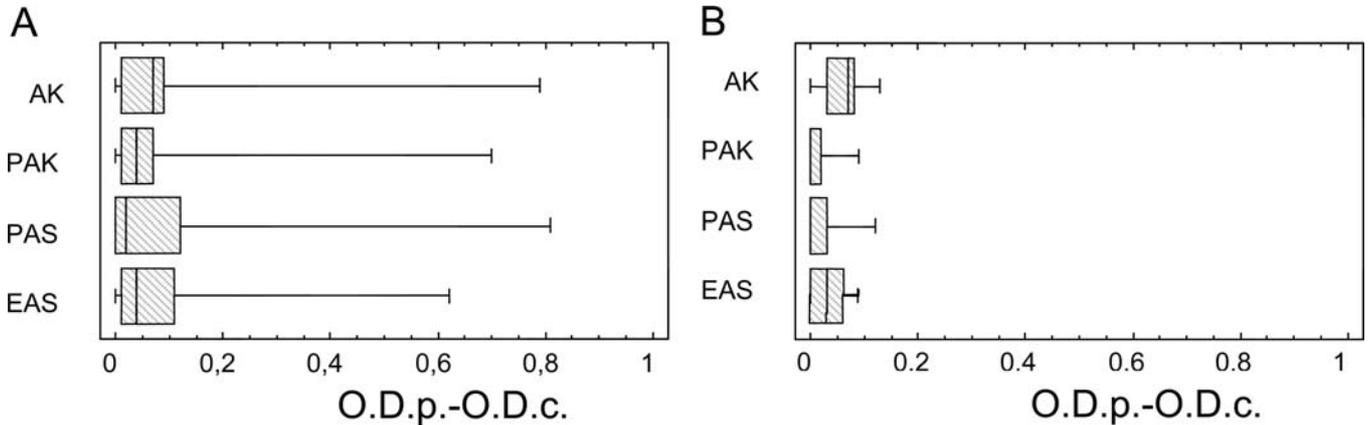


Figure 2. IgE levels in sera from patients diagnosed with (A) Anisakis sensitization and (B) VLM against AK, PAK, PAS, and EAS antigens. Mean and SD are shown.

compared with those observed using the purified antigens (Fig. 1; Table 1).

Sixty-five point seven percent of the VLM patients showed IgM values of O.D.p-O.D.c indexes ≥ 0.2 against the PAK antigen. This value only decreased to 63% when the PAS antigen was used. Finally, 80% were positive when the EAS antigen was tested (Fig. 1; Table 1). Fifteen point seven percent of the patients diagnosed with *Anisakis* sensitization showed IgE levels ≥ 0.2 against the AK antigen. In the case of the purified antigens, 10.5% of the patients showed detectable IgE levels against the PAK antigen (O.D.p-O.D.c. ≥ 0.2). These values were enhanced until 18.4 and 21% against PAS and EAS antigen, respectively (Fig. 2 A; Table 1).

Concordance Determination by κ -Coefficient

To determine actual levels of agreement among the CE of *A. simplex* (AK antigen) and the purified antigens (PAK, PAS, and EAS), the values of the κ -coefficient were calculated. When we compared the concordance between the CE of *A. simplex* and the PAK antigen, we observed that the IgM and IgE immunoglobulins had the higher values (94 and 54%, respectively) and the IgG did not reach 30%. All of these values descended, when the concordance was calculated, when we compared PAK with PAS antigen. Again, the IgM reached the higher values (28%) followed by IgE (16.5%) and finally the IgG (2.5%). Finally, the percentage of con-

Table 2 Values of κ -coefficient to measure concordance of crude and purified *A. simplex* antigens

	AK/PAK	AK/PAS	PAK/PAS
IgG	0.2821	0.0244	0.3254
IgM	0.9474	0.2791	0.3684
IgE	0.5422	0.1659	0.3512

The κ takes on the value of zero if there is no more agreement between two judges or tests as can be expected on the basis of chance. κ takes on the value 1 if there is perfect agreement. It is considered that $\kappa[\rho]$ values < 0.4 represent poor agreement, values between 0.4 and 0.75 represent fair to good agreement, and values > 0.75 represent excellent agreement.

cordance enhanced around 35% for IgM, IgE, and IgG immunoglobulins when we compared PAK and PAS antigens (Table 2).

Determination of Sensitivity and Specificity

When the *A. simplex* CE was used, a variation of sensitivity and specificity was detected according to the immunoglobulin selected. The cut point of O.D. ≥ 0.15 for the IgG was selected as a first reference because this immunoglobulin showed the greater sensitivity (82.7%). When this parameter was applied, 70% of the patients diagnosed with sensitization to *A. simplex*

were positive. When the second determination was performed by using the PAK antigen and the cut point of O.D. ≥ 0.07 for the IgG, a decrease of 20% in the positive group was observed.

DISCUSSION

The aim of this work was to assay, by ELISA, the specificity and the sensitivity of the larval *A. simplex* CE antigen purified by means of affinity chromatography. When the AK antigen was tested using sera from patients diagnosed with *Anisakis* sensitization, we did not detect either IgG or IgM levels in $\sim 40\%$ of the tested sera, despite the fact that all patients had positive CAP values. This difference between the IgE levels obtained by the CAP system method and the other immunoglobulins could be caused by the different techniques and methodologies used to determine the different immunoglobulin class levels. The CAP system assay uses a lower serum dilution and a higher antigenic concentration than the dilution and concentration used by the ELISA method. Another parameter to consider could be the possible competition between IgE and IgG for the same epitopes present in the *Anisakis* CE. In the case of the CAP system assay, the excess of antigen used could decrease this competition and, therefore, the IgE levels could be increased.

The antigenic cross-reactivity has been observed previously by several authors such as, Lorenzo *et al.*³ They detected the presence of IgM anti-*Anisakis* antibodies in sera of healthy and allergic patients to *Anisakis* (30 and 58%, respectively), but after making the deglycosylation of the *Anisakis* antigens, these values decreased to 4.5 and 7%, respectively. The results obtained in our work could be produced by a reaction with the sugared epitopes present in the antigens used in these assays.

When the sera were tested against the PAK antigen, we observed a higher decrease of the IgG levels in comparison with the AK antigen (from 57.8 to 18.4%). However, as it was mentioned previously, the pattern of the PAK antigen showed the same proteins of the AK antigen, but in a different proportion.⁵ This could indicate a reduction of the antibody levels raised to proteins in which their concentrations could have decreased.

When the sera were tested against the PAS antigen, only three of them (7.89%) showed IgG O.D.p-O.D.c indexes ≥ 0.2 . Two of these sera (CAP = 5 and 6) maintained the same values against the PAK antigen. However, a third serum (CAP = 2) had values of O.D.p-O.D.c ≤ 0.2 against the previously assayed AK antigen. Nevertheless, high O.D.p-O.D.c values (>0.5) were seen against the PAS antigen and this could indicate that an increase of the immunogenic proteins able to induce the IgG responses was produced. Finally, 45 and 55% of the sera showed O.D.p-O.D.c \geq

0.2 when the IgG and the IgM immunoglobulins were tested, respectively, against the EAS antigen.

When the IgE levels were tested by the ELISA method, $>75\%$ of the assayed sera, with CAP values between 2 and 5, had no detectable IgE antibodies against the AK antigen. A possible cause of the small proportion of positive sera, as it was previously mentioned, could be the great amount of antigen used by the CAP system assay for the IgE detection. This quantity also could provoke an increase of false positives for an augmentation of the proteins responsible for the cross-reactions among several parasites. Another factor could be the possible competition between the IgE and IgG for the same epitopes present in the AK antigen, because all of the sera, except one, showed high IgG levels. These problems of unspecificity, shown by the CAP system technique, have already been cited by other authors.^{14,15}

Another important factor in the IgE level determination is the clinical history of the patient, to evaluate the possible relation between the appearance of the allergic reaction and the possible ingestion of raw or poorly cooked fish, as well as, the appearance of gastric distresses. We have no evidence of whether these patients had contact with live larvae at a gastric level or if some larvae were gastroscopically extracted from them. According to Sastre *et al.*,¹⁶ the patients only presented allergic episodes after living larvae were ingested, because the allergens present in the ES products did not exist in sufficient amounts in the dead larvae. However, these patients recognized the *A. simplex* proteins present in the CE because the CAP system assay used this antigen and not the ES antigen for specific IgE determination.

When the IgE levels were determined against the PAK and the PAS purified antigens, the number of sera with values ≥ 0.2 varied from 10.5 to 18.4%. This increase of recognition and, thus, of the concentration of immunogenic proteins, could be related to the higher O.D.p-O.D.c values obtained by ELISA. This indicated that an important concentration of specific IgE antibodies had occurred and, consequently, the appearance of allergic episodes. Seventy-one percent of the sera with IgE values ≥ 0.2 against the PAS antigen did not show detectable specific IgE anti-*Anisakis*. All of these sera, except one, did not show high CAP values, which indicates that the greatest number of false negatives was in the patient group with the lowest CAP values (classes 1–3).

We decided to use the κ -coefficient to calculate the concordance among the different antigens used by means of the ELISA technique as a diagnostic test. The κ -values do not indicate which is the method that produced the best results. This statistic method only indicates if there is agreement when two methods are used. Likewise, when we compared the both crude and

PAK antigens, an excellent concordance appeared at the IgM level (near to 1). This indicated that, at such an immunoglobulin level, there were not differences in the case of its determination using the both CE and PAK purified antigens. However, when the CE was compared with the PAS purified antigen, a strong decrease was produced, because there only was concordance in 28% of the patients. This same effect was observed, although in a lower manner, at the IgE level, which fell from 54 to 16.5%, when the CE was compared with the PAK and the PAS antigens, respectively. A stronger diminution was observed at the IgG level, where there was concordance only in 2.5% of the patients when the *A. simplex* CE was compared with the PAS purified antigen. This fact proved that the proteic compounds of both antigens are very different.

To calculate sensitivity and specificity parameters, it is necessary to select the "gold standard," which can evaluate the accuracy of the diagnostic test on the basis that the evaluated disease is truly present or absent in the selected patients. In our case, the CAP value was selected as "gold standard," although it is not a very good diagnostic method but is routinely used to select the *Anisakis*-sensitized patients.

The sensitivity and specificity determinations were performed testing sera from patients diagnosed with *A. simplex* sensitization against the CE (AK antigen), as well as, the purified PAK and PAS antigens, to observe the possible variations produced during the purification process. These evaluations were performed against the tested immunoglobulins, to determine the working conditions (immunoglobulin and antigen) for the human anisakidosis diagnosis.

When the *A. simplex* CE was used, a variation of the sensitivity and the specificity was detected according to the selected immunoglobulin (cut point of O.D. = 0.15, 83% of sensitivity for the IgG). This fact showed the difficulty of the immunodiagnosis of anisakidosis when only a determined immunoglobulin is studied.

In our experimental conditions, we showed the usefulness of testing the sera in a first step against the *A. simplex* CE and then performed a second determination against the PAK and the PAS purified antigens. This does not imply an additional cost of time. For this, the cut point of O.D. ≥ 0.15 for the IgG was selected as a first reference. This immunoglobulin showed the greater sensitivity (82.7%). In the first step of the diagnostic investigations, the sensible tests are the most useful. When this parameter was applied, 70% of sera were positive, despite the fact that all sera except one had a CAP value ≥ 2 . The CAP system has a poor specificity and shows a high rate of false positive. This fact also was observed by Lorenzo *et al.*³ when they evaluated several immunologic techniques to make the diagnosis of *Anisakis*

allergy, observing a 50% of specificity with the CAP system assay.

The second determination was performed by using the PAK antigen and the cut point of O.D. ≥ 0.07 for the IgG. This value was selected because in the middle of the diagnostic investigation, medium values of specificity and sensitivity were established (58.6 and 54.5%, respectively). When all assayed sera were evaluated, a decrease of 20% in the positive group was observed. Only two sera that were previously negative against the *A. simplex* CE, remained positive. These results are in accordance with the high predictive value (80%) observed in the *A. simplex* CE, although a probability of 20% (six sera) of the existence of false positive is always present. In our case, the decrease observed was greater (from 28 positive sera for *A. simplex* CE to 20 for the PAK antigen).

When the sera of the VLM patients were tested against the AK heterologous antigen, we observed cross-reactivity in 20% of them, in the case of the IgG. On the contrary, when the PAK and the PAS purified antigens were evaluated, a sharp decrease in the number of the sera with IgG values ≥ 0.2 was observed (2.8 and 5.7%, respectively). This decrease indicates that in the purification process, most of the proteins responsible for producing the cross-reactivity between *Ascaris* and *Anisakis* were deleted. Contrarily, this purification process did not alter the IgM levels. These levels with O.D.p-O.D.c ≥ 0.2 were very high for both PAK and PAS antigens (66 and 63%, respectively). This fact indicates the poor specificity of this immunoglobulin and its low usefulness in the immunodiagnosis.

When the PAK and PAS purified antigens were evaluated, no sera from these VLM patients reached values of IgE ≥ 0.2 . The results obtained indicate that a deletion of the proteins responsible for unchaining the cross-reactions among *Anisakis*, *Ascaris*, and *Toxocara* were produced after the purification process of *A. simplex* CE.

In conclusion, the purification of the *A. simplex* CE by its elution from an anti-*A. simplex* IgG column and further filtration by an anti-*A. suum* IgG column (PAS antigen) produced an increase of the immunogenic proteins able to unchain the IgG responses, avoiding, besides, the competition for epitopes present in proteins that are poor specific and responsible for the cross-reactions with *Ascaris* and *Toxocara*. In the PAK antigen (eluted from an anti-*A. simplex* column), the proteins of 60, 40, and 25 kDa were concentrated^{4,5} with an increase of the serum recognition toward these fractions and a decrease of the reaction with high molecular weight proteins, properties that were increased in the PAS antigen (eluted from the anti-*A. simplex* IgG column and filtrated by the anti-*A. suum* column). The protein of 14 kDa, which was mainly responsible for the cross-reactions among *Anisakis*, *Ascaris*, and *Toxo-*

cara,² was concentrated in the EAS antigen (eluted from an anti-*A. suum* IgG column).^{4,5} This fact caused the elimination from the PAS antigen of the antigenic epitopes responsible of the cross-reactivity among those ascarids and the production of a false positive in the diagnostic of the sensitization produced by *A. simplex* as well as an important concentration of the proteins responsible for the induction of an increase of specific IgE.

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