Enzyme-linked immunosorbent assay and Western blot antibody determination in sera from patients diagnosed with different helminthic infections with Anisakis simplex antigen purified by affinity chromatography

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An evaluation of the sensitivity and the specificity of the Anisakis simplex antigens purified by affinity chromatography was performed using sera from patients diagnosed with Anisakis sensitisation and sera from patients previously diagnosed with different helminthic infections. Only the sera of the patients diagnosed with Schistosoma mansoni or Onchocerca volvulus parasitic infections were negative against the A. simplex antigen and its purified fractions (PAK antigen: A. simplex antigen purified using columns prepared with anti-A. simplex rabbit IgG and PAS antigen: PAK antigen purified using columns prepared with anti-Ascaris suum suum rabbit IgG). However all the sera were positive against the A. suum antigen. In all the sera from the patients diagnosed with Anisakis sensitisation, the antibody levels detected using the purified antigens (PAK and PAS antigens) were lower than the observed using the A. simplex crude extract with the highest diminution in the case of the IgG. When these same sera were tested against the A. simplex crude extract by Western blot, several bands of high molecular masses were observed as well as, intense bands at 60 and/or 40 kDa. A concentration of these last proteins was observed in the PAK and the PAS antigens. When the sensitivity and the specificity determinations were performed, only seven of the 38 patients diagnosed of Anisakis sensitisation were positive, as well as, the sera from the patients diagnosed with parasitisms by Echinococcus granulosus or Fasciola hepatica.

Key words: Anisakis simplex - enzyme-linked immunosorben assay - Western blot - helminthoses - cross-reactivity

Anisakidosis is a human disease caused by the ingestion of larval nematodes from the family Anisakidae, especially Anisakis simplex. The infection is acquired by eating raw seafood or undercooked fish and squid (Sakanari & McKerrow 1989). Anisakidosis is divided into gastric, intestinal, and ectopic (Ishikura et al. 1993). Due to the vagueness of the symptoms, this disease is often misdiagnosed as appendicitis, acute abdomen, gastric tumor or cancer, ileitis, cholecystitis, diverticulitis, tuberculous peritonitis, cancer of the pancreas or Crohn’s disease (Sakanari & McKerrow 1989).

Immunodiagnostic methods have been developed, but several cross-reactivities have been observed. Although, the introduction of sensitive techniques, such as enzyme-linked immunosorbent assay (ELISA) did not avoid the presence of antibodies that provoke cross-reactivities with Ascaris and Toxocara (Kennedy et al. 1988) when the A. simplex larval crude extract was used as reactive for the determination of specific IgE levels in patients with suspected anisakidosis, so by fluoro enzymo immuno assay (FEIA), immunoblot or prick test, due to the high proportion of false negative results detected (Lorenzo et al. 2000).

In fact, extensive homology between both somatic and excretory-secretory antigens of A. simplex and other ascaridoid nematodes mainly A. suum, A. lumbricoides, and T. canis has been reported (Kennedy et al. 1988).

For these reasons, in this work, an evaluation of the sensitivity and the specificity of the A. simplex antigen purified by affinity chromatography (Rodero et al. 2001, 2002) was carried out using sera from patients previously diagnosed with Antisakids sensitisation and different helminthic infections.

MATERIALS AND METHODS

Parasites - A. simplex L3 were picked up manually from the viscera, flesh, and body cavities of naturally infected blue whiting (Micromesistius poutassou) and exhaustively washed in water (Ferteguer & Cuéllar 1998). A. suum were obtained from swine natural infections (Guillén et al. 1986).

Antigens - For the preparation of the crude extract (CE) of A. simplex (A. simplex CE antigen or AK antigen), L3 were placed at 4°C in phosphate-buffered saline (PBS). This material was homogenised in a hand-operated glass tissue grinder at 4°C, followed by sonication with a Virsonic (Virtis, NY, US) set at 70% output power. The homogenate was extracted in PBS at 4°C overnight and, subsequently, delipidised with n-hexane and then centrifuged at 8497 g for 30 min at 4°C (Biofuge 17RS: Heraeus Sepatech, Gmb, Osterode, Denmark). The supernatant was dialysed overnight at 4°C in PBS. The protein content of the extract was
estimated and the extract was frozen at 20°C until used (Perteguer & Cuéllar 1998). The antigen from *A. suum* adults (*A. suum* CE antigen or AS antigen) was obtained using a modification of the Welch et al. (1983) method by homogenisation and extraction in PBS at 4°C overnight (instead of ultrasonic burst). Its protein content was estimated by the Bradford (1976) method, and then the antigen was frozen at 20°C until use (Águila et al. 1987, Cuéllar et al. 1990).

Hyperimmune sera - New Zealand rabbits of about 3 kg body weight were immunised with 1 mg of larval *A. simplex* or adult *A. suum* CE antigens by the intramuscular route. The animals were bled weekly postimmunization (p.i.) after the first inoculation (week 0) (García-Palacios et al. 1996). The experiments were carried out according to the European Council on applied animals 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.

Human sera - Thirty-eight human anti-Anisakis sera were obtained from the Servicio de Alergia del Hospital del Aire de Madrid. The concentration of circulating anti-Anisakis IgE antibodies was measured by means of the Pharmacia CAP System RAST FEIA (Pharmacia AB, Uppsala, Sweden). The results of this fluoro immuno assay were reported in kU/l and converted to CAP “scores” of 0 (< 0.35), 1 (0.35-0.7), 2 (0.7 - 3.5), 3 (3.5 - 17.5), 4 (17.5 -50), 5 (50 - 100) and 6 (> 100). All the sera showed positive CAP values to Anisakis by FEIA, which varied from values of CAP = 1 (3%), CAP = 2 (26%), CAP = 3 (47%), CAP = 4 (10%), CAP = 5 (8%) and to CAP = 6 (5%). Nine sera from patients diagnosed with Loa loa, Onchocerca volvulus, A. lumbricoides, Trichuris trichiura, T. canis, S. mansoni, S. intercalatum, Echinococcus granulosus and Fasciola hepatica parasitic infections were selected.

Purification of antigens by affinity chromatography - Protein A-Sepharose CL-4B beads (Pharmacia Biotech) columns were prepared according to the manufacturer’s instructions. Anti-*A. simplex* or *A. suum* rabbit antibodies, in sample buffer 0.05 M Tris, 0.5 M NaCl, pH 8.0 were loaded into the columns. Fractions of 1 ml were then collected. Unbound immunoglobulins were washed with washing buffer (0.05 M Tris, 0.5 M NaCl, pH 8.0). Bound immunoglobulins then were eluted with glycine buffer (0.2 M glycine, 0.5 M NaCl, pH 2.8). Fractions were collected onto 100 µl of collection buffer (Tris-base 1 M, pH 8.5) and read on a spectrophotometer at A280 for calculating IgG concentration. A column was prepared with protein A affinity isolated anti-*A. simplex* IgG, at a concentration of 5 mg/ml in NaHCO3 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 3 h at room temperature. Fractions of 1 ml were then collected. Unbound antigens were washed with washing buffer and bound antigens were eluted with glycine buffer followed by 50 mM diethylamine in saline, pH 11.5 collected into glycine to neutralise the eluted fractions. Fractions were read at A290. This antigen was named as *A. simplex* PAK antigen. The same procedure was carried out using columns prepared with rabbit anti-*A. suum* IgG, obtaining the *A. simplex* PAS antigen and the *A. simplex* EAS antigen. In summary: PAK (*A. simplex* CE antigen eluted from CNBr-activated Sepharose 4B coupled to IgG from rabbits immunised with *A. simplex* CE antigen), PAS (*A. simplex* PAK antigen after loading into a CNBr-activated Sepharose 4B coupled to IgG from rabbits immunised with adult *A. suum* CE antigen) and EAS (*A. simplex* PAK antigen eluted from the anti-*A. suum* column).

Immunoblot - Sodium dodecylsulphate-polyacrilamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (1970) and revised by Hames (1986) using a Mini Protein® III cell (Bio Rad). The gels consisted of a 4% stacking gel and 5-20% gradient separating gel. Samples were dissolved in a sample buffer (50 mM Tris-HCl buffer, pH 8.6, containing 2% SDS, 20% glycerol, and 0.02% bromophenol blue) diluted 1:1 in electrode buffer (25 mM Tris, 192 mM 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 mass markers (6.5 - 205 or 7.2 - 209 kDa, Bio Rad) were incorporated into each electrophoresis run. Gels were stained with AgNO3. Following the SDS-PAGE of antigens, the protein bands were transferred onto a 0.22 µm pore size nitrocellulose membrane (Pharmacia) in a Mini Trans-blot Electrophoretic Transfer Cell (Bio Rad) with 25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3. The transblot was carried out at a constant 100 V for 1 h. The membrane was blocked for 3 h at room temperature with PBS containing 5% non-fat dry milk, prior to immunorecognition by incubation for 2 h with the human sera diluted in PBS-Tween containing 1% non-fat dry milk. Each paper was then washed with PBS-Tween 20 (3 × 5 min) and incubated for 3 h with goat anti-human Ig’s (total immunoglobulins), IgM, IgG or IgA, HRP conjugate (Biosource International, Camarillo, CA, US), at the appropriate dilution in PBS-Tween 20, containing 1% non-fat dry milk. To visualize bands the nitrocellulose was washed with PBS-Tween 20 (3 × 5 min) and reacted with the substrate (PBS, containing 0.006% H2O2 methanol, containing 0.03% 4-chloro-1-naftol). The reaction was stopped by exhaustive washing with distilled water.

Determination of specific antibody levels - The antibody levels were measured by indirect ELISA. The 96 well microtitre plates (Nunc-Immuno Plate PolySorp™) were sensitised overnight at 4°C by the addition of 100 µl/well of antigens diluted at 1 µg/ml in a 0.1 M carbonate buffer, pH 9.6. 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 human sera at 1/400 in PBS-Tween, containing 0.1% BSA were added and incubated at 37°C for 2 h. Once the plates were washed, 100 µl per well of goat anti-human Ig’s (total immunoglobulins), IgM, IgG or IgA, HRP conjugate (Biosource International, Camarillo, CA, US), at the appropriate dilu-
tion in PBS-Tween, 0.1% BSA, were incubated for 1 h at 37°C. After adding substrate (phosphate citrate buffer, containing 0.04% H₂O₂ and 0.04% o-phenylenediamine) the reaction was stopped with 3N sulphuric acid and the plates were read at 490 nm (García-Palacios et al. 1996). Results were expressed as O.D.p-O.D.c indexes by subtracting the mean optical density (O.D.) of the control from the mean O.D. of the test sera once the non-specific reaction with the BSA used in the blocking was subtracted.

**Determination of the sensitivity and the specificity of the A. simplex crude extract and the PAK and PAS purified antigens with sera from patients diagnosed with Anisakis sensitisation** - For the determination of the sensitivity and the specificity of the different antigens, 2 x 2 tables were carried out. In these tables, the number of sera from patients diagnosed with Anisakis sensitisation and CAP values ≥ 3 and ≤ 2 against the different antigens were represented for each immunoglobulin (Ig’s, IgG, IgM, IgA, IgE). Further, different O.D. cutting points were selected in function of the positive and negative predictive values, as well as, the sensitivity and the specificity, in the case of each assayed immunoglobulins and antigens (AK, PAK, and PAS) (Fig. 3).

**RESULTS**

**ELISA antibody determinations in the sera from patients diagnosed with Anisakis sensitisation using A. simplex antigens purified by affinity chromatography** - Thirty-eight sera obtained from the Servicio de Alergia del Hospital del Aire de Madrid were assayed. All the sera showed positive CAP values to *Anisakis* by FEIA, which varied from values of CAP = 1 (3%), CAP = 2 (26%), CAP = 3 (47%), CAP = 4 (10%), CAP = 5 (8%), to CAP = 6 (5%).

The 96 well microtiter plates were coated with 100 µl/well (1 µg/ml) of the crude extract (CE) antigens of *A. simplex* or *A. suum* (AK and AS antigens), as well as, the antigens purified using the columns of anti-A. *simplex* or anti-*A. suum* IgG (PAK and PAS antigens, respectively) and the antigen eluted from the latest column (EA antigens). The sera were diluted at 1/400, and, after their incubation, the Ig’s, IgG and IgA were measured. The mean O.D. were calculated after the subtraction of the BSA and negative control values.

With all the assayed sera from the patients diagnosed with *Anisakis* sensitisation, the highest values were obtained when the ELISA test were performed against the A. *simplex* CE (AK antigen). The IgG was the most abundant immunoglobulin, followed by the IgM and the Ig’s, whilst the lowest values were observed in the case of the IgA (Fig. 1).

When the sera were tested against the A. *suum* crude extract (AS antigen) 45% of them showed values equal or superior to 0.2 in the case of the specific Ig’s were detected. Contrarily, these values were modified when the IgG antibodies were tested. In this case, 24% of the sera showed anti-*A. suum* IgG levels equal or superior to 0.2. On the other hand, IgM values were detected in 39% of the sera. In the case of the IgA, the levels were lower, because only 13% of the sera showed O.D. values equal or higher than 0.15 (Fig. 1).

When the sera were tested against the purified antigenic fractions (PAK and PAS antigens), in both cases, the highest values were observed when the IgM immunoglobulins were tested, followed by the Ig’s and the IgG. Finally, the lowest values belonged to the IgA.

All the detected O.D. values using the purified preparations (PAK and PAS antigens) were lower than the observed using the A. *simplex* CE (AK antigen) with the highest diminution in the case of the IgG (0.36 - 0.08/AK-PAS antigens).

When the sera were tested against the “eluted of Ascaris” (EAS antigen) the mean values of the IgG increased (O.D. = 0.26) compared to the observed using the purified antigens (Fig. 1).

**ELISA antibody determinations in the sera from patients diagnosed with different helminth parasitic infections using A. simplex antigens purified by affinity chromatography** - The evaluation by ELISA of the A. *simplex* larval antigens purified by affinity chromatography was completed using sera from patients diagnosed with *L. loa*, *O. volvulus*, *A. lumbricoides*, *T. trichiura*, *T. canis*, *S. mansoni*, *S. intercalatum*, *E. granulosus*, and *F. hepatica*.

These sera were tested against the CE antigens of *A. simplex* (AK antigen) and *A. suum* (AS antigen), as well as the antigen eluted from the column of anti-*A. simplex* rabbit IgG (PAK antigen), the *A. simplex* antigen purified by the column of anti- *A. suum* rabbit IgG (PAS antigen), and the eluted from the mentioned column (EAS antigen). The specific Ig’s, IgG, IgM, and IgA were measured.

Only the sera from the patients diagnosed with *S. mansoni* or *O. volvulus* parasitic infections were negative against the A. *simplex* antigen (AK antigen) and its purified fractions (PAK, PAS, and EAS antigen). However, all the sera showed cross-reactions with the A. *suum* one (AS antigen) when the Ig’s, IgG, IgM and IgA antibodies were measured (Table I).

When the PAK antigen were tested, in all the patients, except *O. volvulus*, *A. lumbricoides*, and *S. mansoni* cases, the IgG levels enhanced with respect to the *A. simplex* antigen (AK antigen). On the contrary, when the sera were tested against the PAS antigen, the IgG levels suffered a reduction higher than 50% for the *T. trichiura*, *S. intercalatum*, and *E. granulosus* patients and were negative for the *O. volvulus*, *A. lumbricoides*, and *S. mansoni* patients. Finally, when the EAS antigen was tested, the IgG levels enhanced more than twice with respect to the PAS antigen in the case of the *T. trichiura*, *S. intercalatum*, *E. granulosus*, and *F. hepatica* patients. The IgM levels unchanged with respect to the PAS antigen, as well as, the total immunoglobulins (Table I).

**Western blot antibody determinations in the sera from patients diagnosed with different helminth parasitic infections using A. simplex antigens purified by affinity chromatography** - When the sera were tested against the A. *simplex* antigen, all the sera except the *O. volvulus* case, reacted with proteins of high molecular masses (> 84 kDa). The sera from the *L. loa*, *A. lumbricoides*, and *S. mansoni* patients reacted with the 60 kDa protein. These same sera also reacted with a 40 kDa protein, as well as, the sera from *T. trichiura*, *T. canis*, and *S. intercalatum*
patients, while the 32 kDa protein only was immuno-
recognised for the F. hepatica serum. When the sera were
tested against the PAK antigen, the immunorecognition
against the 60 kDa protein enhanced in intensity in the
case of the L. loa and A. lumbricoides sera. This reaction
disappeared with the S. mansoni serum and appeared using
the T. canis, E. granulosus, and F. hepatica sera. In
addition, in this last patient, the immunorecognition
against the 32 kDa protein disappeared. Against the PAS
antigen, only was immunorecogniton of the 60 kDa pro-
tein in the case of the L. loa, A. lumbricoides, and T. canis
sera (Fig. 2).

**DISCUSSION**

In this work, the specificity and the sensitivity of the
A. simplex antigen purified by affinity chromatography
previously described (Rodero et al. 2001, 2002), were evalu-
ated by ELISA, using sera from patients previously diag-
nosed with several parasitic infections.

When these three parameters were applied to the sera
from the patients diagnosed with several parasitic infec-
tions, only the sera from the patients diagnosed with
parasitisms of E. granulosus and F. hepatica were posi-
tive.

**Determination of the sensitivity and the specificity of the A. simplex crude extract and the PAK and PAS purified antigens with sera from patients diagnosed with A. simplex sensitisation** - When the A. simplex crude extract was used, variations of the sensitivity and the specificity were detected according to the selected immunoglobulin.

The cut point of O.D. ≥ 0.15 for the IgG was selected as a first reference because this immunoglobulin showed the greatest sensitivity (82.7%). When this parameter was
applied, 70% of the patients diagnosed with A. simplex
sensitisation were positive. When the second determina-
tion 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 produced. Finally, the cut point of
O.D. ≥ 0.05 for the IgA was selected against the PAS anti-
gen. Only seven of the 38 tested sera were positive (Table
II).

When these three parameters were applied to the sera
from the patients diagnosed with Anisakis sensitisation against crude extract of A. simplex (AK antigen), Ascaris suum (AS antigen), and purified antigens PAK, PAS and EAS.
IgA were tested by ELISA, against the A. simplex antigen, as well as its purified fractions, only the sera from the patients diagnosed with S. mansoni or O. volvulus infections were negative. These results were maintained using the serum from an O. volvulus patient when assayed by Western blot as a diagnostic method. Any immunoreactive band did not appear when the total immunoglobulins were tested. However, the S. mansoni serum showed several bands of high molecular masses against the A. simplex antigen (AK antigen) and others immunoreactive bands of around 60 and 40 kDa.

This difference between the antibody determination using both techniques (ELISA and Western blot) could be due to the different techniques and methodologies utilised in order to determine the different immunoglobulins.

**TABLE I**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Ig’s</th>
<th>IgG</th>
<th>IgM</th>
<th>IgA</th>
<th>Ig’s</th>
<th>IgG</th>
<th>IgM</th>
<th>IgA</th>
<th>Ig’s</th>
<th>IgG</th>
<th>IgM</th>
<th>IgA</th>
<th>Ig’s</th>
<th>IgG</th>
<th>IgM</th>
<th>IgA</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Loa loa</em></td>
<td>0.25</td>
<td>0.29</td>
<td>0.69</td>
<td>0.01</td>
<td>0.41</td>
<td>0.61</td>
<td>0.77</td>
<td>0.07</td>
<td>0.32</td>
<td>0.16</td>
<td>0.59</td>
<td>0.00</td>
<td>0.32</td>
<td>0.16</td>
<td>0.59</td>
<td>0.00</td>
</tr>
<tr>
<td><em>Onchocerca volvulus</em></td>
<td>0.05</td>
<td>0.00</td>
<td>0.05</td>
<td>0.00</td>
<td>0.07</td>
<td>0.07</td>
<td>0.08</td>
<td>0.00</td>
<td>0.03</td>
<td>0.02</td>
<td>0.05</td>
<td>0.00</td>
<td>0.05</td>
<td>0.13</td>
<td>0.06</td>
<td>0.00</td>
</tr>
<tr>
<td><em>Ascaris lumbricoides</em></td>
<td>0.26</td>
<td>0.00</td>
<td>0.62</td>
<td>0.04</td>
<td>0.31</td>
<td>0.00</td>
<td>0.66</td>
<td>0.00</td>
<td>0.28</td>
<td>0.00</td>
<td>0.54</td>
<td>0.00</td>
<td>0.35</td>
<td>0.19</td>
<td>0.59</td>
<td>0.00</td>
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<tr>
<td><em>Trichuris trichiura</em></td>
<td>0.89</td>
<td>1.33</td>
<td>0.28</td>
<td>0.12</td>
<td>0.68</td>
<td>1.86</td>
<td>0.38</td>
<td>0.12</td>
<td>0.36</td>
<td>0.82</td>
<td>0.31</td>
<td>0.00</td>
<td>0.80</td>
<td>1.66</td>
<td>0.39</td>
<td>0.08</td>
</tr>
<tr>
<td><em>Toxocara canis</em></td>
<td>0.16</td>
<td>0.00</td>
<td>0.25</td>
<td>0.00</td>
<td>0.55</td>
<td>0.12</td>
<td>0.39</td>
<td>0.00</td>
<td>0.16</td>
<td>0.01</td>
<td>0.25</td>
<td>0.00</td>
<td>0.30</td>
<td>0.12</td>
<td>0.34</td>
<td>0.00</td>
</tr>
<tr>
<td><em>Schistosoma intercalatum</em></td>
<td>0.42</td>
<td>0.57</td>
<td>0.11</td>
<td>0.04</td>
<td>0.54</td>
<td>0.96</td>
<td>0.08</td>
<td>0.00</td>
<td>0.23</td>
<td>0.35</td>
<td>0.05</td>
<td>0.00</td>
<td>0.37</td>
<td>0.67</td>
<td>0.06</td>
<td>0.00</td>
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<tr>
<td><em>Schistosoma mansoni</em></td>
<td>0.07</td>
<td>0.04</td>
<td>0.04</td>
<td>0.00</td>
<td>0.02</td>
<td>0.00</td>
<td>0.04</td>
<td>0.00</td>
<td>0.08</td>
<td>0.00</td>
<td>0.06</td>
<td>0.00</td>
<td>0.06</td>
<td>0.00</td>
<td>0.07</td>
<td>0.00</td>
</tr>
<tr>
<td><em>Echinococcus granulosus</em></td>
<td>0.78</td>
<td>0.36</td>
<td>0.11</td>
<td>0.45</td>
<td>0.55</td>
<td>0.53</td>
<td>0.10</td>
<td>0.52</td>
<td>0.54</td>
<td>0.21</td>
<td>0.06</td>
<td>0.58</td>
<td>0.77</td>
<td>0.51</td>
<td>0.06</td>
<td>0.54</td>
</tr>
<tr>
<td><em>Fasciola hepatica</em></td>
<td>1.49</td>
<td>0.51</td>
<td>0.59</td>
<td>0.38</td>
<td>1.39</td>
<td>0.83</td>
<td>0.52</td>
<td>0.30</td>
<td>1.35</td>
<td>0.36</td>
<td>0.56</td>
<td>0.26</td>
<td>2.10</td>
<td>1.12</td>
<td>0.69</td>
<td>0.31</td>
</tr>
</tbody>
</table>

**TABLE II**

Positive human sera (§) against *Anisakis simplex* AK, PAK, and PAS antigens for the different cut points selected

<table>
<thead>
<tr>
<th>Patient/CAP</th>
<th>IgG ≥ 0.15</th>
<th>Ig ≥ 0.07</th>
<th>PAS ≥ 0.65</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>28</td>
<td>20</td>
<td>7</td>
</tr>
</tbody>
</table>

The difference between the antibody determination using both techniques (ELISA and Western blot) could be due to the different techniques and methodologies utilised in order to determine the different immunoglobulins.
lin class levels, like the serum dilution, as well as, the antigenic concentration.

Noya et al. (1995) detected IgG, IgM, IgA, and IgE specific antibodies by ELISA when tested the sera of 30 *S. mansoni* infected children, while, by Western blot, appeared reactive bands of high molecular masses, as well as, 45, 36, and 30 kDa for IgG and 77 kDa for IgM antibodies, indicating that the 36 kDa protein was useful for immunodiagnostic. In the case of *S. intercalatum* the results obtained, in our experimental conditions, were completely different. High levels of Ig’s and IgG antibodies, and basal levels for IgM and IgA were detected by ELISA. These differences among immunoglobulins could be due to the different parasitic locations in function of the infection.

tion time and the type of immunological response that the worms can provoke in the host.

In the serum from the *T. trichiura* patient the highest antibody levels detected by ELISA against the crude extract (CE) *A. simplex* and its purified fractions were observed when the Ig’s and IgG antibodies were measured. Turner et al. (2002) mentioned that people who live in endemic areas have high levels of IgG1 and IgG4 antibodies when tested against their homologous antigen. This increase of antibodies could be related with the high IgG levels detected by us when the *A. simplex* antigen and its purified fractions were tested by ELISA, due to both parasitic and vector antigens. The different cut points selected corresponded of values of O.D. ≥ 0.05.

<table>
<thead>
<tr>
<th>CAP values</th>
<th>≥ 3</th>
<th>≤ 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>b</td>
<td>c</td>
</tr>
<tr>
<td>O.D.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

% sensitivity = \( \frac{a}{a + b} \times 100 \)

% specificity = \( \frac{d}{b + d} \times 100 \)

% predictive value:

Positive test = \( \frac{a}{a + b} \times 100 \)

Negative test = \( \frac{d}{c + d} \times 100 \)

Fig. 3: calculation of sensitivity, specificity, and predictive value (positive and negative) for the different immunoglobulins (Ig’s, IgG, IgM, and IgA) against crude extract of *Anisakis simplex* and purified antigens PAK, and PAS. The different cut points selected corresponded of values of O.D. ≥ 0.05.

...
its purification using the anti-A. suum column, the unbinding proteins (specific proteins) were of 120, 66-45, 40, 31-21, and 14 kDa. Higher concentrations of specific proteins were seen compared to the unpurified samples (Rodero et al. 2002).

Finally, in both sera (T. canis and A. lumbricoides) a band around 60 kDa appeared against the EAS antigen responsible of the cross-reaction with the A. simplex antigen. Nunes et al. (1997) detected at least one band with molecular weight around 55-66 kDa that seems to be responsible for the cross-reactivity between T. canis and A. suum, which disappears when previous absorption of the serum samples with A. suum antigens was performed.

Previously, the discriminating capability of both antigens (PAK and PAS) were assayed in sera from rabbits immunized with larval A. simplex or adult A. suum CE or inoculated with embryonated T. canis eggs and we observed that this capability for discriminate between A. simplex and A. suum was improved in the case of the PAS antigen (Rodero et al. 2002).

To calculate the sensitivity and the 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 of absent in the selected patients. In our case, the CAP value was selected as a “gold standard”, although is not a very good diagnostic method but is routinely used to select the Anisakis sensitised patients.

The sensitivity and the specificity determinations were performed testing sera from patients diagnosed with A. simplex sensitisation, against the CE (AK antigen), as well as, the purified PAK and PAS A. simplex antigens, in order to observe the possible variations produced during the purification process. These evaluations were carried out against all the tested immunoglobulins (Ig’s, IgG IgM, IgA, IgE), to determine the working conditions (immunoglobulin and antigen) for the human anisakidosis diagnosis.

When the A. simplex crude extract was used, variations of the sensitivity and the specificity were detected according to the immunoglobulin selected. For the same cut point of O.D. = 0.15, the sensitivity varied from values of 83%, for the IgG, to only 20.7%, in the case of the IgA. This fact showed the difficulty of the immunodiagnosis of anisakidosis when only a specific immunoglobulin type is studied.

In our experimental conditions, we showed the useful of testing the sera in a first step against the A. simplex crude extract and then carrying out 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 sensitive tests are the most useful (Fletcher et al. 1998). When this parameter was applied, 70% of the sera were positive, in spite of all the sera except one that had a CAP value ≥ 2. The CAP System has a poor specificity and shows a high rate of false positive results. This fact was also observed by Lorenzo et al. (2000) when evaluated several immunological techniques in order to make the diagnosis of Anisakis allergy, observing 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, showed medium values of specificity and sensitivity (58.6 and 54.5%, respectively). When all the assayed sera were evaluated, a decrease of 20% in the positive group was observed. Only two sera, previously negative against the A. simplex crude extract, were maintained positive. These results are in accordance with the high predictive value (80%) observed in the A. simplex crude extract, although a probability of 20% (six sera) of the existence of false positive results is always present. In our case the decrease observed was greater (from 28 positive sera for A. simplex crude extract to 20 for the PAK antigen).

Finally, the cut point of O.D. ≥ 0.05 for the IgA was selected against the PAS antigen. This immunoglobulin showed the highest specificity (81.8%) and predictive value (88.9%).

Likewise, for the detection of IgA, a little amount of serum (1 µl) is required in contrast to the IgE, in which case 200 µl of serum are necessary.

When these parameters were applied, only seven of the 38 tested sera were positive. All these sera were also positive against both the A. simplex CE and the PAK purified antigens.

When the cut points were selected, we applied these parameters to the sera from the patients diagnosed of several parasitic infections and we observed that, against the AK antigen (O.D. ≥ 0.15 for the IgG), the sera from the patients diagnosed with parasitisms by L. loa, T. trichiura, S. intercalatum, E. granulosus, and F. hepatica were positive. This fact indicated the presence of common antigenic proteins among the Anisakis antigens and other parasites. Likewise, one of the fundamental aspects of the immunodiagnosis is the selection of adequate antigens to carry out the diagnostic tests.

When the sera were tested against the PAK antigen (OD ≥ 0.07 for the IgG), the same sera were positive again but, when the third determination was carried out using the PAS antigen (OD ≥ 0.05 for the IgA), only were positive the sera from the patients diagnosed with parasitisms of E. granulosus and F. hepatica. Previously, we had observed this same fact, when the capability of the different antigens to discriminate among the sera from the patients diagnosed with several parasitic infections were determined, when the lowest values were detected using the anti-Echinococcus or anti-Fasciola sera. It is necessary to mention that, in the purification process of the A. simplex antigen, an hyperimmune serum from rabbits immunised with A. suum was used and no sera from animals immunised with the other parasites that are infecting the different patients utilised in this assay. However, a remarkable augmentation of the sensitivity and specificity in the fractions of A. simplex antigen purified, was appreciated.
REFERENCES


