

## STANDARDIZATION OF DOT-ELISA FOR THE SEROLOGICAL DIAGNOSIS OF TOXOCARIASIS AND COMPARISON OF THE ASSAY WITH ELISA

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### SUMMARY

The dot-enzyme-linked immunosorbent assay (dot-ELISA) was standardized using somatic (S) and excretory-secretory (ES) antigens of *Toxocara canis* for the detection of specific antibodies in 22 serum samples from children aged 1 to 15 years, with clinical signs of toxocariasis. Fourteen serum samples from apparently normal individuals and 28 sera from patients with other pathologies were used as controls. All samples were used before and after absorption with *Ascaris suum* extract. When the results were evaluated in comparison with ELISA, the two tests were found to have similar sensitivity, but dot-ELISA was found to be more specific in the presence of the two antigens studied. Dot-ELISA proved to be effective for the diagnosis of human toxocariasis, presenting advantages in terms of yield, stability, time and ease of execution and low cost.

**KEY WORDS:** Visceral larva migrans; *Toxocara canis*; *A. suum* absorption; immunodiagnosis of toxocariasis; dot-ELISA; ELISA.

### INTRODUCTION

First described by BEAVER<sup>2</sup>, the visceral larva migrans (VLM) syndrome in vertebrate organisms, which may act or not as paratenic hosts, is characterized by an intense eosinophilia and visceral involvement from the migration of larvae. Most frequently caused in humans by *Toxocara canis*, it affects mainly children, those most in contact with soil contaminated with viable eggs of the helminth. These being ingested, tissues may be invaded by larvae, which may remain viable for a long time<sup>17</sup>. However, because larvae are in general hardly detectable, diagnosis is largely based on serology<sup>20,21</sup>. SAVIGNY<sup>12</sup>, in 1979, standardized an ELISA, in search of a more sensitive and specific assay than haemagglutination, flocculation or complement fixation tests, then in use. In Brazil, CHIEFFI et. al.<sup>7,8</sup> developed an ELISA which employed as antigen (S) a crude larval extract of *T. canis*. Non-specific reactions are observed, mainly due to cross-reactivity with *Ascaris sp* antigens. However, these reactions may be mostly removed by previously absorbing sera

with such antigens<sup>9,15</sup>. The use of excretion and secretion antigens (ES) has been referred as providing a high sensitivity, with no such cross-reactions<sup>11,12</sup> but BACH-RIZZATI<sup>1</sup> has not observed significant reactivity differences when comparing somatic and excretion-secretion antigens.

Recently, MATSUMURA et. al.<sup>19</sup> referred good results, for a dot-ELISA to detect antibodies against *Toxocara* in dogs. Easier to read, with no need of a spectrophotometer and requiring much less antigen, it should also be tried for human diagnosis, as we here propose and compare with ELISA, using both a somatic and an excretion-secretion antigen.

### MATERIALS AND METHODS

#### 1. Antigens

##### 1.1. *Toxocara canis*

##### a) Larval Form - Somatic Antigen (S)

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The antigen extracts used in ELISA and dot-ELISA were prepared by the method of CHIEFFI<sup>6</sup>, distributed into aliquots and stored at -20°C. Protein concentration was determined by the method of BRADFORD<sup>4</sup> and the optimum antigen concentration determined by block titration in the presence of positive and negative standard sera.

#### b) Excretion-Secretion (ES)

The excretory and secretory antigen was prepared from 3rd-instar larvae by the method of SAVIGNY<sup>10</sup>, with some modifications. This antigen was dialyzed in distilled water and lyophilized in 2 ml aliquots. The final product was characterized for protein concentration by the method of BRADFORD<sup>4</sup> and optimum antigen concentration determined by block titration.

#### 1.2. *Ascaris suum*

An *Ascaris suum* extract was prepared from adult female specimens obtained from swine intestine. After collection and identification, worms were washed in distilled water, cut with a scissors and ground in a Potter tissue homogenizer. NaOH (1M) was added at the proportion of 1.5 ml/8.5 ml extract, the solution was neutralized with 1 ml of 1 M HCL and centrifuged at 1.500 rpm in a refrigerated centrifuge for 1 hour. The final product was filtered through Millipore membranes (0.22 µm) and ether was added, with intermittent shaking, at the proportion of 1/3 of the final volume. The antigen was then divided into aliquots and stored at -20°C. Protein concentration was measured by the method of BRADFORD<sup>4</sup> and the optimum amount for nonspecific antibody absorption in the sera under study was determined before use.

#### 2. Serum Samples

Twenty-two serum samples from male and female patients aged 1 to 15 years, originating from different parts of the State of São Paulo and showing clinical signs of toxocariasis were studied. The control group consisted of 14 serum samples from apparently normal individuals and of 28 samples from patients with other pathologies but without a clinical suspicion of toxocariasis (5 cases of schistosomiasis, 4 of malaria, 5 of Chagas disease, 4 of leishmaniasis, 5 of syphilis, and 5 of

toxoplasmosis). The serum samples were collected and stored at -20°C for about six months.

#### 3. Serological Tests

3.1. Immunoenzymatic test - ELISA - IgG - standardized according to the methodology described by ENGVALL and PERLMANN<sup>13,14</sup>, modified by CYPESS et. al.<sup>9</sup>, with some alterations:

Procedure: Polystyrene plates with a U-shaped bottom (Inlab, Brazil) were sensitized with S (0.3 ug/ml) or ES (1 ug/ml) *Toxocara canis* antigen diluted in buffered sodium carbonate bicarbonate solution (0.02 M Na<sub>2</sub>CO<sub>3</sub>; 0.03 M NaHCO<sub>3</sub>, pH 9.6) added to each well in a 100µl volume. After one hour at room temperature and 18 hours at 4°C, the plates were washed three times in SF-T (0.15 M NaCl containing 0.05% Tween 20, Sigma, USA).

One hundred microliters of the blocking PBS solution (0.13 M NaCl; 0.02 M Phosphates, pH 7.4) containing 0.5% gelatin (Difco, USA) PBS-G, was added to each well and the plate was incubated for 1 hour at 37°C. The samples were absorbed with *Ascaris suum* antigen extract (final protein concentration, 52µg/ml) at the proportion of 10µl serum/390µl antigen, and submitted to intermittent shaking for one hour at 37°C, in order to remove nonspecific antibodies that might cross-react with *Toxocara canis* antigens.

Plates were then washed three times and 100µl of absorbed and non absorbed serum samples were added, starting from the 1:40 dilution and diluting successively by a factor of 2 in PBS containing 0.05% Tween 20 and 0.5% bovine serum albumin (Sigma, USA), PBS-T-BSA. After 1 hour incubation the plates were submitted to a new washing cycle and 100µl of the anti-human IgG-alkaline phosphatase conjugate (Sigma, USA) were added at the appropriate dilution in PBS-T-BSA and incubated for 1 hour at 37°C. The conjugate was removed and washings were conducted as described above and 100µl of chromogen solution (p-nitrophenyl-disodium phosphate 1 mg/ml in 1 M diethanolamine solution, pH 9.8) were added, followed by incubation for 30 minutes at 37°C. The enzymatic reaction was stopped by the addition of 100µl 2 M NaOH and reactivity was evaluated using a plate

spectrophotometer (Titertek, Multiskan Flow. Lab., USA) at 405 nm wave length. Dilutions with absorbances of 0.30 or more were considered as significant. When the serum titer after absorption and using the S antigen was 1:160 or higher, the reaction was considered to be positive for anti-*Toxocara* antibodies. This standard was established based on the fact that the negative control sera never react in dilution higher than 1:40, after absorption (6). To increase the security of reading, the 1:80 dilution was not considered significant.

When ES antigen was used, the cut-off was determined by the arithmetic mean of the absorbance of the control group (normals and other pathologies), plus 2 SD, the 1:160 dilution.

3.2. Dot-ELISA-IgG test standardized by the method of HAWKES et. al.<sup>16</sup>, modified by BENNETT et. al.<sup>3</sup>, with some modifications.

**Procedure:** Nitrocellulose membranes (Schleicher and Schüller) were sensitized with 1 $\mu$ l of the S (0.005  $\mu$ g/ $\mu$ l) or ES (0.01  $\mu$ g/ $\mu$ l) *Toxocara canis* antigen diluted with sodium bicarbonate buffer, pH 9.6. After 30 minutes at room temperature, the membranes were blocked for 2 hours under constant shaking in 0.02 M tris-(hydroxymethyl)-aminomethane, pH 7.5, TBS 7.5, containing 5% skimmed milk powder. This solution was previously heated on a water bath at 100°C for 15 minutes and filtered to inactivate the milk proteases (S. Hoshino-Shimizu, personal communication).

Samples were diluted with TBS 7.5 containing 1% skimmed milk powder, TBS-M, and 50 $\mu$ l transferred to the membranes. These were placed in an orbital shaker for 1 hour at room temperature under constant shaking and then washed three times (10 minutes each time) with TBS containing 0.05% Nonidet P.40 (Shell -Brazil). The membranes were dried on filter paper, and 50 $\mu$ l of the anti-human IgG-peroxidase conjugate (Sigma, USA) were added, diluted with TBS-M according to titer, and incubated at room temperature under constant shaking. After a new washing cycle, the chromogen 4-chloro-1 naphthol solution (Sigma, USA) was dissolved in p.a. grade methanol (Merck) at the 3 mg/ml proportion. At the time of use, 10 ml TBS, 10  $\mu$ l 30% H<sub>2</sub>O<sub>2</sub> and 2 ml chromogen solution were added and the membranes were soaked in this solution for 10 minutes at room

temperature. The reaction was stopped by washing the membranes 5 times with distilled water. A visual reading was taken and the positive results were indicated by the development of a well defined purple color on the dots.

## RESULTS

Fig. 1 shows the distribution of anti-*Toxocara* antibody titers in the three serum groups for both tests studied (ELISA and dot-ELISA).

Even after absorption with *Ascaris suum*, ELISA with antigen S presented reactivity for *Toxocara canis* in 6 serum samples from the control group (2 from patients with Chagas disease, 2 with schistosomiasis and 2 with malaria. ELISA with antigen ES presented reactivity in serum from 1 patient with Chagas disease, 1 with schistosomiasis and 1 with malaria. When the dot-ELISA antigen S was used for the control sample, serum from 1 patient with Chagas disease and other from 1 patient with malaria presented reactivity, and when the ES antigen was used, only the serum from the patient with Chagas disease continued to be reactive to *Toxocara canis*.

The sensitivity and specificity indices and the respective confidence limits (95%) obtained by the tests are presented in Table 1.

## DISCUSSION

Serological diagnosis of human infection with *Toxocara* larvae antigens was standardized with ELISA in view of the highly sensitive and specific results as well as the good reproducibility of this technique<sup>6,7,8,9,11,12</sup>. These tests are usually performed over a period of 24 hours using antigens with a protein concentration of the order of 0.1 to 3 mg/ml protein.

Under the conditions used in the present study, dot-ELISA, which had already been used for the diagnosis of canine toxocariasis by MATSUMURA et. al.<sup>19</sup>, in addition to shortening the reaction time permitted the use of significantly smaller amounts of antigen. For the sensitization of the nitrocellulose membrane the S and ES antigens were used at protein concentrations of 0.005 and 0.01  $\mu$ g/ $\mu$ l, respectively, amounts 60 and 100 smaller than those utilized in ELISA in the present

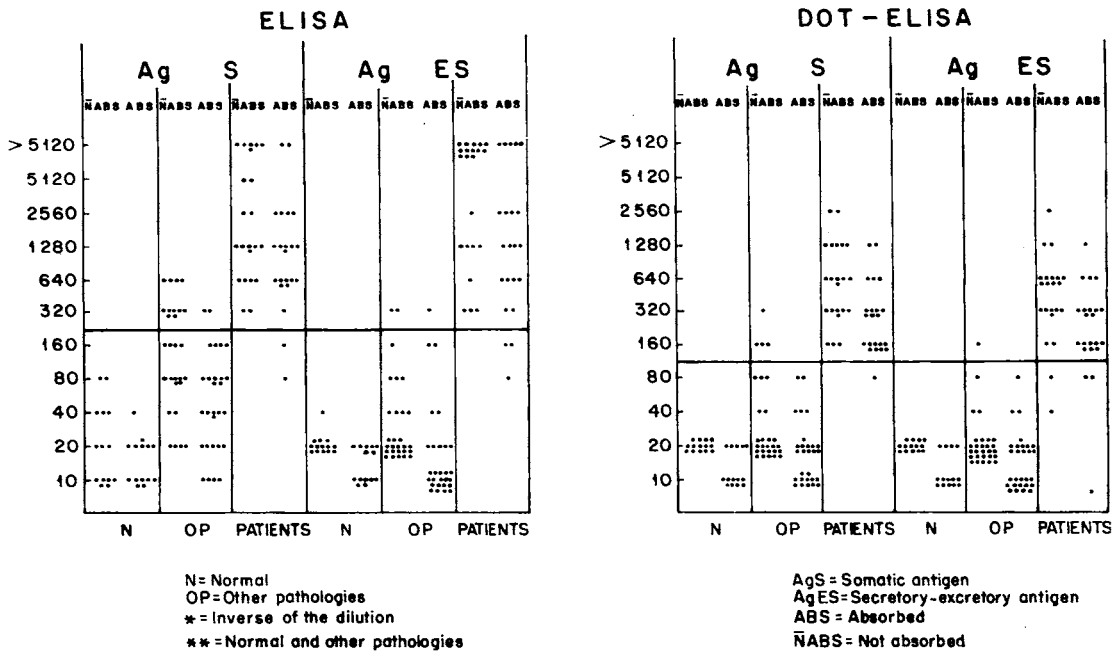


Fig. 1 - Anti-Toxocara antibody titers\* for toxocariasis patients and controls\*\*

Table 1  
Sensitivity and specificity for ELISA and dot-ELISA in human toxocariasis.

Test	Sensitivity	Specificity
ELISA-Antigen S		
sera not absorbed	100%(60-100%)*	57.1%(36.1-100%)
sera absorbed	95.4%(87.4-100%)	83.7%(69.7-100%)
ELISA-Antigen ES		
sera not absorbed	100%(60-100%)	90.5%(78.5-100%)
sera absorbed	95.4%(87.4-100%)	90.5%(78.5-100%)
dot-ELISA-Antigen S		
sera not absorbed	100%(60-100%)	85.7%(71.7-100%)
sera absorbed	100%(60-100%)	95.3%(83.3-100%)
dot-ELISA-Antigen ES		
sera not absorbed	95.4%(87.4-100%)	95.3%(83.3-100%)
sera absorbed	95.4%(87.4-100%)	97.6%(91.6-100%)

\* Confidence Interval (95%)

study and in other reports<sup>9,12,18</sup>. During the washings, can occur the desorption of the antigen proteins from the plastic plates utilized in ELISA<sup>5</sup>. This phenomenon does not occur with the nitrocellulose membrane utilized in dot-ELISA and could be responsible for the need for higher

antigen concentration in ELISA.

As expected, the two tests showed a similar behavior in terms of sensitivity since based on the same methodological principle and the antigens and reagents employed were the same for both.

The antigens differed significantly, with advantages for ES in terms of specificity, as also reported by others<sup>12,17</sup>. This difference, however, was not observed when the sera were previously absorbed with *A. suum* antigen extract, supporting the hypothesis that the components of the S antigen membrane are responsible for most of the cross-reactions with other parasites and indicating the possible use of this type of antigen in epidemiologic serum studies (8).

The false-positive reactions observed, specially with ELISA, support the observations of Lynch et. al.<sup>18</sup> about the occurrence of cross-reactions between *Toxocara* antigens and several other infectious agents commonly occurring in underdeveloped countries.

The results obtained with dot-ELISA demonstrated higher specificity for the two antigens studied.

Sensitized membranes were stored at 4°C in sealed plastic sacks to verify the stability. The same results were obtained during six months.

In addition to the above considerations, it is interesting to point out other advantages of dot-ELISA in the diagnosis of human toxocaríasis, such as the possibility of visual reading with reliable results without the need for special equipment, and the fact that the test be performed at room temperature, thus being easily executed under field conditions and in modestly equipped laboratories.

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#### RESUMO

##### Padronização do teste dot-ELISA para o diagnóstico da toxocaríase, estudo comparativo com o teste imunoenzimático ELISA.

Padronizou-se o teste imunoenzimático dot-ELISA, empregando-se os antígenos somático (S) e excretor-secretor (ES) de *Toxocara canis*, para pesquisa de anticorpos específicos em 22 soros de pacientes com idades entre 5 a 15 anos, com dados clínicos de toxocaríase.

Como grupo controle, foram estudados 14 soros de indivíduos supostamente normais e 28 soros de pacientes com outras patologias. Todas as amostras em estudo foram empregadas antes e após absorção com extrato de *Ascaris suum*.

Os resultados obtidos foram avaliados comparativamente com o teste ELISA evidenciando, nos dois testes estudados, comportamento semelhante quanto à sensibilidade e maior especificidade para o dot-ELISA, com qualquer dos dois antígenos estudados.

O teste dot-ELISA mostrou-se eficiente para o diagnóstico da toxocaríase humana, apresentando vantagens quanto ao rendimento, estabilidade, tempo e facilidade de execução e baixo custo.

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