# Evaluation of the dot enzyme-linked immunosorbent assay in comparison with standard ELISA for the immunodiagnosis of human toxocariasis

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*A dot enzyme-linked immunosorbent assay (dot-ELISA) was standardized using excretory-secretory antigens of* Toxocara canis *for the rapid immunodiagnosis of human toxocariasis. Thirty patients with clinical signs of toxocariasis, 20 cases with other parasitic diseases, and 40 healthy subjects were tested. A total of 0.2 ng of antigen per dot, serum dilution of 1:160 and dilution conjugate of 1:1000 were found optimal. The sensitivity and specificity of the assay were 100 and 95%, respectively. Comparable sensitivity of dot-ELISA and the standard ELISA was obtained, but only 3 cross-reactions occurred in the dot-ELISA, compared with 6 in the standard ELISA. Dot-ELISA is simple to perform, rapid, and low cost. Large-scale screening studies should be done to evaluate its usefulness under field conditions.*

Key words: *Toxocara canis* - toxocariasis - dot enzyme-linked immunosorbent assay - ELISA

Human toxocariasis is a world-wide helminthic zoonosis due to the human infection by larvae of *Toxocara canis*, the common ascarid of dogs, and also by the cat ascarid *T. cati* (Schantz & Glickman 1983, Despommier 2003). The incidence of human toxocariasis is unknown because toxocariasis is not a communicable disease in the majority of the countries. However, many cases of this disease have been reported throughout the world (Glickman & Schantz 1981, Despommier 2003).

Humans are infected by ingestion of embryonated *T. canis* eggs. Children playing in areas contaminated with dog faeces are in higher risk, because of their likelihood of ingesting soil. Prevalence of *Toxocara* infestation of dogs and the resulting contamination of the ground is relatively high in many countries all over the world. Reported data range from 0 to 93% for dog infestation (Glickman & Schantz 1981) and 15 to 78% for soil contamination (Gillespie 1988, Magnaval et al. 2001). It has been determined that in Peru from 24 to 80% of soil samples in public playgrounds and parks are contaminated with *Toxocara* eggs (Buitrón 1976, Guerrero 1995, Lescano et al. 1998, Chavez et al. 2000, Dávalos et al. 2000).

Although the clinical features vary, two syndromes are recognized: visceral larva migrans (VLM) and ocular larva migrans (OLM). VLM is usually detected in young children (1 to 5 years of age) with a history of geophagia and/or exposure to puppies. It is a self-limited, rarely lethal disease characterized by fever, cough, wheeze, pallor, malaise, asthma, weight loss, hepatomegaly, and marked eosinophilia (Schantz 1989). OLM occurs unilaterally in children and young adults and cause visual loss, strabismus and, more rarely, eye pain (Shields 1984). Another clinical manifestations of *Toxocara* infection are "common toxocariasis" in adults and "covert toxocariasis" in children (Schantz 1989, Magnaval et al. 2001).

Diagnosis of toxocariasis is based on clinical and serological data because of the difficulty in detecting larvae from tissues. The test currently employed for the serodiagnosis of toxocariasis is ELISA using excretory-secretory antigens from *T. canis* second-stage larvae (TES) (De Savigny et al. 1979, Jacquier et al. 1991). However, this technique has some drawbacks, including the need for trained personnel, requirement of special equipment, the lack of reproducible reading due to plate-to-plate variation, and may be troublesome to perform under field conditions. Dot-ELISA test, a modification of the standard ELISA test, offers a simple and less expensive tool for toxocariasis detection. The dot-ELISA has been successfully adapted for the detection of parasitic diseases in humans as leishmaniasis, schistosomiasis, toxoplasmosis, and hydatidosis (Pappas et al. 1984, Boctor et al. 1987, Rogan et al. 1991, Elsaid et al. 1995). A dot-ELISA for diagnosis of human toxocariasis was described (Camargo et al. 1992). The present study was conducted to standardize and evaluate a dot-ELISA to establish the optimal conditions for the detection of IgG antibodies to toxocariasis in comparison to the standard ELISA test.

## MATERIALS AND METHODS

*Obtention of fully embryonated Toxocara canis eggs* - *T. canis* eggs were obtained by dissection of gravid adult female worms and left to embryonate for 30 days at 28°C in a mixture of 2% formalin and 1% sodium hypochlorite solution. Embryonated *T. canis* were washed repeatedly with sterile distilled water and decoated for 20 min in 5% sodium hypochlorite solution at 37°C (Espinoza et al. 2003). The egg suspension was then washed repeatedly in sterile distilled water until all traces of chlorine had been removed.

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*Toxocara canis excretory-secretory antigens (TES)* - Fully embryonated *T. canis* eggs were hatched by mechanical treatment with sterile glass beads for 15 min and the larvae were collected in a Baermann apparatus (Lescano 1991). The TES antigen were produced according to De Savigny (1975) modified by Bowman et al. (1987). The supernatant from the *Toxocara* larvae cultures was removed each week, then dialyzed and concentrated using PEG 20,000 (mol. wt) (Sigma, US). The protein content of the TES antigen was estimated (Lowry et al. 1951) and finally kept in aliquots at –20°C until use.

*Ascaris suum adult stage extract* - Adult worms were collected from swine intestine at local abattoir and washed in distilled water. Adult extract was prepared as described (Camargo et al. 1992) and protein concentration was estimated (Lowry et al. 1951) and finally kept in aliquots at –20°C until use.

*Absorption with A. suum extract* - Each human serum to be assayed was absorbed (v/v) with soluble *A. suum* extract containing 52 µg/ml of antigen in order to remove nonspecific antibodies that might cross-react with TES antigen. After incubation at 37°C for 1 h dilutions of the absorbed sera were made and added to the nitrocellulose (NC) strips or microtitration wells.

*Human sera* - A total of 90 serum samples from 3 groups of persons were tested by standard ELISA and dot-ELISA. Group A included 30 sera from patients with clinical diagnosis of toxocariasis. Group B consisted of 20 sera from patients infected with helminths: *Ascaris lumbricoides* (n = 3), *Fasciola hepatica* (n = 3), *Taenia* sp. (n = 3), *Strongyloides stercoralis* (n = 3), *Hymenolepis nana* (n = 3), *Trichuris trichiura* (n = 3), and larval stage of *T. solium* (n = 2). All infections were confirmed parasitologically except patients with cysticercosis, which were diagnosed by computed tomography findings and serological data. Group C comprised 40 serum samples from apparently normal children without evidence of toxocariasis.

Positive and negative reference samples were used to standardize the dot-ELISA. A positive reference serum was prepared by pooling 10 sera from patients with clinical diagnosis of toxocariasis with high (1024) and intermediate (512 to 256) titers in the ELISA test. A negative reference serum consisted of a pool of sera from clinically healthy and parasitologically negative children with no evidence of toxocariasis.

*ELISA procedure* - Standard ELISA was performed as reported by De Savigny et al. (1979) and Espinoza et al. (2003) with slight modifications. Briefly, 96-well microtiter polystyrene plates (Immulon 2 HB, US) were sensitized with a TES antigen solution (100 µl/well) containing 12.5 µg of protein per ml in 0.05 M bicarbonate buffer, pH 9.6, and then maintained for 18 h at 4ºC in a moist chamber. The microtiter plates were washed 3 times with 0.15 M phosphate-buffered saline-0.05% Tween 20 (PBS/T) and then tested with a 1:128-diluted human serum sample (100 µl/well) in PBS/T containing 5% nonfat skimmed milk powder for 60 min at 37ºC. The plates were washed 3 times with PBS/T and then 100 µl of a 1:4000 dilution of anti-human IgG-peroxidase conjugate (Sigma, US) were added, to each well. After 60 min the plates were again rinsed and to each well was added 100 µl of substrate solution, consisting of *o*-phenylenediamine (Sigma) 0.04 and 0.03% hydrogen peroxide diluted in 0.05 M citrate-phosphate buffer, pH 5.0, for approximately 30 min, then stopped by the addition of 2 N sulphuric acid and the plates were read at 492 nm, using an automatic microplate reader (Multiskan plus Labsystem version 2.01). The cut-off point was set at the mean optical density (OD) of the negative reference serum, plus 3 standard deviation (SD). Serum samples with OD > 0.26 (cut-off) were considered as reactives.

*Dot-ELISA procedure* - The optimal conditions for dot-ELISA were standardized according to the procedures described by Boctor et al. (1987) with some modifications. Strips of NC paper (Sigma), 1 cm wide and 6 cm long were placed on a microtiter 96-well plastic plate and after a slight pressure round areas were marked. Two microliters of several different TES antigen concentrations (0.01- 40 µg/ml) were dotted on separate round areas. After drying, the free bindings sites on the paper were blocked by 18 h incubation at 4°C in 0.01 M phosphate-buffered saline (PBS), pH 7.2 containing 5% defatted dry milk. Then, the strips were washed 3 times (5 min/wash) with PBS containing 0.05% Tween 20. Twelve microliters of diluted serum (ranging from 1:40 to 1:320) were tested to determine the optimal working dilution. After 45 min at room temperature, they were washed as described above and incubated with 1:1000 dilution of goat anti-human IgG peroxidase conjugate (Sigma) for 45 min at room temperature. The strips were washed and soaked in freshly prepared substrate solution, which consisted of 0.5 mg of 3,3'-diaminobenzidine (Sigma) per milliliter and hydrogen peroxide (0.01%) in 0.01 M citrate buffer, pH 5.0, for 5 min. The reaction was stopped by washing with water and left to dry. The development of brown dots on NC was considered evidence of positively. The intensity of the color was judged by the naked eye, and numbers were on an arbitrary scale of 0; +; ++; +++; or ++++ in reference to the negative control (0).

*Statistical analysis* - For the determination of the dot-ELISA and micro-ELISA sensitivity, specificity and positive and negative predictive values, $2 \times 2$ tables were carried out.

## RESULTS

*Antigen concentration and serum and conjugate dilution* - The optimal concentration of TES antigen, using standard positive and negative sera, revealed an optimum of 0.1 µg/ml (equivalent to 0.2 ng per dot). The best cut-off for the detection of specific anti-*Toxocara* antibodies in sera was correspondent to 1/160 dilution. However, minimum concentrations of TES 0.01µg/ml (equivalent to 0.02 ng/dot) can be used to detect anti-*Toxocara* IgG at 1:320 serum dilutions. At all antigen concentrations, pooled normal sera gave negative results and nonspecific reactions were not found in either antigen or serum blank round areas. 1:1000 dilution of conjugate were found to be optimal for most preparations because they provide a clear discrimination of positive and negative reactions.

*Incubation time* - The pooled positive and negative sera were incubated with antigen for a period of time varying from 30 to 60 min, at room temperature. Best results could be observed after 45 min. The optimum incubation time for the peroxidase conjugate was also 45 min.

*TES antigen fixation to nitrocellulose paper* - In order to determine the most appropriate type of antigen dilution solution, the antigen was diluted in PBS or in buffered sodium carbonate bicarbonate solution (0.02 M $Na_2CO_3$; 0.03 M $NaHCO_3$, pH 9.6) with or without 2% ovoalbumin (OVA). Sodium bicarbonate buffer with 2% OVA was the better antigen dilution solution since the positive dot increases color intensity without affecting the negative dot color in the same strip.

*Sensitivity and specificity of the dot-ELISA assay* - All 30 serum samples from patients with toxocariasis reacted positively, giving sensitivity of 100%. The specificity was checked on 60 sera. From 20 sera of patients with different parasitic infections, one from a strongyloides case, one from fascioliasis case and one from teniasis case reacted positively, but not a single positive reaction was observed among the sera from 40 apparently normal individuals, giving a specificity of 95%. When sera from all patients with different parasitic infections were repeatedly tested, the same cross-reaction was obtained.

*Comparison of dot-ELISA with standard ELISA* - All 30 sera investigated to detect IgG antibodies anti-*Toxocara* gave positive results by dot-ELISA and by standard ELISA. However, even after absorption with *A. suum* extract, standard ELISA showed positive results in 6 serum samples from the control group (3 from patients with teniasis, 1 with strongyloidiasis, 1 with fascioliasis, and 1 with cysticercosis) while only 3 serum samples from the same control group (1 patient with strongyloidiasis, 1 with fascioliasis and 1 with teniasis) were positive by dot-ELISA (Table I). Likewise, our results showed a good correlation between the visual grading of the dot-ELISA and the absorbance of ELISA (Table II).

TABLE II

Comparison between standard ELISA absorbance and dot-ELISA positivity

| dot-ELISA scale | ELISA $A_{492\ nm}$ | Nr of samples tested |
|---|---|---|
| + 4 | 1.47-1.55 | 2 |
| + 3 | 1.11-1.38 | 10 |
| + 2 | 1.01-1.09 | 8 |
| + 1 | 0.29-0.98 | 16 |
| 0 | 0.01-0.23 | 54 |

## DISCUSSION

The development of specific, sensitive, and reliable techniques to demonstrate the presence of antibody in toxocariasis is an important step towards improving diagnosis. In this study, we evaluated a simple and sensitive dot-ELISA test for the immunodiagnosis of human toxocariasis. Conventional ELISA procedures require larger volumes of soluble antigen (De Savigny et al. 1979, Espinoza et al. 2003), whereas the dot-ELISA uses only minute amounts of TES antigen per test (0.2 ng total protein per dot in contrast to 1.25 µg per microtitration well). We found that the best result to fix TES antigen to NC sheets was obtained with sodium bicarbonate buffer with 2% OVA which could be responsible for the need for lesser antigen concentration than previously reported (Camargo et al. 1992). Simplification of the procedure can be achieved by the use of large NC strips (Boctor et al. 1987), mass production of NC strips (Janitschke et al. 1987), and the use of the commercially available Bio-dot apparatus (Chan & Ko 1988). Another advantage of this method is that it is simple, rapid (about 2 h), does not need expensive equipment, results can be read visually, a large number of samples can be assayed, and the test is performed at room temperature.

The sensitivity of dot-ELISA and standard ELISA was similar (100%) as previously reported (Camargo et al. 1992), whereas specificity of the dot-ELISA was higher than standard ELISA. The healthy control sera were negative by both tests and other parasites-positive group, 3 out of 20 sera gave a positive reaction in the dot-ELISA and 6 out of the same subjects were positive for ELISA. The positive reactions observed specially with ELISA was undetermined but may include the presence of common antigens between *Toxocara* antigens and several other infectious agents commonly occurring in underdeveloped countries and previous unapparent past infections with *Toxocara* species. Previous absorption of sera with *A. suum* antigen extract, does not eliminate all cross-reactions, as reported (Camargo et al. 1992).

ELISA is the most commonly utilized diagnostic serologic test for VLM, OLM, and covert toxocariasis, the most frequent clinical syndromes associated with *Toxocara*

TABLE I

Sensitivity, specificity, positive, and negative predictive values for dot-ELISA and standard ELISA tests

| | Performance index | Serology test |
|---|---|---|
| | Dot-ELISA (%) | Standard ELISA (%) |
| Sensitivity | 100 [a] | 100 |
| Specificity | 95 (84-100) | 90 (82-98) |
| Positive predictive value | 90.9 (81-100) | 83.3 (71-96) |
| Negative predictive value | 100 | 100 |

*a*: confidence interval (95%)

infections. However, the serum ELISA for *Toxocara*-specific IgG is less sensitive for the diagnosis of OLM than for other forms of the disease (Magnaval et al. 2001, Despommier 2003). Therefore, confirmation of ocular toxocariasis can be obtained by testing aqueous or vitreous fluid. Dot-ELISA here presented has not yet been performed with sera or intraocular fluids of patients with OLM but we think this test will not only improve the diagnosis of VLM because its specificity is higher than the indirect ELISA but also the diagnosis of ocular toxocariasis.

The dot-ELISA for toxocariasis, as reported here, is a specific means of establishing serological diagnosis of the disease. The dot-ELISA can be used as a qualitative test to screen large number of samples and can be further developed as a field detection method.

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