An evaluation of the dot-ELISA procedure as a diagnostic test in an area with a high prevalence of human *Toxocara canis* infection

María V Bojanich[1,2]/+, Gioia L Marino[2], María Á López[1,2], José M Alonso[2]

[1]Área de Microbiología, Facultad de Ciencias Exactas y Naturales y Agrimensura, Corrientes, Argentina
[2]Área de Inmunología, Instituto de Medicina Regional, Universidad Nacional del Nordeste, Resistencia, Chaco, Argentina

The aim of this work was to evaluate a dot-enzyme-linked immunosorbent assay (dot-ELISA) using excretory-secretory antigens from the larval stages of Toxocara canis *for the diagnosis of toxocariasis. A secondary aim was to establish the optimal conditions for its use in an area with a high prevalence of human* T. canis *infection. The dot-ELISA test was standardised using different concentrations of the antigen fixed on nitrocellulose paper strips and increasing dilutions of the serum and conjugate. Both the dot-ELISA and standard ELISA methods were tested in parallel with the same batch of sera from controls and from individuals living in the problem area. The best results were obtained with 1.33 µg/mL of antigen, dilutions of 1/80 for the samples and controls and a dilution of 1/5,000 for the anti-human IgG-peroxidase conjugate. All steps of the procedure were performed at room temperature. The coincidence between ELISA and dot-ELISA was 85% and the kappa index was 0.72. The dot-ELISA test described here is rapid, easy to perform and does not require expensive equipment. Thus, this test is suitable for the serological diagnosis of human* T. canis *infection in field surveys and in the primary health care centres of endemic regions.*

Key words: *Toxocara canis* - dot-ELISA - immunoassay - serodiagnosis

Toxocariasis is the clinical presentation of human infection by *Toxocara canis* and (less frequently) *Toxocara cati,* which are roundworms that live in the small intestines of dogs and cats, respectively. Human infection occurs by accidental ingestion of embryonated *Toxocara* eggs present in contaminated soil or on dirty hands. Infection can also occur by eating contaminated meat from paratenic hosts containing encapsulated larvae. The infective larvae hatch in the duodenum, but fail to develop to mature adult stages and migrate through the somatic organs for a period that ranges from months to several years (Despommier 2003).

Human infection is present worldwide and is a consequence of the human habit of keeping dogs and cats for company, which favours the persistence of the parasite in the environment and its transmission (Mizgajska 2001, Delgado & Rodriguez Morales 2009). Despite its extensive geographical distribution, infection is more frequent in tropical and subtropical regions, especially in populations with poor sanitary conditions (Magnaval et al. 2001).

Because of the difficulty of detecting larvae in human tissues, the diagnosis of toxocariasis is based on clinical, epidemiological and serological data (Magnaval et al. 2001, Despommier 2003). The enzyme-linked immunosorbent assay (ELISA) using excretory-secretory antigens from *T. canis* second-stage larvae (TES) is the most widely used test to detect anti-*Toxocara* antibodies, but there are other immunological tests that have also been described, such as the dot-ELISA and immunoblot methods (Camargo et al. 1992, Magnaval et al. 2001, Roldán et al. 2006).

Human *T. canis* infection is a public health concern in the Americas, Europe and in all developing countries. However, a full appreciation of the global burden of the disease may be greatly underestimated (Hotez & Wilkins 2009). For this reason, the development of specific, sensitive and reliable techniques to detect the presence of anti-*Toxocara* antibodies is an important step towards improving and expanding the diagnosis of infection (Roldán et al. 2006). This may be particularly relevant in the case of highly endemic populations, such as the northeastern region of Argentina. This area of South America contains a large number of people living with serious sanitary and socio-economic deficiencies and the seroepidemiological data show high prevalence rates of *T. canis* infection in the adult and infant populations (Alonso et al. 2000, 2004, López et al. 2005b, Bojanich et al. 2008).

The aim of this work was to develop a dot-ELISA test for the serodiagnosis of human toxocariasis and to optimise the conditions for its use in public health centres with basic levels of equipment and few trained personnel. The performance of this test was evaluated in comparison to the standard ELISA test.

SUBJECTS, MATERIALS AND METHODS

Samples - Two hundred sixty-six serum samples from children and adults from the cities of Resistencia and Corrientes (northeastern Argentina) were evaluated. The samples belong to the serum bank of the Regional

Financial support: Alberto J Roemmers Foundation of Argentina, SECYT-UNNE
+ Corresponding author: mavibojanich@yahoo.com.ar
Received 23 May 2011
Accepted 14 December 2011

Institute of Medicine. They were originally obtained to diagnose toxocariasis, but they were used anonymously for this work. The serologic statuses of the sera for *T. canis* infection were evaluated by a standard ELISA test, using pools of positive and negative sera confirmed by Western blot as controls (López 2005a).

Antigens - The TES antigens were obtained according to the technique described by Gillespie (1995). TES were maintained at 37ºC with 5% CO_2 atmosphere and an adjusted pH of 6.4-6.5 in Iscove's modified Dulbecco's culture medium supplemented with HEPES buffer and a Penicillin-Streptomycin solution (St. Louis, MO, USA). The culture supernatant was removed weekly; the supernatant pool was kept at -70ºC. This was concentrated by filtration through polyethersulphone membranes (PM10-Millipore Corp, USA) and dialysed; the protein content was then estimated by the Bradford method with bovine albumin as the standard protein (Bradford 1976).

ELISA-IgG test - Anti-*Toxocara* antibodies were detected by means of an ELISA-IgG test using TES antigens, according to the original procedure described by De Savigny et al. (1979) and with modifications made by Alonso et al. (2004).

Dot-ELISA IgG test - The test was standardised according to the technique described by Roldán et al. (2006), with the following modifications: circular areas 3 mm in diameter were delimited on strips of nitrocellulose paper (NC) (Bio-Rad Labs, Hercules, USA) that was 0.2 µm thick, 2 cm long and 0.5 cm wide. Then, 2 µL of 0.16 µg/mL, 0.30 µg/mL and 1.33 µg/mL TES concentrations were dotted onto each strip. After drying for 30 min, free binding sites were blocked by incubation in TRIS buffered saline (TBS) (pH 7.3) containing 5% fat free dried milk for 12 h at 4ºC. Finally, the strips were washed three times with phosphate buffered saline and were stored at 4ºC wrapped in aluminium foil.

The strips were placed in Western blot holders and 1 mL dilutions of either 1/50, 1/80 or 1/100 of sera and controls in TBS containing 1% fat-free dried milk were poured over the strips. After an incubation for 45 min at room temperature (RT) with constant agitation, the strips were washed three times with TBS containing 0.2% Tween-20 (TBS-T) for 5 min. Dilutions of either 1/1.000, 1/5.000 or 1/10.000 of peroxidase conjugated anti-human IgG (Sigma-Aldrich Co, Saint Louis, USA) in TBS were added to the strips. After incubating for 30 min with constant agitation, the strips were washed for 30 min with TBS-T. Next, 1 mL of a chromogen substrate solution was added to each strip and the mixtures were incubated for 10 min. The chromogen stock solution contained 6 mg of 4-chloro-α-naphthol (Pierce Inc, Rockford, USA) diluted in 2 mL of absolute methanol, 5 µL of 100-vol hydrogen peroxide and 10 mL of TBS. The reaction was stopped by washing with distilled water. All procedures were performed at RT. The strips were dried on filter paper and the development of blue dots was considered evidence of a positive result. The kappa index (k) was used to evaluate the degree of agreement between this method and the standard ELISA test.

RESULTS

The optimal concentration of the TES antigens tested on the NC strips was 1.33 µg/mL. The best dilution for the sera and controls was 1/80 and the ideal dilution for the conjugated anti-human IgG peroxidase was 1/5.000. The criteria used to define a sample as positive was the visual appearance of a bright blue dot of any intensity developed on the antigen area on each of the NC strips. The absence of any colour was considered a negative result (Figure).

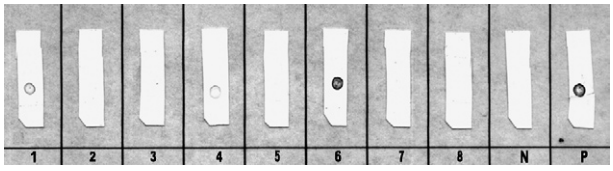
Out of 266 serum samples, 140 were positive in the ELISA-IgG test; 126 were negative. In the dot-ELISA IgG test, 125 were positive; 141 were negative. There were 227 sera (113 positive and 114 negative) that returned the same results by both methods (85% coincidence). The k concordance index was 0.72, meaning that there was a substantial degree of agreement between these two methods (95% confidence interval = 0.61-0.80).

DISCUSSION

The dot-ELISA test has been described as being an appropriate method for the detection of antigens or antibodies in several human and animal infectious diseases. For example, this test has been used to detect *Neospora caninum* antigens in dogs (Pinheiro et al. 2005), as a multiple test for the simultaneous detection of Chagas disease, malaria and syphilis (Coelho et al. 2007), as a quick test for detecting circulating *Mycobacterium* antigens in patients with pulmonary tuberculosis (El-Masry et al. 2008), as a diagnostic test for neurocysticercosis (Shukla et al. 2008) and as a test for the early diagnosis of human leptospirosis (Blanco et al. 2009). The dot-ELISA test has also been used for human toxocariasis by Camargo et al. (1992) and Roldán et al. (2006).

A comparison of the results of this study with the findings of Roldán et al. (2006) reveals that, although similar procedures were employed in both studies, there are some major differences. Roldán et al. (2006) evaluated patients with clinically confirmed toxocariasis and other helminth infections and they fixed 0.1 µg/ml of TES antigen with sodium carbonate-bicarbonate buffer onto the membranes. However, in this study, no fixing buffer was employed and the analysis of the performance of the test was based on results obtained with a previously characterised set of sera from a well-maintained serum-bank instead of sera from patients.

An important problem when attempting to evaluate serological tests for human toxocariasis is that there is no reference laboratory test or parasitological method to definitively diagnose the disease (Chieffi et al. 2009).



Dot-ELISA test for toxocariasis. The blue spots show a positive reaction. Lanes 1-8: serum samples. N: negative control; P: positive control.

Thus, it is not possible to evaluate the performance of a test by comparing it to another with unquestionable results. For this reason, the ELISA-IgG test was chosen as a reference in this work based on the high degree of correlation demonstrated with the Western blot method (López et al. 2005a, Roldán & Espinoza 2009), which is considered a confirmatory test (Magnaval et al. 2001); additionally, the ELISA-IgG test is the internationally accepted diagnostic test. The k concordance index has been described as a way to comparatively analyse the agreement between two diagnostic tests with qualitative results (positive or negative). In this case, the degree of concordance was good (k = 0.72). Therefore, there should be no impediment for the use of the dot-ELISA as a screening test in field surveys for *T. canis* infection.

Even though some differences have been identified regarding the sensitivity and specificity of the dot-ELISA test in its different applications (Camargo et al. 1992, Pinheiro et al. 2005, Roldán et al. 2006, Coelho et al. 2007, El-Masry et al. 2008, Shukla et al. 2008, Blanco et al. 2009), it presents many advantages as a basic diagnostic test. For example, it is highly stable, it does not require specialised tools to analyse the results, it has a lower cost and it can be simultaneously applied to a large number of samples by a basically trained technical staff (Roldán et al. 2006, Chieffi et al. 2009).

In recent years, there have been important methodological advances in the diagnosis of many infectious diseases, but there are innumerable difficulties in the laboratory diagnosis of toxocariasis due to the laborious production of the antigens or the high costs of commercial diagnostic kits (Colli et al. 2010). Toxocariasis is one of the few human parasitic diseases that uses a standardised antigen (i.e. TES) for the serodiagnosis of infection and this assists in determining the global occurrence of the disease and of the infection rates in many countries (Smith et al. 2009). In developed countries, such as the United States, it has been reported that toxocariasis is still under-recognised as a health problem (Hotez & Wilkins 2009). Furthermore, in developing countries, toxocariasis is a largely neglected parasitic infection with serious implications to public health (Delgado & Rodriguez Morales 2009). Recently, it has been reported that the estimated global seroprevalence of toxocariasis in Latin America ranges between 1.8-66.6% (Rodriguez Morales et al. 2011). Therefore, a better understanding of its epidemiologic relevance and an improvement in the availability of serodiagnosis is needed to achieve the control of this zoonosis.

Although the socio-economical and environmental conditions in several regions of Argentina suggest that toxocariasis is an endemic disease in these areas (Martin et al. 2008), accurate serological diagnosis is not available in most of the public health centres and access to medical diagnosis is variable for many segments of the population. For this reason, we believe that the dot-ELISA test described here would be a suitable tool for the diagnosis of toxocariasis, particularly in populations with high levels of exposure to the parasite. This test could be extensively employed in primary health care centres to expand the epidemiological surveillance in the country. The recognition of toxocariasis as an extended parasitic disease would be the first step in national efforts to defeat this neglected infection.

REFERENCES

Alonso JM, Bojanich MVI, Chamorro M, Gorodner JO 2000. Toxocara seroprevalence in children from a subtropical city in Argentina. *Rev Inst Med Trop Sao Paulo 42*: 235-237.

Alonso JM, López MA, Bojanich MV, Marull J 2004. Infección por *Toxocara canis* en población adulta sana de un área subtropical de Argentina. *Parasitol Latinoam 59*: 61-64.

Blanco RM, Takei K, Romero EC 2009. Leptospiral glycolipoprotein as a candidate antigen for serodiagnosis of human leptospirosis. *Lett Appl Microbiol 49*: 267-273.

Bojanich MV, López MA, Fernández G, Azula L, Alonso JM 2008. Infección por *Toxocara canis* en población infantil vulnerable del noreste de Argentina. *Enf Emerg 10*: 60-64.

Bradford, MM 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem 72*: 248-254.

Camargo ED, Nakamura PM, Vaz AJ 1992. Standadization of dot-ELISA for the serological diagnosis of toxocariasis and comparison of the assay with ELISA. *Rev Inst Med Trop Sao Paulo 34*: 55-60.

Chieffi PP, Dos Santos SV, Queiroz ML de, Lescano SA 2009. Human toxocariasis: contribution by Brazilian researchers. *Rev Inst Med Trop Sao Paulo 51*: 301-308.

Coelho JS, Soares IS, Lemos EA, Jimenez MC, Kudó ME, Moraes SL, Ferreira AW, Sanchez MC 2007. A multianalyte dot-ELISA for simultaneous detection of malaria, Chagas disease and syphilis-specific IgG antibodies. *Diagn Microbiol Infect Dis 58*: 223-230.

Colli C, Rubinsky-Elefant G, Paludo M, Falavigna D, Guilherme E, Mattia S 2010. Serological, clinical and epidemiological evaluation of toxocariasis in urban areas of south Brazil. *Rev Inst Med Trop Sao Paulo 52*: 69-74.

De Savigny DH, Voller A, Woodruff AW 1979. Toxocariasis: serological diagnosis by enzymeimmunoassay. *J Clin Pathol 70*: 284-288.

Delgado O, Rodriguez-Morales AJ 2009. Aspectos clínico-epidemiológicos de la toxocariasis: una enfermedad desatendida en Venezuela y América Latina. *Bol Mal Salud Amb 1*: 1-33.

Despommier D 2003. Toxocariasis: clinical, epidemiology, medical ecology and molecular aspects. *Clin Microbiol Rev 16*: 265-272.

El-Masry S, El-Kady I, Zaghloul MH, Al-Badrawey MK 2008. Rapid and simple detection of a mycobacterium circulating antigen in serum of pulmonary tuberculosis patients by using a monoclonal antibody and fast-dot-ELISA. *Clin Biochem 41*: 145-151.

Gillespie SH 1995. Migrating worms. In SH Gillespie, *Medical* parasitology - A practical approach, Oxford University Press, Oxford, p. 177-183.

Hotez PJ, Wilkins PP 2009. Toxocariasis: America's most common neglected infection of poverty and a helminthiasis of global importance? *Plos Negl Trop Dis 3*: e400.

López MA, Bojanich MV, Alonso ME, Alonso JM 2005a. Inmunoblotting para diagnóstico de toxocarosis humana en un área subtropical. *Parasitol Latinoam 60*: 127-131.

López MA, Martin G, Chamorro MC, Alonso JM 2005b. Toxocariasis en niños de una región subtropical. *Medicina (Buenos Aires) 65*: 226-230.

Magnaval JK, Glickman L, Dorchies P, Morassin B 2001. Highlights of human toxocariasis. *Korean J Parasitol 39*: 1-11.

Martin U, Machuca P, Demonte M, Contini L 2008. Estudio en niños con diagnóstico presuntivo de toxocariasis en Santa Fe, Argentina. *Medicina (Buenos Aires) 68*: 353-357.

Mizgajska H 2001. Eggs of *Toxocara* spp in the environment and their public health implications. *J Helminthol 75*: 147-151.

Pinheiro AM, Costa MF, Paule B, Vale V, Ribeiro M, Nascimento I 2005. Serologic immunoreactivity to *Neospora caninum* antigens in dogs determined by indirect immunofluorescence, western blotting and dot-ELISA. *Vet Parasitol 130*: 73-79.

Rodríguez Morales A, Echeverri-Cataño L, Delgado O 2011. Need for a consensus in the diagnosis of human toxocariasis: implications for the Latin American public health. *Rev Peru Med Exp Salud Publica 28*: 161-162.

Roldán W, Cornejo W, Espinoza Y 2006. Evaluation of the dot-enzyme-linked immunosorbent assay in comparison with standard ELISA for the immunodiagnosis of human toxocariasis. *Mem Inst Oswaldo Cruz 101*: 71-74.

Roldán WH, Espinoza YA 2009. Evaluation of an enzyme-linked immunoelectrotransfer blot test for the confirmatory serodiagnosis of human toxocariasis. *Mem Inst Oswaldo Cruz 104*: 411-418.

Shukla N, Husain N, Agarwal GG, Husain M 2008. Utility of *cysticercus fasciolaris* antigen in dot-ELISA for the diagnosis of neurocysticercosis. *Indian J Med Sci 62*: 222-227.

Smith H, Holland C, Taylor M, Magnaval JF, Schantz P, Maizels R 2009. How common is human toxocariasis? Towards standardizing our knowledge. *Trends Parasitol 25*: 182-188.