

Sero-epidemiology of toxocariasis in a rural settlement in São Paulo state, Brazil

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The seroprevalence of *Toxocara canis* and risk factors for infection with this parasite were explored in a rural settlement in São Paulo state, Brazil. Total IgA and IgE levels in 79 subjects were determined by turbidimetry and chemiluminescence, respectively. Total counts of leucocytes and erythrocytes and differential counts of leucocytes were made by flow cytometry. ELISA for the detection of anti-*Toxocara* IgG, IgA and IgE were standardized using *Toxocara* excretory–secretory antigens (TES) obtained from the cultured second-stage larvae of *T. canis*. Seventeen (21.5%) of the subjects were found positive for anti-*Toxocara* IgG, with no significant differences in such seropositivity with age or gender. Thirty (38%) of the subjects showed eosinophilia and 70 (89%) had elevated levels of total IgE. Among the 17 subjects found seropositive for anti-*Toxocara* IgG, the percentage of leucocytes represented by eosinophils ($P=0.0069$) and total levels of IgE ($P=0.0452$) were positively correlated with the levels of anti-TES IgE. Although anti-TES IgA was detected in 10 (59%) of the subjects, there was no significant correlation between the levels of total IgA and those of *Toxocara*-specific IgA. Only one of the 17 subjects found positive for anti-*Toxocara* IgG had attended a secondary school and all but two belonged to households with monthly incomes of <U.S.\$100. In the study community at least, seropositivity may be related to poor living standards and lack of basic sanitary conditions. The presence of anti-*Toxocara* IgE and IgA may facilitate the diagnosis of toxocariasis and may well be useful for monitoring the success of treatment.

Human toxocariasis is one of the most widespread and prevalent helminthiases in developing and industrialized countries, thanks to a large and increasing number of final hosts. The disease is caused by saprozoontic parasites of dogs (*Toxocara canis*) and cats (*T. cati*). Human infection, predominantly with *T. canis*, occurs when embryonated eggs are ingested following their hand-to-mouth transfer. The mammal-infective second-stage larvae (L₂) in the eggs hatch

and penetrate the intestinal wall, migrating through the soft tissues of the body and causing either ocular or visceral larva migrans (Magnaval *et al.*, 2001; Despommier, 2003).

The diagnosis of toxocariasis and sero-epidemiological surveys are largely based on clinical examinations and immunology, since the isolation of the larvae, from liver and eye tissue, for example, is often difficult. The serological test most commonly used is an ELISA for detecting IgG that reacts with *Toxocara* excretory–secretory antigens (TES; Magnaval *et al.*, 2001; Despommier,

2003). Because of the complexity of the surface of *Toxocara* sp., infective larvae elicit an immediate (acute) and persistent (chronic) immune response to TES and stimulate the production of IgE and IgA antibodies, as well as IgG. It is this response that permits the serodiagnosis of toxocariasis and it can also be useful in monitoring the disease and the efficacy of any chemotherapy (Smith, 1993; Kayes, 1997; Magnaval *et al.*, 2001; Elefant *et al.*, 2006).

In Brazil, the environmental and epidemiological aspects of *Toxocara* infection have been investigated in several studies, mainly in urban settings (Anaruma Filho *et al.*, 2002; Campos Junior *et al.*, 2003; Alderete *et al.*, 2003; Coelho *et al.*, 2004; Figueiredo *et al.*, 2005). Seropositivity for *Toxocara* has been found to be associated with socio-economic variables, becoming less likely with increasing level of education, increasing family income, improving water treatment, and decreasing contact with soil. Thus, in Brazil, as in most developing countries, lack of education and poor sanitary conditions contribute to a high incidence and prevalence of human toxocariasis, which often becomes a major public-health problem.

Pontal do Paranapanema is one of the poorest and most undeveloped regions in the state of São Paulo, in south-eastern Brazil. It is characterised by large cattle farms, has been occupied by landless peasants since 1982, and has the highest density of rural settlements in Brazil (Prestes-Carneiro *et al.*, 2006). Before forming settlements, the peasants camp in plastic huts on marginal lands or next to highways, near cattle farms and water sources. They live in precarious conditions, without even minimal sanitation, in environments that favour the transmission and survival of parasites such as *Toxocara*.

The aims of the present study were to determine the seroprevalence of *Toxocara* infection in one rural settlement in Pontal do Paranapanema, and explore some of the associated risk factors.

SUBJECTS AND METHODS

Study Design

The study was carried out in the Padre Josimo settlement in Teodoro Sampaio county (22°17'12"S, 52°36'12"W). The study area has been occupied by the *Movimento dos Trabalhadores Rurais sem Terra* (the Landless Workers Movement) since 2000, and was expropriated by the *Instituto Nacional de Colonização e Reforma Agrária* (the Brazilian National Institute for Land, Settlement and Agrarian Reform), for land-reform purposes, in 2003. The region has a tropical climate with a rainy summer season and a dry winter season. The sandy top-soil is mostly covered by cattle pasture, and summer temperatures range from 28–36°C.

Most of the subjects of the present study had lived in plastic huts, in marginal camps, from 2000 until 2003, when the expropriated area (2290 ha) was divided into 96 plots with the co-ordination of the *Instituto de Terras do Estado de São Paulo* — São Paulo's Land Institute (Prestes-Carneiro *et al.*, 2006). The blood and stool specimens used in the present study were collected in February–April 2004, from the 79 inhabitants of Padre Josimo who agreed to take part in this study; at that time the settlement had a population of 325. The subjects found seropositive for *Toxocara* were clinically examined, by a physician from the *Programa de Saúde da Família* (PSF; the Family Health Programme), in September–December 2005. All subjects gave their written informed consent and each was given a short interview in which a questionnaire was used to collect data on the subject's age, gender, place of birth, educational level, source of water supply, sanitary facilities, household income, clinical manifestations compatible with toxocariasis, and the presence of dogs and/or cats in the subject's household. The study protocol was approved by the Ethics Committee of the *Universidade do Oeste Paulista*, in Presidente Prudente, Brazil.

Haematology

Blood was collected into VacutainerTM tubes (BD, Franklin Lakes, NJ) with and without EDTA. Serum was separated from the blood in the EDTA-free tubes, by centrifugation, and stored at -20°C until use. The blood in the EDTA tubes was used for total counts of erythrocytes and leucocytes and differential counts of leucocytes. All these cell counts were made by flow cytometry, using a Pentra 80 automated haematology analyser (Horiba Diagnostics, Montpellier, France), with the differential leucocyte counts being confirmed by the microscopical examination of stained blood-smears. The level of eosinophilia was assessed as the percentage of leucocytes represented by eosinophils (Williams *et al.*, 1991).

Serology

The levels of total IgE and total IgA in each serum sample were determined using automated chemiluminescence (Immulite; Diagnostic Products, Los Angeles, CA) and turbidimetric assays (SERA-PAK immuno IgA; Bayer Corporation Diagnostics Division, Tarrytown, NY), respectively. Each serum was also tested, in ELISA, for anti-TES IgG, IgA and IgE (see below).

PRODUCTION OF TES

The TES antigen extract was prepared by the method of De Savigny (1975), as modified by Rubinsky-Elefant *et al.* (2001). In brief, *T. canis* eggs were collected from the uteri of female worms that had been dissected out of naturally infected dogs at the Centro de Controle de Zoonoses de São Paulo. The eggs were incubated in 2% formalin at 28°C , for approximately 1 month, and then hatched in serum-free Eagle's medium. The resultant suspension was placed on a loose plug of cotton wool in a Baermann apparatus and the L_2 were collected from the bottom of the apparatus 4 h later. Cultures of the larvae were incubated at 37°C and, at weekly intervals,

the culture fluid was removed and replaced with fresh medium. Protease inhibitor ($5\text{ }\mu\text{l}$ 200 mM phenyl-methyl-sulphonyl-fluoride/ml) was added to each sample of old medium (which contained TES) before the sample was concentrated in an Amicon concentration unit (Amicon, Lexington, MA), dialysed against distilled water, and centrifuged at $18,500\times g$ and 4°C for 60 min. The resultant supernatant solution was passed through a $0.22\text{-}\mu\text{m}$ -pore membrane filter (Millipore, Danvers, MA) and then used as the final TES extract. The protein content in the extract was determined by the Lowry method (Lowry *et al.*, 1951) before the rest of the extract was stored, in aliquots, at -20°C .

PRODUCTION OF *Ascaris lumbricoides* ADULT-WORM EXTRACT

Non-specific antibodies were removed from the serum samples by absorption with an extract of adult *Ascaris lumbricoides*. This adult-worm extract (AWE) was prepared using a slight modification of the method of Kanamura *et al.* (1981). In brief, adult *A. lumbricoides* were macerated in distilled water before 1.5 M NaOH was added to give a final concentration of 0.15 M. After 2 h at room temperature, the mixture was neutralized with 6 M HCl and centrifuged at $18,500\times g$ and 4°C for 20 min. The resultant pellet was discarded. The protein content of the supernatant solution was estimated, at 5 mg/ml, by the Lowry method before the solution was delipidized with ether (1:3, by volume).

STANDARDIZATION OF THE ELISA

Levels of anti-*Toxocara* IgG, IgA and IgE in each serum sample were determined in ELISA based on the method of De Savigny *et al.* (1979), as modified by Elefant *et al.* (2006). Flat-bottomed, 96-well, polystyrene microtitration plates — Costar[®] (Corning, New York, NY) for the detection of IgG and IgE and PolySorpTM (Nunc, Rochester, NY) for IgA — were loaded ($100\text{ }\mu\text{l}$ /well)

with the TES extract (1.9 µg/ml in 0.1 M carbonate-bicarbonate buffer, at pH 9.6). The plates were left for 2 h at 37°C and then 18 h at 4°C, in a humid chamber, before being washed three times with 0.01 M phosphate-buffered saline (pH 7.2) containing 0.05% Tween 20 (PBS-T). The wells were then blocked, for 1 h at 37°C, with either 1% bovine serum albumin in PBS-T (IgG- and IgA-ELISA) or 5% skim-milk (Molico®; Nestlé, Vevey, Switzerland) in PBS-T (IgE-ELISA). After another three washes, the plates were incubated, for 40 min at 37°C, with serum samples that had been pre-absorbed with AWE (25 µg/ml, in PBS-T, for 30 min at 37°C) and diluted 1:320 for the IgG-ELISA (100 µl/well), 1:50 for the IgA-ELISA (100 µl/well), and 1:50 for the IgE-ELISA (50 µl/well). [For the IgA- and IgE-ELISA, the serum samples were also pre-absorbed with AWE (25 µg/ml) and RF-Absorbent v/v (Dade Behring, Marburg, Germany) for 18 h at 4°C.] After incubation with the sera, the plates were washed three times and then incubated, for 40 min at 37°C, with the appropriate peroxidase conjugates: a 1:10,000 dilution of peroxidase-anti-human IgG (Sigma), at 100 µl/well; a 1:5000 dilution of peroxidase-anti-human IgA (Sigma), at 100 µl/well; or a 1:500 dilution of peroxidase-anti-human IgE (Sigma), at 50 µl/well. After a final three washes with PBS-T, ortho-phenylenediamine (0.4 mg/ml) and H₂O₂-urea (0.4 mg/ml) in 0.05 M citrate buffer were added, as substrates, for 15 min at 37°C. The reaction was stopped with 2 M H₂SO₄ (50 µl/well) before absorbances at 492 nm were determined in an automatic microplate reader (Multiskan MCC/340; Titertek, Huntsville, AL). The cut-off values for seropositivity (0.300 for the IgG-ELISA, 0.200 for the IgA-ELISA and 0.220 for the IgE-ELISA) were calculated as the mean absorbance for the negative-control sera plus either three S.D. (IgG- and IgA-ELISA) or two S.D. (IgE-ELISA). An ELISA reactivity index (RI) was calculated for each serum and each type

of ELISA by dividing the absorbance value of the sample by the relevant cut-off value. Samples with RI >1 were thus considered positive. Negative and positive controls and a serum-free blank were run in each ELISA plate.

Stool Samples

Stool samples were collected, from some of the subjects who were found seropositive, and checked for cysts, proglottids and other stages of parasites, using the Faust-Hoffmann and Pons-Janner methods (Pessoa and Martins, 1982).

Soil Samples

Since soil contaminated with embryonated eggs is the main source of human *Toxocara* infection, eight soil samples together weighing about 100 g (two samples from in front of a house, two from each side, and two from behind the house) were collected from each of 24 of the 96 plots in the settlement. The samples were taken to Presidente Prudente where they were stored in refrigerator until they could be checked for *Toxocara* eggs (Nunes *et al.*, 1994). As each sample was checked three times, there were 24 egg counts for each of the sampled plots.

Statistical Analysis

Independent-samples *t*-tests were used to see if the levels of anti-*Toxocara* IgG were significantly affected by the presence of dogs, household income or the level of education achieved by the subject. Where appropriate, the data were also compared in χ^2 and Fisher's exact tests. Correlation coefficients (*r*) were calculated using Sigma-Stat software (Systat Software, Richmond, CA) but version 3.0 of the GraphPad software package (GraphPad, San Diego, CA) was used for all the other data analysis. A *P*-value of <0.05 was considered indicative of a statistically significant difference.

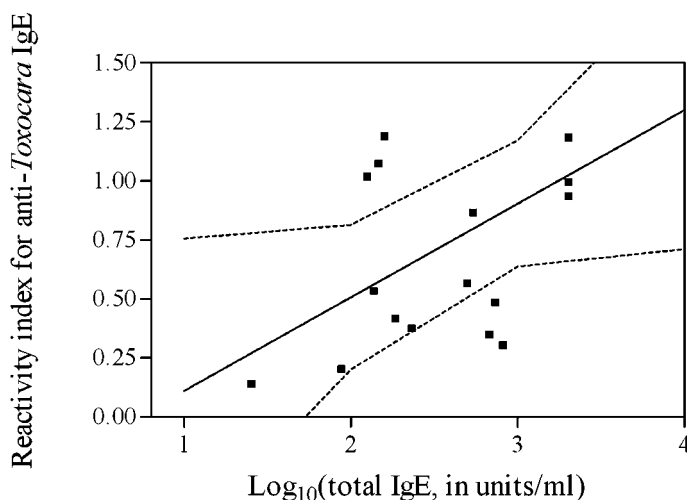


FIG. 1. The total IgE levels seen in 17 subjects from Teodoro Sampaio county, São Paulo, Brazil, plotted against the corresponding reactivity indices for anti-*Toxocara* IgE. The lines indicate the linear regression and its 95% confidence intervals.

RESULTS

The seroprevalence of toxocariasis indicated by IgG antibodies (see Table) was 21.5% (17/79) overall, and was similar among the female and male subjects (24% *v.* 10%; $P>0.05$) and among the children (subjects aged ≤ 15 years) and adults investigated (30% *v.* 19%; $P>0.05$). The 17 subjects found seropositive for anti-*Toxocara* IgG were deemed 'the seropositives'.

Seventy (89%) of the 79 individuals investigated had elevated levels of total IgE, and, among the 17 subjects found seropositive for anti-*Toxocara* IgG, there was a positive correlation between the levels of total IgE and the reactivity index for anti-TES IgE ($r=0.491$; $P=0.0452$; Fig. 1).

Thirty (38%) of the subjects had elevated eosinophil counts in their peripheral blood. The 17 seropositives showed a positive correlation between the level of anti-TES IgE and level of eosinophilia ($r=0.646$; $P=0.0069$; Fig. 2).

Although 10 (59%) of the 17 seropositives were also found positive for anti-*Toxocara* IgA, the levels of total IgA in the 17 were not correlated with the levels of *Toxocara*-specific IgA. Just five of the

subjects were found seropositive for *Toxocara*-specific IgA, IgE and IgG.

Only one (6%) of the 17 seropositives had attended secondary school, the other 16 having only completed elementary education. Family monthly income was low (<U.S.\$100) for 15 (88%) of the seropositives.

Of the 12 stool specimens investigated, only two (17%) were found positive for protozoal cysts and none was found positive for helminths. Sixteen (94%) of the seropositives reported that they used latrines; the other one had no toilet facilities whatsoever and simply defecated on the ground, outdoors.

Thirteen of the seropositives shared a house with a dog and five of these 13 also had cats. Four (67%) of the six seropositive children had anaemia (<10 g haemoglobin/dl).

Sixteen of the 17 seropositives were clinically examined by a PSF physician and three (19%) were found to have non-specific symptoms of toxocariasis: one had cutaneous manifestations (pruritic ulcer on the arms and legs), one a pulmonary manifestation (productive cough), and the other paroxysmic dyspnoea.

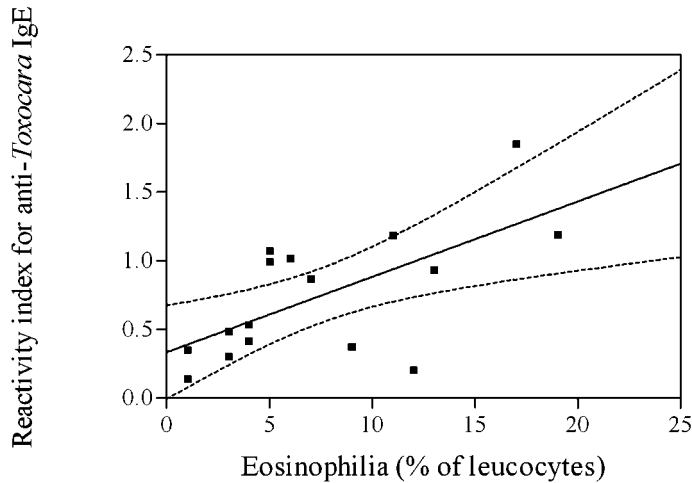


FIG. 2. The levels of eosinophilia seen in 17 subjects from Teodoro Sampaio county, São Paulo, Brazil, plotted against the corresponding reactivity indices for anti-*Toxocara* IgE. The lines indicate the linear regression and its 95% confidence intervals.

When the soil samples were checked, embryonated *Toxocara* eggs were found in samples from five (21%) of the 24 plots investigated. A sampled plot in which a seropositive subject lived was no more likely to be found positive for *Toxocara* eggs than a sampled plot in which no seropositives had been detected.

DISCUSSION

This appears to be the first study focussing on *Toxocara* infection in a rural settlement in Brazil. The seroprevalence of *Toxocara* in the subjects from Padre Josimo, 21.5%, was

not surprisingly high, given the rural setting, generally poor sanitation and large numbers of dogs and cats.

Serological tests and surveys are of considerable importance in the detection of human infection with *Toxocara* sp. because the clinical symptoms of the disease are frequently non-specific. Seroprevalences vary with factors such as geography, ethnicity and economic status. Although results from studies based on different assays may not be directly comparable, the prevalence of toxocariasis appears to be generally lower in urban areas than in rural settings. In the poor rural area of Vale do Ribeira, in the state of São Paulo, for example, 58.0% of subjects aged <20 years were found seropositive (Vicente *et al.*, 2005). In Argentina, 23% of a rural community showed evidence of infection (Chiodo *et al.*, 2006), and in Taiwan almost half (46%) of the subjects investigated from five aboriginal tribes were found to be seropositive (Fan *et al.*, 2004). Human toxocariasis tends to be rarer in developed countries, where there is generally good sanitation, than in the developing world. Magnaval *et al.* (2001) reported that seroprevalence in adults from western countries ranges from 2%–5% in

TABLE. The seroprevalence of human toxocariasis in Padre Josimo peasants' settlement, Teodoro Sampaio county, São Paulo, Brazil, split by age and gender

		No. and (%) of subjects:	
Gender	Age (years)	Investigated	Found seropositive
Male	≤15	9	3 (33)
	>15	32	5 (16)
Female	≤15	11	3 (27)
	>15	27	6 (22)
Both	All	79	17 (21.5)

urban centres but reaches levels of 14.2%–37% in rural areas.

In the present study, children were more likely to be found seropositive than adults but this trend did not reach statistical significance, perhaps because of the relatively small numbers of subjects. In most surveys of toxocariasis, the seroprevalence in children has been found higher than that in adults and this difference has been attributed to geophagy, onychophagy and greater levels of contact with dogs and cats in the children (Chieffi *et al.*, 1990; Coelho *et al.*, 2004; Figueiredo *et al.*, 2005). Of the individuals interviewed in the present study, most (77%) lived in households in which there were dogs and many (29%) shared their homes with both dogs and cats. Semi-wild cats and dogs also wander all over the study area (unpubl. obs.) and these probably make a major contribution to the *Toxocara* sp. eggs in the local soil (Fan *et al.*, 2004; Chiodo *et al.*, 2006).

Although many studies in urban and rural areas have indicated that the predominant mode of transmission of *Toxocara* to humans is by the ingestion of embryonated eggs in soil, the ingestion of embryonated eggs by paratenic invertebrate hosts, such as earthworms and ants, and by vertebrates, such as rabbits and lambs, provides other potential sources of human infection that cannot be ignored (Fan *et al.*, 2004). Most of the land around Padre Josimo is, however, used for cattle-raising and there is no local consumption of the beef produced. Human exposure in this area is therefore probably entirely via contact with contaminated soil or with the contaminated fur of dogs and cats (Wolfe and Wright, 2003). The environment plays a crucial role in maintaining and distributing the human-infective eggs (Overgaauw, 1997; Macpherson, 2005).

The surface of helminths such as *T. canis* is a dynamic structure, which turns over quite rapidly and serves as a renewable source of large quantities of antigens (Kennedy *et al.*, 1989; Kayes, 1997;

Ishida *et al.*, 2003) such as TBA-1, which is abundant in both somatic and excretory–secretory extracts (Maizels and Page, 1990; Yahiro *et al.*, 1998; Schabussova *et al.*, 2007). In a murine model, Del Prete *et al.* (1991) found that *T. canis* infection induced a dominant Th2-type immune response. In both experimental models and humans, this response includes the increased expression of interleukin-4, interleukin-5 and interleukin-10, which is followed by persistent eosinophilia and the production of IgE (including anti-TES IgE), while the production of interferon- γ , interleukin-12 and tumour necrosis factor decreases (Elefant *et al.*, 2006; Pinelli *et al.*, 2005). In the present study, in subjects with anti-TES IgG, there were positive correlations between the levels of total and *Toxocara*-specific IgE and between the level of specific IgE and the level of peripheral-blood eosinophilia. These are not specific characteristics found in patients with clinical signs of *Toxocara* infection, however, and only 19% of the seropositives identified in the present study had any clinical manifestations that might indicate toxocariasis (cutaneous or pulmonary manifestations or paroxysmic dyspnoea). Most seropositive individuals detected in epidemiological surveys probably represent past rather than current or even recent infections (Magnaval *et al.*, 2001; Despommier, 2003).

Children with clinical and laboratory signs of toxocariasis sometimes show eosinophilia (Jacob *et al.*, 1994; Altcheh *et al.*, 2003) and, as in the present study, Figueiredo *et al.* (2005), studying the paediatric population of Capela do Socorro, on the outskirts of São Paulo, found levels of seropositivity for *Toxocara* to be positively correlated with levels of IgE and eosinophilia. The association between elevated levels of total IgE and peripheral-blood eosinophilia seen in the present study appeared to be independent of intestinal parasite infection, since no helminths were seen in any of the stool samples.

The role of IgA antibodies in combating parasites is not completely understood and there are few studies on their efficacy against nematodes, especially *Toxocara* infection. During an outbreak of trichinellosis, asymptomatic carriers were found to have higher titres of IgA reacting with a total soluble extract from *Trichinella spiralis* than the symptomatic cases (Chapa-Ruiz *et al.*, 2002), and patients with hookworm infection seem to have elevated levels of IgA (Musa and Onyemelukwe, 2000). In the present study, 10 of the 17 individuals with anti-TES IgG also had *Toxocara*-specific IgA in their serum, although a positive correlation between the levels of total and specific IgA was not found. Recently, Elefant *et al.* (2006) described a post-treatment decrease in levels of anti-TES IgA in children with toxocariasis. Remarkably, the large amounts of anti-*Toxocara* IgG seen in many individuals seem to mask both the IgA and IgE, through a competitive mechanism with some parasite epitopes (Elefant *et al.*, 2006). In the present study, sera were pre-absorbed with RF-Absorbent to facilitate the detection of the IgA and IgE. The presence of specific IgE and IgA antibodies could be exploited in the diagnosis of toxocariasis and monitoring the efficacy of treatment (Elefant *et al.*, 2006). In human toxocariasis, there is also a non-transient IgM response that occurs in both the acute and chronic phases of the infection (Smith, 1993).

Most of the subjects found seropositive for anti-*Toxocara* IgG in the present study kept dogs and had low family incomes and low levels of education. It is the generally poor living conditions, the lack of sanitary infrastructure and the large number of dogs that probably make Padre Josimo a high-risk area for human toxocariasis. There are many established settlements in the surrounding area and many others being formed. In order to develop effective measures to decrease human exposure to *Toxocara* in São Paulo state, others studies to determine the true seroprevalence of

toxocariasis and other zoonoses in a number of counties in the region, as well as the possible risk factors involved, are being carried out.

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