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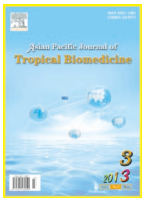

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Anti-*Toxocara* spp. antibodies in an adult healthy population: serosurvey and risk factors in Southeast Brazil

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PEER REVIEW

Peer reviewer

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Comments

The article was well-written, interesting, and well- conducted in respect to determination of the sample, the laboratory analysis methodology and analysis of the results. The study brought interesting data. Even though it is primarily applicable to a local situation and on a specific population group, it makes important methodological contributions in its research area, especially the data analysis.

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ABSTRACT

Objective: To evaluate the frequency of anti-*Toxocara* spp. antibodies in an adult healthy population. **Methods:** The study was performed by interviewing 253 blood donors, from 19 to 65 years of age, in a hematological centre in Presidente Prudente, São Paulo, southeast Brazil. A survey was applied to blood donors in order to evaluate the possible factors associated to the presence of antibodies, including individual (gender and age), socioeconomic (scholarship, familial income and sanitary facilities) and habit information (contact with soil, geophagy, onycophagy and intake of raw/undercooked meat) as well as the presence of dogs or cats in the household. ELISA test was run for detection of the anti-*Toxocara* spp. IgG antibodies. Bivariate analysis followed by logistic regression was performed to evaluate the potential risk factors associated to seropositivity. **Results:** The overall prevalence observed in this study was 8.7% (22/253). Contact with soil was the unique risk factor associated with the presence of antibodies (P=0.017 8; OR=3.52; 95% CI=1.244-9.995). **Conclusions:** The results of this study reinforce the necessity in promoting preventive public health measures, even for healthy adult individual, particularly those related to the deworming of pets to avoid the soil contamination, and hygiene education of the population.

KEYWORDS
Toxocariasis, Larva migrans, Seroprevalence, Diagnostic, Epidemiology

1. Introduction

Toxocariasis is a widespread zoonosis caused by the ascarid nematodes *Toxocara canis* (*T. canis*) and *Toxocara cati*, which primarily infect dogs and cats, respectively[1]. Human toxocariasis is a soil-transmitted helminthic infection. *Toxocara* eggs are released into the environment with the faeces of parasitised pets, and these eggs may be embryonate and accidentally be ingested by humans, particularly children who often play with contaminated soil. Many authors have reported different rates of *Toxocara* infections in both children and adults in different countries. Although human toxocariasis is highly prevalent in disadvantaged countries, some authors have focused

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on the global importance of this zoonosis, which remains underestimated and neglected, even in developed countries[2].

The clinical spectrum of human toxocariasis is broad and ranges from asymptomatic infection to severe organ injury, including hepatic, pulmonary, ophthalmic and neurological disturbances. Some risk factors have been associated with toxocariasis, including gender, age, socioeconomic status, contact with pets, and ingestion of raw meat. Nevertheless, the results of different studies on the toxocariasis risk factors have been largely inconsistent until now[3].

Blood donors have been considered as a model to study the seroprevalence of infectious diseases in the healthy adult population[4-6]. The prevalence of anti-*Toxocara* spp. antibodies in this population has been studied on some continents. In Europe, the seroprevalence ranged from 1% in Spain to 13.65% in the Slovak Republic[7,8]. While in Oceania, the seroprevalence varied from (0.70±1.65)% in New Zealand to 7.0% in Australia[5,9]. In South America, the rates varied from 10.6% to 38.9% in Argentina, respectively[10,11]. In Brazil, there is a reported rate of 46.3% in northeast Brazil[12]. However, little is known about the risk factors for toxocariasis in voluntary blood donors.

Based on these statements, this study was conducted to assess both the seropositivity and risk factors for *Toxocara* spp. infection in an adult healthy population from southeast Brazil.

2. Materials and methods

2.1. Study area

The study was conducted from January to May of 2010 at a haematological centre in the municipality of Presidente Prudente, within the state of São Paulo, southeast Brazil (22°10′30″S, 51°25′28″W). The estimated population of this municipality in 2010 was approximately 207 610 inhabitants that were living in both urban and rural areas[13].

2.2. Subjects

A total of 253 voluntary blood donors ranging from 19 to 65 years old were included in this survey. The number of individuals to be enrolled was established using the statistical software Epi Info, version 6.0, with an estimated seroprevalence of 15%, an absolute error of 4.5 and a 95% confidence interval (95% *CI*).

To avoid the possibility of participation of multiple people from the same family, the individuals were included in the study by using a systematic sampling selection from the record numbers at the haematological centre.

The criteria for the inclusion of subjects followed the recommendations of the Brazilian National Health Vigilance Agency (ANVISA-Resolution 153/2004) that coordinates the Program of Blood and Blood Products in Brazil. All individuals included were considered as healthy individuals. During the clinical trial, all blood donors were asked to provide informed consent for their participation in the study, and a short questionnaire interview was conducted to gather information to determine the epidemiology of toxocariasis, including factors such as gender, age, educational or academic level, family income, sanitary facilities, pet ownership (dogs and/or cats), behavioural habits (onychophagy or geophagy), and intake of either raw or undercooked meat.

2.3. Sample collection

After standard blood collection in a polyethylene donation bag, the residual blood in the tubing was collected into 5.0 mL serum collection vacuum tubes. The tubing was manually clamped at the bag to prevent backflow of blood and/or anticoagulant from the bag into the tubing. The samples of blood were centrifuged at 3 300 r/min for 7 min, and the obtained serum was mixed immediately with a buffered glycerin solution of the same volume (Merck, USA) and stored at −32 °C.

A universal flask container (120 mL) was provided to each individual for stool collection. The stool samples were collected at the household by a researcher on the day after the interview.

2.4. Antigen preparation

T. canis excretory-secretory larval antigens (TES) were obtained according to the method described elsewhere[14], with some modifications[15]. Briefly, *T. canis* eggs were collected from the uterus of female adult worms and were embryonated by incubating them in 2% (v/v) formalin at 28 °C for approximately 1 month. Infective eggs were artificially hatched, and the larvae were recovered and maintained *in vitro* at 37 °C in serum-free Eagle's medium. At weekly intervals, the culture supernatant containing the TES was collected in sterile flasks and replaced with fresh culture medium. All of the supernatants were treated with 200 mmol/L of the protease inhibitor phenyl-methyl-sulfonyl fluoride (Sigma, St. Louis, USA), concentrated with Amicon Ultrafiltration units (Millipore, Danvers, USA), dialysed against distilled water, centrifuged at 12 000 r/min for 60 min at 4 °C, and filtered with 0.22 μm Millipore membranes.

2.5. Preincubation of sera with Ascaris suum adult worm extract (AWE)

To remove antibodies elicited by exposure to *Ascaris* that could cross-react with *Toxocara* antigens, the test samples were preincubated with an AWE of *Ascaris suum*[15]. Briefly, adult worms recovered from a porcine intestine were macerated in distilled water, and NaOH was added to a final concentration of 0.15 mol/L. After a 2 h incubation at room temperature, the mixture was neutralised with 6 mol/L HCl, the lipids were removed from the extract with ether, and the extract was centrifuged at 12 000 r/min for 20 min at 4 °C. The aqueous phase was removed and filtered through a 0.22 μm Millipore membrane. All sera were pre-incubated with a final concentration of 25 μg/mL AWE in 0.01 mol/L PBS (pH 7.2) containing 0.05% (v/v) Tween 20 (PBS-T) (Sigma, St. Louis, USA) for 30 min at 37 °C before use in the ELISA.

2.6. ELISA

Serum samples were tested for IgG antibodies to TES by ELISA at a dilution of 1:320, as previously described

elsewhere[15]. Polystyrene 96-well microplates (Corning, Costar, New York, USA) were coated for 1 h at 37 °C followed by an 18 h incubation at 4 °C with 1.9 μg/mL of TES dissolved in 0.06 mol/L carbonate-bicarbonate buffer (pH 9.6), 100 μL/well, and then blocked for 2 h at 37 °C with PBS-T containing 2.5% (v/v) bovine serum albumin (Sigma, St. Louis, USA). After a 40 min incubation at 37 °C, the serum samples were removed and horseradish peroxidase-conjugated goat anti-human IgG (Sigma, St. Louis, USA) was added at a 1:10 000 dilution (40 min at 37 °C) prior to the addition of the o-phenylenediamine substrate (0.4 mg/mL, Sigma, St. Louis, USA). Absorbance readings were made at 492 nm, and a cut-off absorbance value was defined as the mean absorbance reading for 96 negative control sera plus three standard deviations. Standard positive and negative control serum and a threshold reactive serum were used in all tests. The antibody levels were expressed as reactivity indices that were calculated as the ratio between the absorbance values of each test sample and the cut-off value. A serum sample was considered positive when its reactivity index was greater than 1.

2.7. Stool examinations

Stool were examined using the Faust method to recover protozoan cysts and helminths eggs, the Rugai method to detect helminth larvae, and the Vallada method to identify *Taenia* spp. proglottids[16].

2.8. Data analysis

A database was created with the Statistical Package for Social Science (SPSS) 14.0 for Windows (Chicago, USA) following the instructions published elsewhere[17].

Prevalence rates are given with exact binomial 95% *CI* and compared using *Chi*-squared or Fisher exact tests. Multiple logistic regression was conducted to assess the contribution of the risk factors studied to the likelihood of *Toxocara* seropositivity. Initially, a univariate model was developed with the inclusion of all variables (age and family income were categorised).

From the initial design model, the significant variables in the *Chi*-squared or Fisher exact test with a $P<0.20$ were selected for the final model. The regression coefficients and odds ratios (*OR*) estimated by point and interval with 95% *CI* were calculated for each predictor variable. The model data were adjusted by the Hosmer and Lemeshow test[18]. The predictive performance of the final model was evaluated by measuring the area under the ROC (receiver operator characteristic) curve.

To improve the final model, the predictor variables were tested for colinearity and for the presence of influential values. The accuracy of the model was evaluated by an 80-20 cross-validation[19]. All tests were performed with a significance level of 5%.

2.9. Ethical considerations

The study was approved by the local Institutional Ethic Committee (protocol 184/2009).

3. Results

About 8.7% (22/253) of subjects were seropositive for anti-*Toxocara* spp. IgG antibodies (95% *CI* 5.2-12.2). In the univariate analysis for risk factors related to the seropositive tests (Table 1), the variables that were significant ($P<0.2$) in the final logistic regression were "family income" and "contact with soil". A residual analysis identified eight influential variables. However, elimination of the observations did not significantly improve the indicator accuracy of the final model. The final model was able to correctly classify 91.3% of the observations, and the discriminatory capacity was considered fair (ROC curve 0.703, $P<0.002$).

Table 1

Bivariate analysis including the associated risk factors for anti-*Toxocara* spp. antibodies detected by ELISA in an adult health population (n=253), from January to May of 2010, Presidente Prudente, São Paulo, Brazil.

Variables			Positive (%)	Negative (%)	Odds ratio (95% CI)	*P*
Individual	Gender	Female	10 (4.1)	95 (37.5)	1	
		Male	12 (4.7)	136 (53.8)	0.838 (0.348-2.019)	0.694[a]
	Age (years)	19-30	9 (3.5)	90 (35.6)	1	
		31-40	6 (2.4)	70 (27.7)	0.857 (0.291-2.523)	0.994[a]
		>40	7 (2.8)	71 (28.0)	0.986 (0.349-2.778)	0.979[a]
Socioeconomic	Scholarship	Elementary	19 (7.5)	146 (57.7)	1	1.000[b]
		High school	3 (1.2)	85 (33.6)	1.059 (0.341-3.285)	
	Monthly income	1 to 2 salaries	19 (7.5)	146 (57.7)	1	
		>2 salaries	0 (0.0)	14 (5.5)	0.271 (0.078-0.943)	0.034[a]
Household	Location	Urban	21 (8.3)	221 (87.3)	1	
		Rural	1 (0.4)	10 (4.0)	1.052 (0.128-8.627)	1.000[b]
	Backyard	No	4 (1.6)	25 (9.9)	1	
		Yes	18 (7.1)	206 (81.4)	0.546 (0.171-1.742)	0.295[b]
	Dogs	No	8 (3.2)	94 (37.2)	1	
		Yes	14 (5.5)	137 (54.1)	1.201 (0.485-2.975)	0.692[a]
	Cats	No	17 (6.7)	196 (77.5)	1	
		Yes	5 (2.0)	35 (13.8)	1.647 (0.571-4.754)	0.352[a]
Behavioural	Soil contact	No	5 (2.0)	126 (49.8)	1	
		Yes	17 (6.7)	105 (41.5)	4.080 (1.456-11.431)	0.004[a]
	Geophagy	No	13 (5.1)	153 (60.5)	1	
		Yes	9 (3.6)	78 (30.8)	1.358 (0.556-3.315)	0.500[a]
	Onycophagy	No	19 (7.5)	174 (68.8)	1	
		Yes	3 (1.2)	57 (22.5)	0.482 (0.138-1.689)	0.304[b]
	Eating raw meat	No	12 (4.7)	131 (51.8)	1	
		Yes	10 (4.0)	100 (39.5)	1.092 (0.453-2.628)	0.845[a]

95% CI: Confidence interval 95%; [a]: *Chi*-squared; [b]: Exact Fisher test.

According to the final model of the logistic regression, "contact with soil" was the unique risk factor associated with seropositivity to *Toxocara* spp. (Table 2).

Almost all of interviewed subjects (96.4%) reported that they had access to a potable water supply and wastewater treatment (P=0.604 3, *OR*: 0.851 4, 95% *CI*: 0.102 8-7.05).

Because most of the individuals refused to give their stool samples, the parasitological study included only 94 subjects. Of these subjects, 9.6% (9/94) had intestinal protozoan infections, including *Entamoeba coli* (55.6%), *Endolimax nana* (33.3%) and *Giardia duodenalis* (11.1%). No helminthic structure (egg/proglotid) was detected in the faecal samples.

Table 2

The final logistic regression model for the analysis of the risk factors associated with anti-*Toxocara* spp. antibodies (detected by ELISA) in an adult healthy population (n=253), from January to May of 2010, Presidente Prudente, São Paulo, Brazil.

Predictor	β	SE β	Wald's χ^2	*df*	*P*	Exp (β): odds ratio (95% *CI*)
Constant	−3.9665	0.6813	33.8919	1	0.0000	NA
Family income	1.0885	0.6452	2.8460	1	0.0916	2.9697 (0.8385-0.5174)
Contact with soil	1.2601	0.5316	5.6183	1	0.0178	3.5258 (1.2438-9.9947)
Omnibus tests of model coefficients	NA	NA	12.0000	2	0.0020	NA
Hosmer & Lemeshow	NA	NA	2.0870	2	0.3520	NA

All statistics reported herein use 4 decimal places to maintain statistical precision. Cox and Snell R^2=0.046; Nagelkerke R^2=0.104; β: standardized regression coefficient; SE: standard error; *df*: degree of freedom; Exp (β): antilog β (indicates the change in the odds ratio associated with a 1 unit change in the predictor variable); NA: not applicable.

4. Discussion

Studies regarding the prevalence of diseases have been performed in blood donors because this group of individuals may be employed to characterise a healthy population or to determine the risk factors associated with blood-borne infectious diseases.

The prevalence of toxocariasis varies among countries and even among regions within a country. The global prevalence of toxocariasis in blood donors ranged from 0.70%±1.65% in New Zealand to 46.3% in northeast Brazil[5,12]. The overall prevalence in our study (8.7%) is comparable to the 7.0% and 10.6% verified, respectively, in blood donors from Australia and Argentina[9,10].

Diverse factors may influence the reported infection rate in a population, including the technique used to detect the infection. The indirect ELISA tests using TES antigens is the most commonly employed test to assess the epidemiological status of toxocariasis in human populations[3]. Our study was based on an ELISA (sensitivity 78%; specificity 92%) and used 96 negative samples to calculate the cut-off value.

The main variables surveyed to evaluate the risk factors associated with toxocariasis in humans include individual traits (gender and age), socioeconomic status (residence, household head educational level and wealth index), the presence or absence of pets in the household, and some habits, such as geophagy, onycophagy or eating raw/undercooked meat. However, these risk factors may be inherent to the characteristics of a particular population. Thus, inconsistent results remain abundant[3].

For instance, while some researchers have verified either an increase of the toxocariasis prevalence in the male population[20,21], others have shown that females are more closely associated with the zoonosis[8]. No association between gender and an ELISA positive result for toxocariasis has also been observed[22].

Our results are also inconsistent regarding the association of age with toxocariasis. In spite of the observation that children are prone to infections with *Toxocara* spp.[3], studies, including the survey involving blood donors in Spain[7], have shown no influence of age on toxocariasis prevalence[22]. In Iran, the highest prevalence of infection was observed in adults[23].

In our study, none of the individual characteristics evaluated were associated with toxocariasis. The number of blood donors and the proportion of positive ELISA test were well distributed between genders and across the age groups. Socioeconomic status has been shown to influence the risk of human infection with *Toxocara* spp. Infections are more prevalent in populations at low socioeconomic levels, characterised by a low education level of the head of household and decreased family income due to poor sanitation and water quality[10,24]. Populations living in rural and poverty areas are more likely to become infected by toxocariasis agents as a consequence of an increased number of dogs in the household or the environmental conditions that allow the maintenance of eggs in the soil[8,25,26]. In Presidente Prudente, no significant difference was observed concerning the seroprevalence of anti-*Toxocara* spp. antibodies in middle-class (9.5%) and disadvantaged (12.7%) children[27]. However, seropositivity was inversely proportional to the family income.

Most of the interviewed subjects reported having access to a water supply, wastewater treatment and drinkable water. Additionally, 91.3% of them lived in an urban area. In this study, family income was identified as the unique factor associated with toxocariasis in the univariate model. However, the variable was not considered to be a risk factor by the logistic regression. The low number of subjects infected by intestinal parasites, represented exclusively by protozoan species, demonstrating the good sanitary status of the studied population.

According to the haematological centre in which the survey was performed, most of the voluntary blood donors live in the city, most likely because of the difficulty of travel and the distance from rural areas to the collection site.

The fact that most of the studied population live in urban area may indicate a low risk of infection by *Toxocara* spp. This argument is refuted by a study with blood donors in Argentina that showed no association between seropositivity and living in a rural area. In other study, the risk factor was contact with contaminated soil and water[11]. These findings were partially in agreement with our results. Our logistic regression final model identified contact with soil as the sole variable that was a risk factor for the population in spite of the increased hand-to-mouth and geophagic habits of children, especially at parks and playgrounds, areas that are frequently contaminated with dog and cat faeces. In our study, even though contact with soil was considered a risk factor for toxocariasis, geophagy and onycophagy were not associated with a positive ELISA test for *Toxocara*. A pertinent question is whether the infection in the studied population was recently acquired or if it occurred during childhood. The duration of the human IgG responses triggered by *Toxocara* larvae remains undetermined. Viable larvae may persist in tissues and excrete/secrete antigens for several years, and no simple method is available to confirm parasite death even after chemotherapy. As a consequence, a single-sample IgG-ELISA titre cannot distinguish between past and current infections[28].

Another relevant question to our study is whether the infection was acquired in the household or occurred due to the type of work performed by the individuals. In a recently published review, toxocariasis was included in a list of occupational diseases[29]. In Austria, the risk of infection by *Toxocara* spp. among farmers, veterinarians, slaughterhouse workers and hunters was, respectively, 38-, 18-, 16-, and 9-fold greater than the unexposed control group[26]. Our study included subjects that worked in a wide variety of professions. Therefore, the inclusion of a workplace variable into the statistical model could result in a confounding element.

The majority of the studied population had at least one dog (59.7%), and a minority indicated that they owned a cat (15.8%). It has suggested that direct contact with dog hair containing *Toxocara* spp. embryonated eggs is a potential source for human toxocariasis[30]. However, having a pet was not considered a risk/protective factor for toxocariasis. Keeping a dog in a household has been recognised as a risk factor in some[31,32] but not all surveys[21,33]. The influence of cat ownership on a positive ELISA test has been less studied. In recent serosurveys in Brazil, having a cat was identified as both a protective factor and a risk factor[33,34]. A third survey in Poland[31], but not a large country-wide study in the United States[25] found cat ownership to be a significant predictor of *Toxocara* seropositivity. In northeast Brazil and in southeast Brazil[27,35], the presence of dogs and cats was identified as a risk factor for seropositivity in children. These discrepancies are not surprising, particularly in tropical settings where dogs and cats roam freely and spread eggs across large areas[3].

Eating raw or undercooked meat from paratenic hosts, particularly bovine hosts, has been considered as a risk factor for toxocariasis. In South Korea, it was observed that patients presenting eosinophilia had a recent history of consuming raw cow liver[32,36]. In our study, despite the absence of an association between this habit and positive ELISA for *Toxocara* spp., 45.0% of the subjects reported eating undercooked or raw bovine meat.

Although we identified contact with soil as the only risk factor associated with *Toxocara* spp. seropositivity, it is possible that infection by direct contact with soil is in turn associated with poor personal hygiene. Based on these results and in spite of the limitations of this survey, it is essential to promote preventive public health measures, even for adult healthy individuals, particularly those related to the deworming of pets to avoid soil contamination, and educate the population about hygiene.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

This study deals with the prevalence of antibodies to anti-*Toxocara* in a specific sector (blood donors) of an urban population, as well as the determination of the risk factors associated with this infection. The human toxocariasis, also known as visceral larva migrans, is considered a disease neglected for their short- and long-term effects with other underlying syndromes that make the diagnosis difficult. However, they have important effects on affected individuals, and some authors have been considering this infection as one of the most common and neglected geohelminthiasis, mainly on poor people in the Americas.

Research frontiers

T. canis has been already established as an agent of visceral larva migrans in humans. However, other parasites of animal origin can also perform similar erratic migrations and result in serious clinical syndromes like emerging *Baylisascaris* infections in North America, including cases of fatal encephalitis.

Related reports

In spite of the low frequency of epidemiological studies about this infection in humans, the consolidation of research groups in this area has been bringing out alarming information about the frequency of infections. We must determine, however, the degree of clinical and neurological and ocular development of affected individuals, widen field for research, especially in developing countries.

Innovations and breakthroughs

The seemingly simple study carries bulge in its innovations with regard to the processing of epidemiological data collected. In this way, the use of logistic regression analysis is one of the innovations in this field of study, since most studies are limited to analysis of epidemiological factors by univariated analysis. The use of ROC curves to test the predictive value of the final logistic model is also an interesting innovation, and that deserves a closer look at the validation of these tests.

Applications

The study of the prevalence of infections has inherent value for the constant need to monitor the occurrence of illnesses (epidemiological surveillance). On the other hand, the delimitation of risk factors in a population in particular is very important so that specific measures are taken in relation to the studied population, and it is also good for health policy decisions, which may be relevant in decision making of specific health promotion policies.

Peer review

The article was well-written, interesting, and well-conducted in respect to determination of the sample, the laboratory analysis methodology and analysis of the results. The study brought interesting data. Even though it is primarily applicable to a local situation and on a specific population group, it makes important methodological contributions in its research area, especially the data analysis.

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