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Seroprevalence and risk factors for *Toxocara* infection in children from an urban large setting in Northeast Brazil

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ABSTRACT

Objectives: This study aimed to standardize an "in house" immunoassay to detect anti-*Toxocara* IgG antibodies in human serum to estimate the seroprevalence of *Toxocara* infection, and to identify its potential risk factors in children living in poor areas of Salvador, a large northeastern Brazilian city.
Methods: Parents of 1309 children answered a questionnaire containing possible risk factor for acquisition of this infection. Blood was collected and the presence of anti-*Toxocara* IgG antibodies was detected by indirect ELISA using *T. canis* larval excretory–secretory antigens in sera previously absorbed with *Ascaris lumbricoides* antigens.
Results: Seroprevalence of *Toxocara* infection was 48.4%. Children's age, low maternal schooling, contact with dogs and cats, and household located in paved streets were shown to be risk factors for *Toxocara* infection.
Conclusions: The seroprevalence of *Toxocara* infection is high among children living in a poor urban setting of Brazil. The association of low maternal education with higher *Toxocara* infection supports studies showing that low socioeconomic status is a risk factor for the acquisition of this infection as a reflection of hygiene habits of the family. And both infected-dogs and cats may be involved in this parasite transmission in this children population.

1. Introduction

Visceral larva migrans (VLM) is a syndrome of human beings, transmitted mainly by accidental ingestion of embryonated eggs of *Toxocara canis* (dog round worms) or *T. cati* (cat round worm). In humans, the hatching larvae do not migrate to intestine as occur in the definite hosts, and remain migrating through the organs leading to several clinical pictures varying from asymptomatic to severe systemic forms such as prolonged fever, hepato-splenomegaly, meningoencephalitis and asthma-like symptoms (Despommier, 2003; Haralambidou et al., 2005; Saporito et al., 2008). *Toxocara* infection also causes a hypersensitivity reaction status, even in asymptomatic subject, leading to high eosinophilia, increase in total IgE and high susceptibility to asthma (Ferreira et al., 2007; Dattoli et al., 2011). Although this infection occurs worldwide, its prevalence is higher in non-affluent populations (Coelho et al., 2004; Espinoza et al., 2008), where its diagnosis is rarely performed, being considered a neglected disease. The toxocariasis serodiagnosis depends on the cultivation of *T. canis* larvae to produce excretory-secretory products used in ELISA as antigens. Currently, a few kits for serodiagnosis are commercial available, but is rarely used in Brazil due to their high cost. This infection is also prevalent

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in many developed countries and its global importance may be underestimated. In the United States of America, it is the most common helminthic infection, affecting millions of individuals (Hotez and Wilkins, 2009).

Stray and domiciliated dogs and cats from low income population play an important role in the transmission of *Toxocara* spp. providing environmental contamination, which perpetuates the spreading of the infection among the human populations (Regis et al., 2011). The contact with grounds contaminated with embryonated eggs is the most common *Toxocara* spp. transmission pathway, but contact with dogs and cats, presenting eggs in their fur, as well as the consumption of raw vegetables grown in contaminated gardens and raw or undercooked meat from paratenic hosts (Abougrain et al., 2009) have also been described as important ways of transmission of this zoonosis (Amaral et al., 2010). Studies in Brazil (Alcântara-Neves et al., 1999; Almeida et al., 2007; Tiyo et al., 2008) and worldwide (Mizgajska, 1997; Devera et al., 2008, Martin and Demonte, 2008) showed that soil of public areas such as plazas, parks, and beaches are important foci of *Toxocara* ssp. transmission and to frequent such places poses as an important risk factor to the human being to become infected. In addition, factors such as age, maternal education, low socioeconomic conditions, have also been related to this zoonosis (Wolfe and Wright, 2003). Most of these works however were carried out in small sample population of limited areas.

In this work, we aimed to standardize an "in house" immunoassay to detect anti-*Toxocara* IgG antibodies in human serum and determine the seroprevalence of *Toxocara* spp. infection in a large set of children living in poor areas of a large Brazilian city and to investigate the risk factors associated with the infection, helping to understand the epidemiology of *Toxocara* infection in this city and similar settings around the world.

2. Materials and methods

2.1. Study population

The present work is a transversal study, which evaluated whether the *Toxocara* spp. infection status assessed in 2005 was associated with exposures to potential risk factors for acquisition of the infection. It was conducted in the city of Salvador with nearly 2,800,000 inhabitants, mostly of mixed African descent, located in Northeast Brazil. Briefly, we studied 1309 children aged 4–11 years old, enrolled in a cohort recruited from 1997 to 2003 for evaluating the impact of a sanitation program on the incidence of childhood diarrhea, in different city areas, selected to represent the population without sanitation at that time (Strina et al., 2003). In 2005 these children were resurveyed and social (maternal schooling), demographic (age and gender) and environmental (presence of street pavement, presence of dog and/or cat at home, daycare attendance) data were collected. This children came from a typical urban poor population characterized by: absence of public sewage system in most of their household and 51.7% of the kids were from families having mensal income equal or less than 147 USD and only 3.3% had the family income equal or more than 500 USD. Informed consent was obtained from the children's parents or guardians. Ethical approval was granted by the Instituto de Saúde Coletiva at Universidade Federal da Bahia and the National Commission on Ethics in Research (CONEP), Brazil.

2.2. Blood collection

Blood collection was carried out in laboratory facilities established in each studied area. A blood sample of 5 mL was collected from each child and the sera were kept at −20 °C until use.

2.3. Obtaining excretory-secretory T. canis larval antigens

T. canis excretory/secretory larval antigens were obtained following the de Savigny (1975a) technique, modified by Alcântara-Neves et al. (2008).

2.4. Characterization of the excretory-secretory T. canis larval antigens by sodium duodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Laemmli (1970) using a Mini-PROTEAN III Electrophoresis Cells (Bio-Rad Laboratories, Hercules, CA) and a 12% polyacrylamide gel in the presence of 10% sodium dodecyl sulphate (Merck & Co., Inc., White house Station, NJ, USA). Protein bands were stained with Coomassie Brilliant Blue R 250 (Sigma Chemical Co., San Louis, MO, USA). The relative molecular weights were calculated using prestained protein (Sigma Chemical Co., San Louis, MO, USA) of standard molecular weight according to the relative electrophoretical mobility (REM), using the following equation: REM = distance of the protein migration/distance of bromophenol blue migration.

2.5. Sera absorption with Ascaris lumbricoides antigens

In order to remove nonspecific antibodies that might cross react with excretory-secretory *T. canis* larval antigens each serum was pre-absorbed with adult *A. lumbricoides* somatic antigens in the presence of 3% polyetilenglycol (PEG 15,000; Sigma Chemical Co., San Louis, MO, USA) and 0.1% sodium azide diluted in PBS. After 30 min upon agitation at 4 °C, the samples were centrifuged for 6082 × *g* during 10 min. Some samples were double absorbed with another incubation with the same conditions described above. Because 10.7% of the children were infected with *Trichuris trichiura*, a sample of the studied sera was also absorbed with antigens of this parasite and compared to the same sera absorbed with *A. lumbricoides* antigens alone or with both parasites. Since absorption with *A. lumbricoides antigens* alone or with both parasites provided comparable titers of anti-*Toxocara* spp. IgG, the remaining sera were absorbed with *A. lumbricoides* antigens only. Because this assay does not discriminate infection by T. canis or T. cati, we used the results of this assay as marker of past or present infection by both Toxocara species (Kennedy et al., 1987).

2.6. Immunoassay for detection of anti-Toxocara IgG antibodies

Anti-*Toxocara* IgG antibodies were detected in sera by indirect ELISA assay using excretory-secretory *T. canis* larval antigens according to de Savigny and Tizard (1975b). The only modification introduced was that the reaction was developed using an anti-human biotinylated IgG conjugate (BD Pharmingen, San Diego, CA, USA) instead of a peroxidase conjugate. It allowed a serum dilution of 1:1000 instead of dilution of 1:40–1:160 as reported in the literature (de Savigny and Tizard, 1975b; Nunes et al., 1999; Lynch et al., 1988). To determine the avidity of the antibodies binding to the antigen, the assay was performed in duplicate, and for each serum two wells were washed after the serum incubation with PBS-Tween 20, containing 6 M of urea (Urea P.A.–VETEC, São Paulo, Brazil). *Toxocara*-specific IgG avidity was calculated according to Dziemian et al. (2008) using the following formulae: relative avidity index (RAI) = O.D. in sera treated with urea/O.D. in sera non-treated with urea multiplied by 100. RAI up to 50 was considered as low avidity. The cut-off obtained (0.23) was calculated by the OD from the mean of the 14 negative controls (children without history of contact with dogs and cats) plus three standard deviations of this mean. Five previously assayed sera samples were used as positive controls.

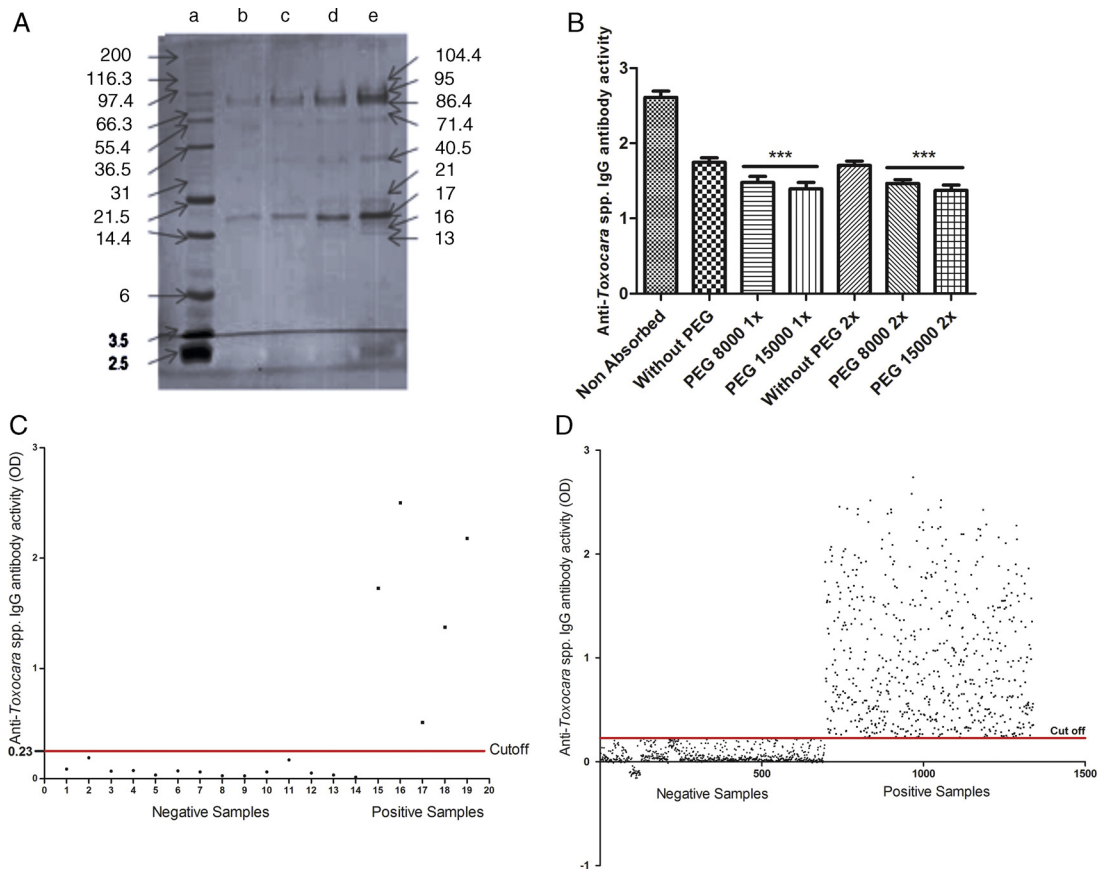

Fig. 1. *In house* immunoassay to detect anti-*T. canis* IgG and results of the tested sera: (A) 12% polyacrilamide gel electrophoresis of *T. canis* excretory/secretory larval antigens for stained with blue Coomasie (a) molecular weight markers; (b–d), antigen amount of 0.1, 0.2, 0.4 and 0.8 μg/well respectively); (B) results of the absorption of the children sera with somatic antigen of adult *A. lumbricoides* in the presence of polyetilenglycol as described in Section 2; (C) determination of the ELISA cut-off for anti-*T.canis* IgG as described in Section 2 and (D) dispersion of the anti-*T.canis* IgG in serum samples of the study population. Numbers in *x*-axis represent each sample.

2.7. Statistical analysis

Only children for whom complete data were available were included in the analysis. The following variables were studied as risk factors for acquisition of *Toxocara* spp. infection: whether the child attended nursery, maternal schooling, presence of dogs or cats at home, if the children houses were served by a paved road. We first performed a univariate analysis between each potential risk factor and outcome. Second, we built a multivariable model with standard logistic regression including only significant variables from the univariate analysis. Variables that remained statistically significant using a stepwise process remained in the final multivariate logistic regression model (Hosmer and Stanley, 2000). The association between outcome (seropositivity to *Toxocara* spp IgG antibodies) and risk factors was estimated with odds ratio and 95% confidence interval. Absorption test with *A. lumbricoides* antigens was analized by Dunn's test.

3. Results

A total of 1309 children with complete data were used in the analysis. Fig. 1A shows the quality of the *T. canis* excretory/secretory larval antigens used as antigen to detect anti-*Toxocara* IgG in an indirect ELISA, determined by SDS-PAGE showing bands of 104, 95, 86, 71, 40, 21, 17, 16 and 13 kDa. Fig. 1B shows the results of the sera absorption with somatic antigens of adult stage of *A. lumbricoides*. Sera absorbed twice with PEG 15.000 had the best performance taking out the non-specific antibodies and decreasing optical density of the reaction up to 54%, Fig. 1C shows the determination of the assay cut-off. Fig. 1D shows the results of the determination of anti-*Toxocara* IgG antibodies in the whole population by the "in-house" indirect ELISA.

Forty eight percent of the children were seropositive for anti-*Toxocara* IgG and only 2.8% of the 633 seropositive children had IgG of low avidity, indicating a recent infection (data not shown).

The following variables were positively associated with an increased prevalence of *Toxocara canis* infection at both univariated and multivariated analyses: to be ≥8 years old; living in house placed in paved streets; presence of a dog at home and presence of a cat at home. Day care attendance was positively associated with *Toxocara* infection only at univariated analysis and negative and statistically significant associations were found between *Toxocara* infection and mother with both, incomplete high school and complete high school or more, when compared with mothers with middle school or less, respectively (Table 1).

4. Discussion

The diagnosis of toxocariasis is based on clinical findings associated with the demonstrations of IgG specific antibodies in serum. Many laboratories have developed "in house" assays for research purpose in order to determine the prevalence of this zoonosis (Aguiar-Santos et al., 2004). The *T. canis* excretory/secretory larval antigens obtained in our laboratory are similar in composition to antigens previously described, according to the size of the bands found in the SDS-PAGE (Rubinsky-Elefant et al., 2006; Iddawela et al., 2007; Roldán and Espinoza, 2009) and the standardization carried out in our laboratory provided an assay with a greater

Table 1
Logistic analyses of the association between seropositivity for anti-*Toxocara* IgG and the studied risk factors for acquisition of this infection in 1309 children.

Variables	Anti-*Toxocara* IgG		
	Positivity *n* (%)	Bivariate OR (95% CI)	Multivariate OR (95% CI)
Gender			
Female	289 (47.6)	1	–
Male	344 (49.0)	1.06 (0.85; 1.31)	
Age			
≤5 years	152 (45.2)	1	1
6–7 years	245 (46.2)	1.04 (0.79; 1.37)	1.04 (0.79; 1.38)
≥8 years	236 (53.3)	**1.38 (1.04; 1.83)**	**1.43 (1.07; 1.92)**
Maternal schooling			
Middle school or less	177 (60.1)	1	
Incomplete high school	322 (51.3)	0.69 (0.52; 0.91)	0.72 (0.54; 0.96)
Complete high school or more	134 (34.5)	**0.35 (0.25; 0.47)**	**0.36 (0.26; 0.50)**
Daycare attendance			
No	514 (46.9)	**1.42 (1.05; 1.90)**	–
Yes	244 (52.6)		
Street paving			
No	389 (46.0)	1	1
Yes	244 (52.6)	**1.30 (1.04; 1.63)**	**1.26 (1.00; 1.60)**
Dog at home			
No	354 (44.7)	1	1
Yes	279 (54.0)	**1.45 (1.16; 1.81**	**1.36 (1.07; 1.72)**
Cat at home			
	498(46.2)	1	1
	135 (58.2)	**1.62 (1.21;1.91)**	**1.40 (1.03;1.91)**

Boldface numbers are statistically significant.

specificity when compared with the literature, since the serum dilution was 1:1000 instead of 1:200 (Nunes et al., 1997). Another point was the utilization of a biotin instead of a peroxidase-conjugate secondary antibody. Even using a cut-off determined by the mean of the negative controls ($n = 14$) plus 3 standard deviation we had a low cut off of 0.23 OD while some positive sera reached optical density values above the upper detection limit of the assay. Furthermore we absorbed the sera with *A. lumbricoides* antigens instead of *A. suum* that is customary used (Lynch et al., 1988, Nunes et al., 1997; Campos Junior et al., 2003; Roldán et al., 2006). Absorption of sera with antigens from other parasites is a practice that increases the specificity of the test, since many parasite species share similar antigens giving rise to cross-reactivity between these antibodies (Ishida et al., 2003). In our study population helminth infection are caused mainly by *A. lumbricoides* and *Trichuris trichiura* which occurs in 16.1% and 10.8% respectively. The absorption with *Ascaris lumbricoides* antigens decreased the reactivity of some sera up to 76.39% indicating a higher removal of specific antibodies to *A. lumbricoides* avoiding cross-reactions between this helminth and anti-*T. canis* antibodies and it also removed the cross-reactivity with *T. trichiura*. We have also absorbed some of the sera with *Ancylostoma braziliensis* antigens and there was no decrease of the anti-*Toxocara* IgG, showing that this hookworm does not share antigens reactive with anti-*T. canis IgG* (data not shown).

In our work we found a prevalence of IgG anti-*Toxocara* of 48.4%, this prevalence is similar to a previous work conducted by our group where we have reported 46.3% of *Toxocara* spp. seroprevalence in blood donors in the same city of the present study (Dattoli et al., 2011). Others studies conducted in Latin America had reported smaller prevalences. In Argentina, Alonso et al. (2000) reported a positivity of 37.9% in children younger than 14 years and Radman et al. (2000) observed a prevalence of 39% infection. Espinoza et al. (2008) determined a seroprevalence of 32.4% in Peru and in Brazil, Chieffi et al. (2009) in a review, cited prevalence of *T. canis* varying from 3.72% to 40%.

Only 2.8% of the 633 seropositive children had IgG of low avidity. The avidity assay can distinguish between a chronic or past infection (high avidity) and a recent infection (low avidity). Because kids are exposed to *Toxocara* early in life it is very unusual to have recent infection by the age when these children were examined (4–11 years old).

In this work and others from our group (Alcantara-Neves et al., 2011; Mendonça *et al.*, 2012), maternal education was used as an indicator of socioeconomic status of the family since it is highly associated in this population. It also reflects the hygiene habits of the family which will be rely on greater chance of infection. The results of the seroprevalence of IgG anti-*Toxocara* found in this study were similar to those observed in other low-income populations, where prevalence of infection in children of mothers with fewer years of education was higher. Alderete et al. (2003) diagnosed a prevalence of 38.8% in children with a mean age of 9.4 years and determined that *Toxocara* infection was inversely proportional to family income.

Contact with dogs has been shown in several studies as an important risk factor for toxocariasis. A cross-sectional study estimated a frequency of 52% seropositivity for *Toxocara spp.* in 34 families in the Amazonas state in Brazil. Individuals who had contact with adult dogs at home, 60% were positive ($chi^2 = 14.317$, $p = 0.026$), and who had contact with puppies at home, 66.6% were positive ($chi^2 = 22.149$, $p = 0.008$), demonstrating the association between contact with dogs and the presence of anti-*T.canis* IgG (Damian et al., 2007). In Argentina, Chiodo et al. (2006) evaluated 100 individuals for IgG anti-*Toxocara* and 23% were positive, and all had contact with dog at home. Our results confirm these findings and the presence of dogs at home is a risk factor for *Toxocara* infection in this study population.

Several epidemiological studies indicate contamination of soil as a determinant in infection by *Toxocara* spp. In the present study was noted that paved street increased the chance of infection, which makes us hypothesizes that we could have higher egg concentrations in soil with paving then you would if you had no paving since in urban areas we would have less area for water to soak into the soil (unpaved areas) and concentrate soil contamination during rainfall and flooding. Another possibility it may occurs is that maybe cats which were also a risk factor in this study, may be polluting the environment with *Toxocara* eggs since they cannot bury their

feces in paved streets leaving them exposed increasing the chance of infection. Few studies were conducted to estimate the infection of cats and their potential to cause VLM. Martínez-Barbabosa et al. (2003) determined a prevalence of 42.5% of *T. cati* infection in cats which makes one think that this parasite may be common and raises the importance of the development of a specie-specific ELISA for detection anti-*Toxocara* spp. IgG, useful for further studies about the epidemiology of VLM caused by both *Toxocara* species.

Some studies refer that soil contamination is not the only effective route in human toxocariasis and eggs of *Toxocara* spp. can be sprouted in fur and direct contact between humans and dogs and cats may be an alternative route of human infection (Wolfe and Wright, 2003). Aydenizöz-Ozkayhan et al. (2008) collected 51 fur samples and observed that 21.56% of the dogs had eggs in their fur. Roddie et al. (2008) examined 100 dogs for the presence of eggs in fur and found *Toxocara* spp. eggs in 67% of adult dogs and 95% of puppies. In the Netherlands, Overgaauw et al. (2009) found *Toxocara* eggs in 4.4% of dog fecal samples and in 12.2% of their fur samples. Moreover, many of the owners allowed their dogs to climb and sleep in their beds, and only 15% washed their hands after contact with your pet. Therefore, this close physical contact between pets and their owners possibly increase the risk of transmission of *Toxocara* spp. and point to the need for greater attention to the potential risk to which humans are exposed.

In conclusion this work shows that *Toxocara* infection is highly prevalent in the studied population and we postulate that it is closely related to social status and hygiene. The presence of the dog at home proved to be an important risk factor for this disease and is necessary to adopt sanitary measures more specific for resident dogs, since the control program of stray dogs is not the only way to control the disease. The association with presence of cats in house confirms previous work showing antigenic similarities between *T. canis* and *T. cati* ESLA and that anti-*T. cati* antibodies have influenced the outcome of the study. Although cats have a habit of burying their feces, paving the street may be influencing the increased exposure to cat feces as well as dogs feces since this variable was associated with increased risk of *Toxocara* infection. So, the importance of cat as a disseminator of this disease was suggested and deserves further attention in programs for this disease control.

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