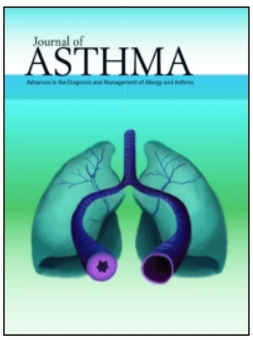

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ORIGINAL ARTICLE

Toxocariasis and childhood asthma: a case-control study

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ABSTRACT

Objective: The objective of this study is to investigate the association between anti-Toxocara IgG seropositivity and asthma in children. *Methods:* This was a case-control study conducted in a university hospital in south Brazil between May 2012 and June 2013. Were recruited 208 children up to 12 years old of whom 156 had asthma (cases) and 52 did not have asthma (controls), with a case-control ratio of 3:1 matched by age. Children's parents or guardians were interviewed using a structured questionnaire with closed questions. Serology was performed using enzyme-linked immunosorbent assay (ELISA) with excretory-secretory antigen of *Toxocara canis* (TES). *Results:* The seroprevalence of IgG anti-*T. canis* antibodies was 12.8% in the cases and 7.7% in the controls. There was no significant association between seropositivity to *T. canis* and risk of asthma (adjusted odds ratio [OR]: 1.89, 95% CI: 0.52 to 6.89, $p = 0.33$). Household income < 2 minimum salaries, paternal school years < 9, allergic rhinitis in children, a positive family history of asthma and rhinitis and contact with cats were significantly associated with asthma, with adjusted ORs (95% CIs) of 3.05 (1.21 to 7.73), 2.83 (1.11 to 7.18), 10.5 (4.32 to 25.6), 2.65 (1.14 to 6.17), 2.49 (1.07 to 5.78) and 2.73 (1.03 to 7.27), respectively. *Conclusions:* This study did not find a statistically significant association between seropositivity to *Toxocara* sp. and risk of asthma in children. Low family income, low paternal education level, concomitant allergic rhinitis, family history of asthma and allergic rhinitis and contact with cats were independent factors associated with childhood asthma.

Keywords

Anti-*Toxocara* IgG, asthma, children, *Toxocara canis*, seroprevalence

History

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Introduction

Human toxocariasis is a neglected parasitosis with a cosmopolitan distribution and a higher prevalence in developing countries with tropical climates [1]. However, the global prevalence and the impact on human health of this anthropozoonosis have generally been underestimated due to the limitations of diagnostic laboratory methods and the lack of standardised clinical diagnostic criteria [2]. The main causative agents of human toxocariasis are the nematodes *Toxocara canis* and *Toxocara cati,* which are intestinal parasites of dogs and cats, respectively [3,4].

Children are more susceptible to human toxocariasis due to immunological immaturity and repeated exposure to the risk of infection by close contact with infected dogs and cats [5], with sandboxes for recreation [6], with dog hairs [7,8] and with soil contaminated with the embryonated eggs of *Toxocara* sp. [9].

The detection of specific antibodies (IgG) is extremely relevant for the diagnosis of main clinical forms of human toxocariasis (visceral, ocular and covert) [2]. Enzyme-linked immunosorbent assay (ELISA), using the excretory-secretory antigen of *T. canis* (TES), is considered the standard diagnostic method in epidemiological studies [10]. In Brazil, serological studies using ELISA-TES with sera pre-absorbed with *Ascaris* sp. antigen have reported seroprevalence rates ranging from 4.2% to 51.6% in normal children [5,11–14].

Since the 1990s, human toxocariasis has been believed to be associated with the occurrence of asthma and hospitalization for asthma in children [15]. There are common characteristics for asthma and toxocariasis, such as airway inflammation, eosinophil accumulation and induction of the production of IgE [16]. A limited number of observational studies have been conducted to investigate the associations between human toxocariasis and childhood asthma. Some studies showed a statistically significant association between *Toxocara* sp. seropositivity and presence of asthma [17–19]. However, other studies failed to show such association [20–23].

One study performed in Mexico found false-positive serological results for *Toxocara* sp. due to cross-reaction with *Ascaris lumbricoides* among asthmatic children [24]. However, most of the previous studies investigating the association between asthma and human toxocariasis in children have not used the pre-adsorption step for sera [18,22].

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Given the conflicting results across previous studies and methodological limitation in identifying *Toxocara* sp, we performed this study to investigate the association between human toxocariasis and asthma in children, using ELISA-TES with pre-adsorption of sera with somatic antigens of *Ascaris suum* (SaAs).

Methods

Study design

We conducted a case–control study at the General Pediatric Outpatient clinic and Pediatric pulmonary clinic of a university teaching hospital in southern Brazil between May 2012 and June 2013. The research project was approved by the Research Ethics Committee of the Federal University of Rio Grande (FURG) (CEPAS 205/2011) and the informed consent forms were obtained from all parents or guardians.

Participants

The cases were children aged 6 months to 12 years old with diagnosis of asthma attending the Pediatric pulmonary clinic. The controls were children without asthma who attended the General Pediatric Outpatient clinic for routine health care. We have had difficulty enrolling the controls because parents were usually reluctant to allow blood sampling in their healthy children. Thus, we recruited only one control for three cases, matched by age groups: 6 months to <3 years, ≥3 years to <7 years, and ≥7 years to 12 years.

The diagnosis of asthma was performed by two senior pulmonologists, based on the criteria of Global Initiative for Asthma – GINA (update 2010) [25]. The diagnosis of asthma was made if all the following criteria were met: (1) recurrent episodes (≥3 episodes) of one or more of the following symptoms – wheeze, cough, breathing difficulties and chest tightness, particularly at night or in the early hours of the morning; (2) respiratory symptoms improve spontaneously or after treatment (bronchodilators associated or not with corticosteroids); (3) presence of triggers or aggravating factors such as exposure to allergens or irritants, physical exercise, weather changes or emotional stress; (4) personal history of atopy (allergic rhinitis or eczema) and/or family history of atopy (asthma, allergic rhinitis or eczema) in first-degree relatives.

We excluded from the study children with bronchopulmonary dysplasia, cystic fibrosis, congenital cardiopulmonary diseases, immunodeficiency and chronic encephalopathy.

Data collection and variables

Data collection was done by three trained investigators through interview with patient's parents or guardians using a standard pre-coded questionnaire. The questionnaire consisted of closed questions concerning demographic and socioeconomic data (gender, age, skin color, family income, household size and parental educational level), environmental factors and life habits (contact with dogs, contact with cats, and sanitary conditions of the residence) and child's medical history.

Blood and serum sampling

Blood collection and haemography were performed in the clinical laboratory of the university hospital (FURG), and the results of haemography were obtained from the medical records of each child. The serum of children was held at −20°C until analysis in the Laboratory of Parasitology of the School of Medicine (FURG).

Production of excretory/secretory products of *T. canis* larvae (TcESLA)

The production of TcESLA was performed in the Laboratory of Parasitology of the School of Medicine (FURG), using the methodology described by De Savigny (1975) and modified by Maizels et al. (1991) and Shoenardie et al. (2012) [5,26,27]. After a month of incubation of *T. canis* eggs in formalin 2%, at 28°C ventilation and relative air humidity greater than 90%, we incubated infective larvae using RPMI-1640 at 37°C and CO_2 at 5–8% (supplemented with HEPES 25 mM, glucose 1%, penicillin 100 IU/mL, streptomycin 100 mg/mL, ofloxacin 0,4 mg/mL and fungizone 50 mg/mL) for 3 months. During this period, the depleted supernatant from the larval culture was collected weekly, after adding the protease inhibitor phenyl methylsulfonylfluoride (PMSF) 1 mM and stocking at −20°C. After the time of incubation, the supernatant was thawed and concentrated by ultrafiltration at 4°C, using *Stirred cell* (Sigma Chemical Co., St. Louis, MO) and PTGC membrane (U-4255) (Sigma Chemical Co., St. Louis, MO), with an exclusion limit of 10 kDa. This material was dialysate in ultrapure water at 4°C, over 24 h and was concentrated by primary lyophilisation. The protein concentration was determined by the bicinchoninic acid method (BCA) [28]. We obtained two aliquots of TES antigen with protein concentrations of 0,318 mg/mL and 0,122 mg/mL.

Somatic antigens of *Ascaris suum*

For production of this antigen, we collected female *Ascaris suum* from the intestines of pigs in a slaughter house in the municipality of Pelotas, located in southern Brazil. The somatic antigen of *A. suu* (SaAs) was produced with the methodology used by Mendonça et al. [29]. The protein concentration was determined by BCA [28], yielding a final concentration of 6.348 mg/mL.

Detection of serum anti-*Toxocara* IgG antibodies

The serum samples were pre-adsorbed with SaAs, being submitted to intermittent agitation for one hour at 37°C [29,30]. Subsequently, 96-well plate wells were sensitized using TcESLA (2 μg/mL) in carbonate bicarbonate buffer (pH 9.6–9.8) and were incubated overnight at 4°C. Blocking was performed with casein at 5% in PBS-Tween-20 at 0.05% (PBS-T), pH 7.2, for 1 h inside of a moist chamber at 37°C. The pre-adsorbed sera with SaAs were tested in duplicate at a dilution of 1:50 in PBS-T and with the conjugate (anti-human IgG [Fc specific] peroxidase antibody produced in goat [Sigma Chemical Co., St. Louis, MO) at a dilution of 1:6.000 in PBS-T. Both the sera and conjugate were incubated for 45 min inside a moist chamber at 37°C. Between each phase, the plates were washed five times with PBS-T for 10 min. All the reagents were used in volumes of 100 μL. We used the orthophenylenediamine

chromogen (OPD) at a concentration of 0.4 mg/mL in citrate-phosphate buffer, pH 4.0, plus hydrogen peroxide at 0.1%. We performed readings (450 nm) 15 minutes after the addition of chromogen.

A cut-off (0.118) was established with the mean absorbance of negative control sera (children without symptoms of human toxocariasis, with eosinophils <3% and without contact with dogs) plus two standard deviations.

Statistical analysis

The data were entered into EPI-data software, version 3.1 (EpiData Association, Odense, Denmark), and the statistical analyses were performed using Stata software, version 11 (Stata Corp, College Station, TX). The descriptive analyses of the data consisted of the calculation of absolute and relative frequencies of categorical variables and means with 95% confidence intervals (95% CI) of continuous variables. Given the design used (case–control study matched by age), crude and adjusted Odds ratios (OR) and 95% CI were calculated by conditional logistic regression. To define the final model of multivariate analysis, we used the *stepwise backward* method, removing variables with p value > 0.05. The variable of IgG for *Toxocara* remained in the final model given that it was the main exposure factor. The p value <0.05 in two-tailed tests was defined as statistically significant.

Results

A total of 208 patients included in the study, of whom 156 were classified as the cases (children with asthma) and 52 as the controls (children without asthma). Table 1 shows general characteristics of 208 patients. Only low household income (<2 minimum salaries) was significantly associated with asthma, with OR (95% CI) of 2.79 (1.43–5.58).

The seroprevalence of *Toxocara* sp. was 12.8% (20/156) in asthmatic children and 7.7% (4/52) in children without asthma ($p = 0.32$) (Table 2). Bivariate analysis showed that personal history of allergic rhinitis, family history of asthma, family history of rhinitis and contact with cats were significantly associated with asthma, with OR (95% CI) of 8.94 (4.10–19.51), 3.94 (1.58–5.84), 2.68 (1.39–5.15), and 2.19 (1.05–4.55), respectively. No significant association was found between seropositivity to *Toxocara* sp. and asthma (OR: 1.76, 95% CI: 0.59–5.93).

The multivariate analysis showed that low household income, low parental schooling (<9 yr), personal history of allergic rhinitis, family history of asthma, family history of rhinitis, and contact with cats were independent factors associated with asthma (Table 3). Again, no significant association was found between seropositivity to *Toxocara* sp. and asthma (OR: 1.89, 95% CI: 0.56–6.89).

Discussion

One recently published meta-analysis with 10 observational studies involving a total of 1530 participants (723 cases of asthma and 807 controls) showed a significant association between Toxocara seropositivity and asthma (OR: 3.36, 95% CI: 1.76–6.42) [31]. The association remained significant in subgroup analysis of seven studies in children with a total of 1161 participants (508 cases and 653 controls) (OR: 2.80, 95% CI: 1.35–5.82). However, there was high heterogeneity in the results across seven studies in children with OR varying from 0.89 to 13.7 (I-squared = 81.7%). Toxocara seropositivity varied from 14.7% to 61.3% among case groups and from 2.2% to 52.8% among control groups. Of seven studies, four did not show a statistically significant association between Toxocara seropositivity and asthma [20–23]. The overall results of this meta-analysis may not significantly change even if our study is included in the analysis; however, one more study with "negative results" reinforces the existence of high heterogeneity in the results across studies which investigate association between toxocariasis and childhood asthma. The heterogeneity between studies could be due to differences in study design, sample size,

Table 1. General characteristics of the study sample ($n = 208$).

	Children with asthma ($N = 156$)		Children without asthma ($N = 52$)				
Variables	*n*	%	*n*	%	OR	95% CI	*p*
Gender							
Male	89	57.1	24	46.2	1.54	0.82–2.88	0.18
Female	67	42.9	28	53.8	1		
Skin colour							
White	95	60.9	28	53.8	1.34	0.71–2.52	0.37
Not white	61	39.1	24	46.2	1		
Age, months (mean, 95% CI)	60.8 (54.9–66.8)	61.4 (49.8–72.9)	0.99	0.99–1.0	0.93		
Maternal schooling							
<9 years	92	58.9	26	50.0	0.96	0.87–1.08	0.26
≥9 years	64	41.1	26	50.0	1		
Paternal schooling							
<9 years	95	60.9	35	67.3	0.76	0.39–1.47	0.41
≥9 years	61	39.1	17	32.7	1		
Household income							
<2 minimum salaries	121	77.6	29	55.8	2.79	1.43–5.48	0.003
≥2 minimum salaries	35	22.4	23	44.2	1		
Agglomeration							
<1 room/person	90	57.7	26	50.0	1.38	0.73–2.62	0.33
≥1 room/person	66	42.3	26	50.0	1		

Table 2. Serology for *Toxocara* sp. and other factors associated with asthma in children: bivariate analysis ($n = 208$).

	Children with asthma ($N = 156$)		Children without asthma ($N = 52$)				
Variables	*n*	%	*n*	%	OR	95% CI	*p*
Toxocara (IgG)							
Positive	20	12.8	4	7.7	1.76	0.57–5.39	0.32
Negative	136	87.2	48	92.3	1		
Eosinophils in blood							
> 10%	23	14.7	3	5.8	2.79	0.81–9.67	0.09
≤ 10%	133	85.3	49	94.2	1		
Allergic rhinitis							
Yes	104	67.1	10	19.2	8.94	4.10–19.51	0.000
No	51	32.9	42	80.8	1		
Family history of asthma							
Yes	100	65.4	20	38.5	3.04	1.58–5.84	0.001
No	53	34.6	32	61.5	1		
Family history of rhinitis							
Yes	93	61.6	19	37.3	2.68	1.39–5.15	0.003
No	58	38.4	32	62.7	1		
Contact with cats							
Yes	61	39.1	12	23.1	2.19	1.05–4.55	0.036
No	95	60.9	40	76.9	1		
Contact with dogs							
Yes	102	65.4	32	61.5	1.19	0.61–2.31	0.616
No	54	34.6	20	38.5	1		

age range of participants, geographic and environmental conditions of study locations, definition of asthma, and diagnostic accuracy (sensitivity and specificity) of serologic tests for Toxocara.

This study showed that children with low socio-economic status (SES) characterized by low family income and low paternal education level were more likely to have asthma. Previous studies produced conflicting results regarding association between SES and childhood asthma. Some studies are consistent with our findings showing an inverse association (i.e. increased likelihood of having asthma with decreased SES) [32–34] whereas others showed a positive association or no association at all [35–37]. This confusing picture may be due to different definitions of asthma and different classification of SES used by studies. The pathway linking SES and childhood asthma still remains unclear.

Table 3. Serology for *Toxocara* sp. and other factors associated with asthma in children: adjusted analysis ($n = 208$).

Variables	Adjusted OR	95% CI	*p*
Toxocara (IgG)			
Positive	1.89	0.52–6.89	0.34
Negative	1		
Household income			
<2 minimum salaries	3.05	1.21–7.73	0.01
≥2 minimum salaries	1		
Paternal schooling			
<9 years	2.83	1.11–7.18	0.02
≥9 years	1		
Allergic rhinitis			
Yes	10.52	4.32–25.64	<0.001
No	1		
Family history of asthma			
Yes	2.65	1.14–6.17	0.02
No	1		
Family history of rhinitis			
Yes	2.49	1.07–5.78	0.03
No	1		
Contact with cats			
Yes	2.73	1.03–7.27	0.04
No	1		

This study confirmed the association between allergic rhinitis and childhood asthma as shown by numerous previous studies. Several prospective cohort studies have showed that presence of allergic rhinitis in infancy is a predictive factor for subsequent onset of asthma [38–41]. However, it remains unclear whether allergic rhinitis is an earlier clinical manifestation of allergic disease in atopic children who eventually develop asthma or if the nasal disease itself is causative for asthma.

This study showed that presence of asthma and allergic rhinitis in first-degree relatives was an independent factor associated with childhood asthma. Familial aggregation of asthma and atopic diseases has been well known, suggesting that genetic factors may play an important role in asthma etiology [42–44].

Previous epidemiologic studies showed inconsistent results regarding association between contact with domestic pets and childhood asthma. Some studies have found that early contact with dogs and cats may protect a child against allergic sensitization and development of asthma [45–47], but others suggest such exposure may increase the risk of allergic diseases [48–50]. This study showed that contact with cats rather than dogs was significantly associated with childhood asthma. The time when a child is exposed to pets, the quantity of exposure and intrinsic properties of pet allergens may determine different roles of contact with pets on the development of childhood asthma and other allergic diseases.

This study has at least two strengths. Firstly, we used somatic antigen of *A. suum* for pre-adsorption of sera that may increase the specificity of serological testing for toxocariasis, especially in developing countries with tropical climates where ascaridiasis is endemic [30]. Secondly, asthma was diagnosed by physicians based on the criteria of international guidelines.

However, the diagnosis of asthma can be difficult in children, especially in those under six years of age [51]. The other limitation of this study is a relatively small sample size which may have no enough power to find the association between Toxocara seropositivity and childhood asthma.

In conclusion, this study did not find a statistically significant association between seropositivity to *Toxocara* sp. and risk of asthma in children. Low family income, low paternal education level, concomitant allergic rhinitis, family history of asthma and allergic rhinitis, and contact with cats were independent factors associated with childhood asthma.

Declaration of interest

The authors report that they have no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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