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Title: **Study of *Toxocara* seropevalence among patients with allergy and healthy individuals in Bulgaria**

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Abstract

Data in the literature addressing the ability of *Toxocara* infection in humans to induce development of atopic disease are controversial.

Aim of our study was to determine the seroprevalence of anti-*Toxocara* antibodies in three groups of people: subjects with allergic symptoms and presence of allergen-specific IgE, subjects with allergic symptoms and absence of allergen-specific IgE, and clinically healthy blood donors. Serum samples from all subjects were tested by ELISA and Western blot for presence of specific antibodies against *Toxocara canis*. The results of our study did not support the link between toxocariasis and allergic manifestations in atopic patients. Among subjects with allergic symptoms and absence of atopy was found seroprevalence of 2.2% in Western blot. Same index in patients with atopy was 0.8%, and in clinically healthy blood donors 4.0%.

Our study gives us grounds to consider that it is appropriate persons with allergic reactions, without evidence of atopy to be tested for presence of anti – *Toxocara* antibodies in the course of their diagnostic evaluation. Data from clinically healthy persons suggest that there is a "hidden" infection among the population, which is not clinically manifested.

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Keywords: Toxocariasis, allergy, ELISA, Western blot, seroprevalence

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Introduction

Human toxocariasis is a parasitic zoonotic disease with prolonged relapsing course and polymorphic clinical manifestations. Causative agents are larvae of dogs' and cats' nematodes *Toxocara canis* and *Toxocara cati*. For first time toxocariasis (Larva migrans visceralis) was described by Beaver[1] in 1952 who defined hepatomegaly, pneumonitis and eosinophilia as main clinical signs of the disease. Currently toxocariasis is one of the most commonly reported zoonotic diseases with worldwide distribution[2]. Most often the diagnosis is conducted with serological tests and determination of specific IgG antibodies. ELISA and Western blot (WB) are used predominantly for routine diagnosis of *Toxocara* infection. The spread of toxocariasis among humans depends on several factors: place of residence (urban or rural), age, socio-economic status and professional exposure[3]. Study in Austria recorded seroprevalence rate among farmers of 44%, 27% among veterinarian workers, 25% in employees of slaughterhouses and 17 % among hunters[4]. In Ireland seroprevalence rate of up to 16.6% was found among school age children living in rural areas[5], and in USA the recorded rate reached 25% among African Americans living in rural areas[3]. In regard to the socio-economic status a study in Spain found seroprevalence rate of 37% among poor children living in the province of Guipuzkoa[6]. From the countries with tropical climate higher seroprevalence rate was found in the Marshall Islands (86.75%)[7].

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In Bulgaria toxocariasis is not notifiable disease and for this reason we do not have available data for the exact prevalence of the infection in the country, but from 2004 to 2013 in the Department of Parasitology and Tropical Medicine at the National Centre of Infectious and Parasitic Diseases (NCIPD) were studied 300 serum samples from patients suspected of toxocariasis with symptoms of allergy, collected from different parts of the country. Seroprevalence of 8% was found among them. Study of healthy blood donors (n=350) revealed seroprevalence of 8.6% (n = 30) in ELISA and 3.4% (n = 12) in WB[8].

The immune response of the host to the migrating *Toxocara* larvae is directed against larval excretory-secretory antigens, which consist of glycoproteins with a potent allergenic component[9,10]. According to some epidemiological and experimental studies *Toxocara* infection assists in the development of various allergic reactions, including asthma and this relationship was described by Desowitz et al. in 1981 and later by some other authors[11-13]. Study performed in Malaysia reported data for significantly higher level of anti - *Toxocara* antibodies in children with bronchial asthma than in children without asthma[14]. In a meta-analysis of ten studies published in 2014, the authors found higher prevalence of *Toxocara canis* infection in patients with asthma than in controls. Similar results were found when meta-analysis was restricted to the studies considering only children or adults[15]. Other authors do not confirm this connection and their research found no association between positive tests for toxocariasis and history of asthma or other allergic manifestations[16,17].

According to data from the Ministry of Health in Bulgaria 15-18% of the population suffers from allergic rhinitis and 9-10% from asthma[18].

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Aim of present work was to find out is there a link between toxocariasis and allergic symptoms in patients with or without proven atopy and also to establish the prevalence of asymptomatic toxocariasis among the healthy population in the country.

Patients and methods

Study sample

The study encompasses three groups of subjects: group #1 (n = 125), people with IgE-mediated allergy; group #2 (n = 136), people with symptoms, but without evidence of increased allergen-specific IgE and control group #3 (n =50) of clinically healthy blood donors whose blood samples were obtained from the Centre of Transfusion Hematology in the city of Rousse. Subjects included in groups #1 and #2 were directed from different medical professionals to the Department of Immunology and Allergy at NCIPD for consultation and diagnostic evaluation. In regard to age, we categorized all subjects in four classes: between 0 and 19 years old; between 20 – 39 years old; between 40 – 59 years old and between 60 – 80 years old.

Serum samples

Serum samples from 261 persons with allergic symptoms were tested with a system ImmunoCAP (Phadia AB) for allergen specific IgE. Depending on the results the subjects were divided into two groups: group # 1 - subjects with clinical symptoms of IgE-mediated allergy (n = 125) and group #2 - subjects with symptoms, but without evidence of increased allergen-specific IgE (n = 136). From the persons of group #1 in 57 IgE reacted against grass pollen (gx1); in 18 against house dust (h2); in 6 against mold mix (mx1); in 33 against *Dermatophagoides pteronyssinus* (d1); in 7 against food (fx∗) and in 4 against bee venom (i1).

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Parasitology

Sera of all subjects included in the study were tested for presence of specific IgG anti-*Toxocara* antibodies with commercial kit ELISA (R - Biopharm, Germany), according to the manufacturer's instructions. Cut-off of the reaction was calculated from the extinction value of the negative control plus 0.150. Sample ratio (SR) was obtained by dividing the extinction value of the sample by the cut off value. Samples with SR < 0.9 were considered negative, with values between 0.9 and 1.1, borderline, and with SR > 1.1 were rated positive for presence of anti-*Toxocara* antibodies. For verification all serum samples with positive or borderline ELISA were tested additionally by Western blot (LD BIO, France), according to the manufacturer's instructions. The appearance of two or more low molecular weight bands with size of 24-35 kDa was confirmative for presence of specific anti-*Toxocara* IgG in the subject's sample.

Data analysis

Data were processed with statistical program GraphPad Prism 6 (GraphPad Software, Inc.). We performed comparison 1x1 of age distribution between the 3 groups using Mann-Whitney non-parametric test and Kruskal-Wallis test for qualitative variables (class of age). Concerning sex, we compared the sex-ratio between groups using chi-square test in 2x2 tables and Dunn's multiple comparisons test.

Ethical considerations

For this study were not used any personal data and approval by a local Human Investigations Committee and obtaining informed consent from the subjects were not necessary.

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Results

Toxocara seropevalence

The subjects of group #1 (n = 125) were with clinical symptoms of atopy, presence of specific IgE to at least one of the allergens included in the test panel. The group consisted of 67 males and 58 females. Depending on the age these patients were also divided into 4 classes: from 0 to 19 years old (n=40), from 20 to 39 (n=56), from 40 to 59 (n=23) and between 60 and 80 years old (n=6). The group was tested by ELISA for presence of anti-*Toxocara* antibodies. Positive results were not found, but borderline values were obtained in four of the subjects. One of the subjects with borderline result in ELISA (19 years old male) was confirmed positive by Western blot. The seroprevalence in Western blot among this group was 0.8%.

The group #2 of subjects without specific IgE to a different allergens included 136 individuals of whom 50 were men and 86 were women. Distributed by age, 37 of them were between 0 and 19 years old; 45 – from 20 to 39; 40 - between 40-59 years old and 14 were between 60 and 80 years old. Positive in ELISA were seven subjects of this group. All positive in ELISA samples were tested by Western blot and three of them confirmed positive. They were from a female person (57 years old) and from two male persons (70 years old and 59 years old). Established by Western blot the seroprevalence among subjects without atopic allergy was 2.2%.

The examination by ELISA for presence of anti-*Toxocara* antibodies of serum samples from 50 clinically healthy subjects (32 males and 18 females) showed 3 positive and 1 borderline results, from which two were positive by confirmatory WB. Thus the seroprevalence for toxocariasis among clinically healthy individuals was 4%.

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Statistical analysis

To exclude possible influence of factors such as age we did one by one comparison of age distribution between the three groups using the non-parametric Mann - Whitney test. Such influence was excluded. Because we divided the subjects of the three groups into four age classes we used also Kruscal - Wallis test for qualitative variables and it also showed no statistically significant difference and rejected the influence of age on the results (Table 1). To estimate the influence of gender on the results we compared the sex ratio between groups using Chi - Squared test (Table 2) and Dunn's multiple comparison test (Table 3) and statistically significant difference was not found.

Disscusion

Studies discussing the possible role of *Toxocara* for inducing allergy symptoms were conducted mostly among children from different countries arround the wourld. After Desowitz et al. in 1981, positive association was established by Hakim et al. (1997) and Chan et al. (2001) in Malaysia, Buijs et al., (1997) in Netherlands.[11,14,19,20] The described groups of children by these authors were aged between 1 to 12 years and after the epidemiological inquiry a relation between *Toxacara* infection and allergy in children with atopic predisposition (bronchial asthma/recurrent bronchitis) was found. Other authors do not confirm the relation in chidren between seropositivity for toxocarosis and allergic conditions.[16,17]

Studies on the incidence of toxocariasis among adult patients (18-65 years) with allergic manifestations were also with contradictory results, although most researchers have found positive relationship between *Toxocara* seropositivity and allergic sensitization.[21,22]

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Our survey included children and adults and was performed in two stages. Initially, all sera from the surveyed groups of individuals were tested by ELISA. Due to the possibility of cross reactions with other helminth infections all borderline and positive in ELISA sera were tested by Western blot for confirmation or rejection of the results obtained by ELISA.[23-25] The discrepancy of the results obtained by ELISA and Western blot is may be due to cross reactivity with other helminthiases. Our study was limited to processing of serum samples from patients selected because of certain medical conditions. We were not in direct contact with any of the subjects, and we were not able to examine them for presence of other helminth infections.

Although the statistical processing of data did not confirm statistically significant difference among the subjects of the three groups, highest seroprevalence in Western blot was found among the persons of group #3, followed by group #2 and group #1. Our study did not found significant relation between age and sex of the surveyed persons and the presence of anti-*Toxocara* antibodies.

Unlike some publications[15] according to which there is a significant connection between atopy (especially asthma) and toxocariasis, our results do not allow us to confirm that such connection exists. In contrast, we consider that individuals with allergy but without evidence of specific IgE mediated allergy should be tested for toxocariasis in the course of their diagnostic evaluation. The present and our previous studies showed seroprevalence of about 4% among clinically healthy people which is sign for presence of hidden asymptomatic infection among the population.

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Founding: None

Conflicts of interest: None

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Table 1 Distribution by groups and class of age

Table 2 Distribution by gender

Table 3 Multiple comparisons test of gender distribution

Table 1 Distribution by groups and class of age

Class of age	**Group #1**	**Group #2**	**Group #3**
0 – 19 years old	40	37	0
20 – 39 years old	56	45	21
40 – 59 years old	23	40	29
60 – 80 years old	6	14	0
Total	125	136	50
Mann - Whitney test	Group #1 vs. Group #2	Group #2 vs. Group #3	Group #3 vs. Group #1
P value	0.9714	0.1143	0.1714
Significantly different? (P < 0.05)	No	No	No
Mann-Whitney U	7.5	2	3
Kruskal-Wallis test for class of age distribution	P value = 0.1563		

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Table 2 Distribution by gender

Gender	**Group #1**			**Group #2**			**Group #3**		
	WB +	WB -	Total	WB +	WB -	Total	WB +	WB -	Total
male	1	66	67	1	49	50	2	30	32
female	0	58	58	2	84	86	0	18	18
Total	1	124	125	3	133	136	2	48	50
Chi-square, df	0.8727, 1			0.01554, 1			1.172, 1		
z	0.9342			0.1246			1.083		
P value	0.3502			0.9008			0.279		
Statistically significant? (alpha<0.05)	No			No			No		
Odds ratio	2.639			0.8571			3.033		
95% confidence interval	0.1054 to 66.09			0.07570 to 9.705			0.1378 to 66.76		

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Table 3 Multiple comparisons test of gender distribution

Dunn's multiple comparisons test			Rank sum diff.	Significant?	Summary
males group #1	vs.	females group #1	-8	No	ns
males group #1	vs.	males group #2	-1.5	No	ns
males group #1	vs.	females group #2	-8	No	ns
males group #1	vs.	males group #3	-1.5	No	ns
males group #1	vs.	females group #3	-5	No	ns
females group #1	vs.	males group #2	6.5	No	ns
females group #1	vs.	females group #2	0	No	ns
females group #1	vs.	males group #3	6.5	No	ns
females group #1	vs.	females group #3	3	No	ns
males group #2	vs.	females group #2	-6.5	No	ns
males group #2	vs.	males group #3	0	No	ns
males group #2	vs.	females group #3	-3.5	No	ns
females group #2	vs.	males group #3	6.5	No	ns
females group #2	vs.	females group #3	3	No	ns
males group #3	vs.	females group #3	-3,5	No	ns

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