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ANTI-TOXOCARA ANTIBODIES IN PATIENTS WITH SUSPECTED VISCERAL LARVA MIGRANS AND EVALUATION OF ENVIRONMENTAL RISK OF HUMAN INFECTION IN BELGRADE, SERBIA

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Abstract – Toxocariasis is a frequent zoonotic parasitosis in Serbia. The aim of the study was to examine anti-*Toxocara* IgG antibodies in serum and cerebrospinal fluid (CSF) in patients with suspected visceral larva migrans (VLM) in Belgrade, and to estimate the prevalence of *T. canis* eggs in dog fecal deposits collected in green public areas. A total of 145 patients were examined by ELISA. In 26.39% (38/144) of serum samples, *T. canis* IgG were detected, and in 6 CSF samples, 2 were positive for antibodies. Seropositivity was detected in 26.1% (34/130) adults and 33.3% (5/15) children. A total number of 155 fresh dog feces were collected in five public parks in Belgrade where *T. canis* eggs were identified with a prevalence of 33.55% (52/155). Based on the high prevalence of parasite eggs, we estimated a high risk of human infection by *T. canis* eggs in public green areas in Belgrade.

Key words: Visceral larva migrans, anti-*Toxocara* antibodies, *Toxocara canis*, dogs, eggs, feces; Belgrade

INTRODUCTION

Visceral larva migrans (VLM) is a clinical form of human toxocariasis caused by larvae of the nematode *Toxocara canis*. Other forms of human toxocariasis are covert toxocariasis, ocular larva migrans (OLM) and neural larva migrans. Covert toxocariasis is the most common form of human toxocariasis (Despommier, 2003). The eggs of *T. canis* can be found in environments contaminated with dog feces. Human infection can occur by ingesting viable embryonated eggs from contaminated sources (e.g., soil). Humans are paratenic hosts and after ingestion, the eggs hatch to release larvae that penetrate the small intestine, enter the circulation with the possibility of invading different organs and encyst in tissues. Wandering of the second-stage larvae through the tissues produces hemorrhage, necrosis and infiltration of lymphocytes and eosinophils (Despommier, 2003). Granulomatous foci are produced around dead larvae.

Diagnosis of human toxocariasis relies on clinical, laboratory (hypereosinophilia) and epidemiological data, including immunological findings. Immunoassays, such as ELISA (enzyme-linked immunosorbent assay), as well as Western blot analysis, that have been used to detect the presence of specific anti-*Toxocara* IgG antibodies in serum samples, have acceptable sensitivity and specificity (Watthanakulpanich, 2010; Kranjčić-Zec et al., 2003). Studies in Europe have shown prevalence rates from 2 to 5% in apparently healthy adults in urban areas, while

in rural areas the seroprevalence ranged from 14 to 37% (Magnaval et al., 2001). Won et al. (2008) determined a seroprevalence of *T. canis* infection of 13.9% at the national level in the United States by detection of specific IgG antibodies to *T. canis* in human sera. The sensitivity and specificity of ELISA in serum samples from patients with OLM is much lower than in patients with VLM and covert toxocariasis (Despommier, 2003).

Evaluation of the prevalence of *T. canis* eggs in canine feces contaminating urban green public areas may be useful in replacing or supplementing the studies that estimate the risk of environmental contamination (Maśnik, 2000; Azian et al., 2008). Examination of dog fecal samples is more convenient than examination of soil samples. Environmental contamination with *T. canis* eggs in a given area may be associated with the occurrence of human toxocariasis (Gawor et al., 2008).

The aim of this study was to determine the occurrence of anti-*Toxocara* IgG antibodies in the serum and cerebrospinal fluid (CSF) samples of patients with suspected VLM in Belgrade, Republic of Serbia. Our aim was to determine and analyze the presence of *T. canis* eggs in dog fecal samples collected in five public parks in Belgrade, and to estimate the environmental risk of human infection.

MATERIALS AND METHODS

Patients

The study covered the period from December 2011 to December 2012 in which 145 people with suspected VLM aged 6 to 53 years were included on the basis of their medical history and clinical findings. In the investigated patients, other intestinal helminths (*Strongyloides, Ascaris, Tichuris*) and tissue parasites (*Trichinella, Cysticercus, Echinococcus*) were excluded. The most common clinical features in the patients were abdominal discomfort, nausea, hepatomegaly, cough, sleep disturbances, myalgia, recurrent urticaria and fever. Epidemiological data collected from patients suspected of VLM stated that they were Belgrade citizens.

Serological analysis

To determine the presence of specific anti-*Toxocara* IgG antibodies in the serum and CSF samples of the patients, a commercial ELISA Toxocara IgG (NovaTec Immundiagnostica GmbH, Germany) was chosen for its sensitivity and specificity higher than 95% (Schantz, 1989). The presence of specific IgG antibodies was investigated in 144 serum samples and in 6 CSF samples. In three patients, serum and CSF samples were analyzed, while in three patients the tests were carried out only in CSF. In 139 patients, analyses were performed in one serum sample, except in 2 patients, where tests were repeated because of borderline test values. Samples were obtained according to the standard protocol and stored at +4°C no longer than 7 days before the examination. The serum samples were diluted (1:100) while CSF specimens were not diluted. Values higher than 1.1 were interpreted as positive, those less than 0.9 as negative, whereas values between 0.9 and 1.1 were considered borderline. In the cases of borderline test values, according to the manufacturer's instructions, the analyses were repeated after 2-4 weeks in new serum samples taken from the same patients.

Questionnaire

Patients with suspected VLM filled out a questionnaire regarding contact with dogs. Patients were divided into two groups: children (under 18 years) and adults (18 and over).

Coprological study

Study area and collection of samples

Belgrade is the capital of the Republic of Serbia (44°49'14" N, 20°27'44" E). It is located 116.75 m above sea level and lies in the humid continental climate zone with an average temperature of 11.7°C. During October and November 2012, 155 fecal samples from different dogs were collected in five public parks (Zemun Park, Tašmajdan, Pionirski Park,

Čuburski Park, Karađorđev Park) in Belgrade. All samples of canine origin were judged by their size, aspect and deposition place (on the surface and not buried). The public places from which the fecal samples were taken are not fenced and dogs had twenty-four hour access to them. Fresh fecal samples were taken at random from whole park surfaces. The deposits were collected in plastic bags, 100-150 g per sample, and stored at +4° C for a maximum of 5 days until examination.

Fecal flotation technique

Coprological samples were examined by routine flotation technique. Between 5-10 g of fecal sample were mixed thoroughly with a saturated NaCl solution in polyvinyl chloride containers, yielding a homogenous suspension that was filtered through a 60-mesh sieve. Flotation was performed with test tubes filled to the top with the fecal suspension. A cover glass was placed on top for 15 min, then removed, placed on a microscopic slide and examined under a light microscope (Carl Zeiss Jena GmbH, Germany) at 10× and 40× magnification. During microscopy, focus was directed at the identification of *T. canis* eggs, while the presence of other intestinal parasitic elements was considered secondary. *T. canis* eggs and other intestinal parasites were identified by morphological characteristics. Fecal samples in which at least one egg of *T. canis* was found were considered positive.

Statistical analysis

Fisher's exact test was used to test the difference between seropositive patients (adults and children) who made a positive or negative response to contact with dogs. Statistical significance was defined as $p<0.05$. Data analysis was performed using Graph Pad Prism 6.0.

RESULTS

In the investigated group of patients, there were 130 adults and 15 children with 77 (53.1%) males and 68 (46.9%) females with an average age of 23.5 years, ranging from 6 to 53 years. Out of 144 serum samples, 38 (26.39%) samples were positive for anti-*Toxocara* IgG antibodies, while 95 (65.97%) were negative. In 11 (7.64%) serum samples borderline test values were determined. In two patients the analysis was repeated, one out of which was found positive, and the other negative. Positive and negative results of ELISA tests that were repeated were included in the total of positive and negative results of serum samples tested. Positive values of the test ranged from 1.13 to 7.20. In most of the serum samples (23/144), the values were in the range from 1.13 to 2.10, while in 15 samples they ranged from 2.18 to 7.20. A positive test result was observed in two samples of CSF (Table 1).

Seropositivity was detected in 26.1% (34/130) of adults, while in the children's group anti-*Toxocara* antibodies was detected in 33.3% (5/15). Information about contact with dogs obtained from the questionnaire filled out by the patients clearly indicates that the majority of seropositive patients denied contact with dogs (Table 2).

Out of the total number of positive fecal samples for helminth eggs in Zemun Park, *T. canis* eggs were found in 85.71% (36/42) of samples, 73.33% (11/15) in Tašmajdan, 0% in Pionirski Park, 33.33% (2/6) in Čuburski Park and 27.27% (3/11) in Karađorđev Park. The results of the examination of fecal samples of dogs for the presence of *T. canis* eggs collected in five public parks in Belgrade are shown in Table 3.

DISCUSSION

VLM is an important multidisciplinary medical problem that requires cooperation between medical and veterinary professionals to be successfully treated and prevented. Contamination of public parks with eggs of *T. canis* originating from the feces of infected dogs is an issue of public importance and correlates with human toxocariasis. Our study covered *Toxocara* infection in people and dogs, aiming to increase the understanding of the epidemiology of VLM in Belgrade. Laboratory tests, including detection of anti-*Toxocara* IgG antibodies and eosinophil count

Table 1. ELISA seropositivity of *Toxocara* antibodies in samples of patients with suspected VLM.

Results	Serum		CSF		Total
	No. of samples (%)	Test value	No. of samples (%)	Test value	
Positive	38 (26.39 %)	1.13-7.20	2 (33.33 %)	1.33-2.40	40 (26.67 %)
Borderline	11 (7.64 %)	0.9-1.1	0 (0 %)	-	11 (7.33 %)
Negative	95 (65.97 %)	<0,9	4 (66.67 %)	<0,9	99 (66 %)
Total	144 (100 %)	-	6 (100 %)	-	150 (100 %)

Table 2. Number of seropositive patients reported contact with dogs in the questionnaire survey.

Patients	Contact with dogs No (%)	No contact with dogs No (%)	Total No (%)
Children	3 (7.7)	2 (5.1)	5 (12.8)
Adults	5 (12.8)	29 (74.7)	34 (87.5)
Total	8 (20.5)	31 (79.5)	39

p=0.0491

Table 3. *T. canis* eggs in dog fecal samples collected in five public parks in Belgrade.

Park	Positive findings of helminth eggs		Negative findings of helminth eggs No (%)	Total
	Toxocara canis No (%)	Other helminths No (%)		
Zemun Park	36 (60)	6 (10)	18 (30)	60
Tašmajdan	11 (33.3)	4 (12.1)	18 (54.5)	33
Pionirski Park	0	9 (41)	13 (59)	22
Čuburski Park	2 (10)	4 (20)	14 (70)	20
Karađorđev Park	3 (15)	8 (40)	9 (45)	20
Total	52 (33.5)	31 (20)	72 (46.5)	155

in patients' blood help to diagnose this zoonotic infection.

Our study showed a seroprevalence of 26.7%. Hakim et al. (1993) in Malaysia during 1989-1991, showed 19.6% patient seropositivity. The seroprevalence of people in different areas of the Czech Republic ranged from 5.8% to 36.0% (Uhlikova, 1998). In Brazil, the regional seroprevalence in people was in the range of 13.7 to 26.8% (Rubinsky-Elefant et al., 2008; Prestes-Carneiro et al., 2009). The observed seroprevalence in people at the national level was 1.6% in Japan, while a study in Denmark showed a seroprevalence of 2.4% (Akao, 2007; Stensvold et al., 2009). Zarkovic et al. (2007) showed seropositivity of 0.7% in New Zealand. Serologic tests for *Toxocara* infection should be interpreted with caution because commercial the ELISA kits that use excretory-secretory antigens exhibit diverse sensitivity and specificity (Turrientes et al., 2011; Fillaux et al., 2013).

Veterinarians have an important role in preventing the spread of human toxocariasis (Despommier, 2003). *T. canis* is widespread in canine populations around the world, and the prevalence of infection ranges from 3.06% to 82.6% depending on the age of dogs, and our study indicated a relatively high percentage (33.55%) of infected dogs (O'Lorcain, 1994; Awoke et al., 2011).

The main source of human infection is soil contaminated with embryonated *T. canis* eggs (Magnaval et al., 2001). Public areas show a high contamination of *T. canis* eggs in a range of 1%-78% of soil samples around the world (Matsuo, 2005; Dubná et al., 2007; Tavassoli et al., 2008; Kłapeć, 2009). In Latin American countries that have a high density of dogs, contamination of public areas was observed to be high in Mexico City (60%), Peru (70.6%), Brazil (68%), and Venezuela (60%) (Cazorla et al., 2007; Romero et al., 2009). Other sources of infection may be dog and cat hair, contaminated food, dirty hands, where the eggs of this parasite may be found. *T. canis* eggs are very resistant in the environment and survive temperatures of -25° to +40° C, but they do not embryonate below 12°C. It takes 10-14 days for the eggs to become viable and infectious to humans. Azian et al. (2008) showed that *T. canis* eggs were much more resistant in the environment than the eggs of other parasites. Climate conditions in Belgrade favor the development of infective eggs, as the average temperature is higher than 17.5°C seven months of the year.

There are a few studies related to the presence of this parasite in dogs and contamination of public green places in Belgrade. Kulišić et al. (1998) investigated infection with intestinal parasites through coprological and pathoanatomical examination of dogs. The study found that *T. canis* stands out, with a high percentage (33.27%) of infection and increased levels of contamination of public areas with *T. canis* eggs. Nikolic et al. (2007) showed *T. canis* infection in 30.5% of dogs in the Belgrade area. The prevalence of *T. canis* eggs of 33.55 % in our study clearly shows the sustainability of a high prevalence of *Toxocara* eggs and thus the contamination of public areas in Belgrade due to the large amount of stray dogs. Romero et al. (2010) through examination of dog feces showed the prevalence of *T. canis* infection of 34.22% in Mexico. In other studies, Soriano et al. (2010) in Argentina determined a prevalence of 6.35% and a survey in Italy reported the prevalence rate of 3.6% in dog feces from public places in Florence (Papani et al, 2012). Woodruff (1976) established the axiom that any pollution of the environment with more than 5% by *Toxocara* spp. eggs is a significant human health problem.

Statistical analysis of seropositive patients exposed to dogs compared to seropositive patients without contact to dogs showed a statistically significant difference ($p<0.05$). This emphasizes the importance of the negative response to contact with dogs for *T. canis* infection. Contact with dogs is not the main source of human infection caused by *T. canis* eggs. Green public places contaminated with dogs feces containing *T. canis* eggs are an important source of human infection. Children and adults are in daily contact with green public places and there is a high risk of contact with the parasite's eggs and developing toxocariasis (Hayashi et al., 2005; Doğan

et al., 2007). VLM is predominately seen in children (it may be associated with pica), but our study has shown that seropositivity in adults and children with suspected *Toxocara* infection was similar, 26.1% and 33.3% respectively.

Examination of dog feces contaminating urban areas can provide useful data on the risk of environmental occurrence of *T. canis* as a potential causative agent of human disease (Maśnik, 2000; Stensvold et al., 2009). The highest rates of fecal samples infected by eggs were found in Zemun Park (60%), probably because of the large number of stray dogs in that area. There are two possible reasons for the absence of contamination in Pionirski Park, which is downtown close to the Parliament and City Hall. The first is that dog owners take care of their pets (regular dehelminthization) and collect the feces after defecation, while the second is the low presence of stray dogs in this part of town. Some parts of the park are fenced for dog walking, however, fecal mass collected for the purpose of our study was taken from unfenced public spaces, where children and adults play and walk.

A large number of fecal samples collected in the unfenced parts of public parks showed that it is necessary to take educational measures. It is very important to advise citizens to walk their pets in parts of the park where they are intended to and to explain the importance of collecting fecal deposits. Supervising organs should be advised to increase their control of public parks and penalize dog owners who do not comply with the regulations. It is advisable to clean green public places from the fecal deposits of dogs in order to reduce contamination of parks in Belgrade and prevent the spread of infection caused by *T. canis*. Our results suggest that the contamination of public parks with *T. canis* eggs is an important source of human VLM in the urban environment of Belgrade area. In order to control human and animal toxocariasis in the territory of Belgrade, it is necessary to fulfill the following conditions: (i) education of dog owners by medical and veterinary professionals about the disease and preventive measures, (ii) veterinary supervision in order to reduce infections, and thereby contamination of public areas, and (iii) extensive seroepidemiological investigation in humans to obtain complete data about the prevalence of human toxocariasis.

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