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Study of IgG avidity and the level of specific IgA antibodies and their significance in the diagnosis of human toxocarosis

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ABSTRACT

Toxocarosis is a zoonotic disease caused by migration and subsequent localization of nematode larvae of *Toxocara* spp. in human organs and tissues, which is manifested with development of various non-specific clinical symptoms. Main diagnostic methods are serological and consists in proving the presence of anti-*Toxocara* IgG antibodies in patient's sera. In humans, anti-*Toxocara* IgG has been shown to persist in the serum for a long time and cannot be used to distinguish between past and recent infection.

Aim of the present work is to investigate the diagnostic significance of the specific IgG avidity level, determined by an immuno enzyme test developed by us, and the presence of anti-*Toxocara* IgA for distinguishing between acute and chronic toxocarosis.

The study included 130 patients with positive results in routine serological ELISA and Western blot tests and with clinical symptoms of visceral and ocular toxocarosis. The results revealed low IgG avidity (≤40%) in nine (7.3%) and presence of anti-*Toxocara* IgA antibodies in 36 (26.2%) of the subjects. Low avidity of IgG antibodies was found only in the first tests, and a presence of specific IgA for up to 9 months. The results of our study give us reason to believe that determination of the IgG avidity in toxocarosis is of greater diagnostic value than the presence of IgA to establish the stage of the disease.

1. Introduction

Toxocarosis is a parasitic disease caused by migration of *Toxocara* spp. larvae (*Toxocara canis* and *Toxocara cati*, canine and feline roundworms, respectively). So far, several clinical forms have been described - visceral ("Larva migrans visceralis") in 1952 by Beaver et al. ocular ("Larva migrans ocularis") reported by Wilder (1950) as nematode endophthalmitis, and identified in 1956 by Nichols (Rey, 1962), and latent (Covert Toxocarosis) in 1987 by Taylor et al. (1987). In recent years, the neurological manifestations of the disease have been isolated in a separate form - neurotoxocarosis (Pawlowski, 2001).

Because morphological detection of the parasite is most often impossible, the diagnosis of the disease is difficult. Currently, the main diagnostic methods are serological and are used to detect specific IgG antibodies, most often by immunosorbent assay (ELISA) and Western blot as a confirmatory test (De Savigny et al., 1979; Jacquier et al., 1991; Magnaval et al., 1991). Human infection is characterized by long-term persistence of specific IgG antibodies (Ree et al., 1984), which does not allow determination of the disease stage and the efficacy of anthelmintic therapy (Arpino et al., 1988). In addition, cross-reactions with antigens of other parasites, usually nematodes, have been observed in ELISA (Lynch et al., 1988). Therefore, in recent years, active work has been done to identify new markers that make it possible to determine the age of the infection.

The avidity of antibodies expresses the binding strength between them and the multivalent antigen (Hubner et al., 2001). It is known that the avidity of antibodies increases with time and its measurement is used in diseases where the differentiation of recent from past infection is important, as is for example in toxoplasmosis (Holliman et al., 1994). Inclusion in one of the ELISA steps of a denaturing urea agent that dissociates at low avidity antigen-antibody binding can also be used in toxocarosis (Hubner et al., 2001). According to some authors, the use of this test could help to distinguish acute from past infection (Rychlicki, 2004).

On the other hand, immunoglobulin A is the major antibody class of first-line mucosal surface protection and has a significant serum

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concentration (15%–20% of total human immunoglobulins) (van Egmond et al., 2001). Induction of IgA synthesis has been established during the migration of *Toxocara* larvae through the lung (Buijs et al., 1994). Changes in its levels were found in the second year after treatment of 27 children with toxocarosis (Rubinsky – Elephant, 2004). Despite these studies, the significance of specific IgA antibodies in human infection is still unclear and requires further research.

2. Patients and methods

2.1. Patients

There is a difference between disease (symptoms, pathologic laboratory parameters) and infection (only detection of specific antibodies). Our study included 130 patients with clinical and serological signs of toxocarosis. Of them, 66 (50.8%) were males and 64 (49.2%) females, in a ratio of 1: 0.97. The age of the subjects ranged from one to 88 years (mean age 36 years ± 2 years), of which 41 (31.5%) were children and adolescents up to 18 years of age and 89 (68.5%) were adults. All patients tested positive for toxocarosis in ELISA IgG (commercial kit R-Biopharm) and in confirmatory Western blot test (LD BIO). According to the clinical form of the disease, 120 (92.3%) of the patients were with symptoms of visceral (VLM) and 10 (7.7%) with ocular (OLM) forms of toxocarosis. Patients with clinically pronounced allergy and eosinophilia (Table 1) dominated the group with visceral infection.

2.2. Serum samples

Serum samples were obtained by venipuncture according to standard methods from all 130 patients and were stored at −20 °C until their testing.

2.3. Methods for parasitological diagnosis

Enzyme-linked immunosorbent assay (ELISA) for determination of specific *Toxocara* IgG antibodies. Serum samples from patients with toxocarosis were tested in a commercial kit from RBiopharm, Germany (Ridascreen *Toxocara* IgG) according to the manufacturer's instructions. The reaction was read with an ELISA reader by determining the Sample ratio (SR), which was calculated from the ratio between the obtained extinction of the sample (measured at a wavelength of 450 nm) and the threshold value of the test (cut off). The cut off value is obtained by adding to the extinction of the negative control 0.150. We reported a positive result at SR > 1.1, borderline between 0.9 and 1.1 and below 0.9, negative.

Western blot (WB) Toxocara IgG, commercial kit (LDBIO, France) was used as a confirmatory method of the obtained positive and borderline results in ELISA. According to the manufacturer's instructions, the presence of bands between 24 and 35 kDa on the tested samples are specific for toxocarosis.

ELISA for determination of anti-Toxocara IgA antibodies (EIA Toxocara IgA - TestLine Clinical Diagnostics. R. O., CR). According to the manufacturer's instructions, we interpreted the obtained results by positivity index - IP, as IP < 0.9 was reported as a negative result, IP between 0.9 and 1.1 as a borderline, and over 1.1 as a positive.

To determine the avidity of specific Toxocara IgG antibodies, we used a developed laboratory method utilizing ELISA antigen and 6 M urea as a denaturing agent (Rainova, 2011). Serum samples diluted at 1: 200 in serum and conjugate dilution buffer (0.01 M phosphate buffer containing 0.15 M NaCl, 0.1% Tween 20, 1% Bovine Serum Albumin and merthiolate 1:10 000) were dripped a 100 μl into each of the wells of a flat-bottomed ELISA plates loaded with excretory-secretory (E/S) antigen from *T. canis* larvae. Each serum was placed in two separate wells and incubated for 1 h at 37 °C. After washing three times with washing buffer (0.2 M phosphate buffer with 1% Tween 20), 100 μl of 6 M urea was added dropwise to the first well and in the second 100 μl buffer for dilution of the sera and conjugate and incubated them again for 1 h at 37 °C. After repeated washing with washing solution (3 × 300 μl) in each well we placed 100 μl solution of the specific conjugate - anti-human IgG - peroxidase-bound antibody (Sigma - Aldrich, Germany), diluted at 1:20 000 in dilution buffer and incubated them for 30 min at 37 °C. The reaction was visualized by adding a chromogenic substrate - TMB (100 μl per well) and left for 20 min at room temperature under protection from sunlight. The reaction was stopped by adding 100 μl per well 0.2 M H2SO4 and read by photometry at 450 nm using an ELISA reader (BioTek). We calculated the avidity index as a percentage, multiplying by 100 the ratio between the obtained optical density (OD) of the serum treated with urea and the OD value of untreated serum. We assumed values up to 40% as low avidity (indicating a recent infection), values between 40 and 50% we defined as borderline, and those over 50% as high avidity.

2.4. Statistical methods

Statistical package SPSS, Version 20 (Descriptive statistics - Frequencies, means, x^2). Values of $p \leq 0.05$ are statistically significant.

Table 1
Distribution of patients with VLM and OLM by sex, age and clinical symptoms.

Clinical symptom	Number/ %	Males		Females	
Age	130/ 100%	<18 years	>18 years	<18 years	>18 years
Leading symptom eosinophillia	43 (33,1%)	16	9	5	13
Clinically manifested allergy (urticaria, asthma, etc.)	47 (36,2%)	2	18	1	26
Fever	5 (3,8%)	0	1	1	3
Gastrointestinal symptoms	6 (4,6%)	1	4	0	1
Pulmonary symptoms (pneumonitis)	3 (2,3%)	0	2	0	1
Renal symptoms	1 (0,8%)	0	0	0	1
Neurological manifestations (epilepsy, meningitis)	2 (1,5%)	0	1	0	1
Skin manifestations (eczema, dermatitis, pruritus)	2 (1,5%)	0	1	0	1
Anemia	10 (7,7%)	5	0	5	0
Alopecia	1 (0,8%)	0	1	0	0
Ocular manifestations (chorioretinitis, iridocyclitis, uveitis)	10 (7,7%)	2	3	3	2

3. Results

3.1. Determination of the avidity of specific anti-Toxocara IgG antibodies in patients with VLM and OLM

The study of the avidity of specific IgG antibodies in patients with toxocarosis showed values of up to 40% (low avidity) in nine (7%) of the studied patients. With an avidity index between 40 and 50% (intermediate avidity) were five (4%) of the patients, and with data for high avidity over 50% were 116 (89%). Among the persons with low and intermediate avidity, seven were females (five with low and two with intermediate) and seven were males (four with low and three with intermediate avidity). Of those with high avidity, 59 (50.8%) were females and 57 (49.1%) were males. Compared to the age of the subjects with avidity up to 40% were 4 children (44.4%) and 5 adults (55.6%), with borderline values were only patients over 18 years, and with high avidity over 50% were 37 (90.2%) of children and 79 (88.8%) of adults. According to the clinical form of the disease, out of 120 patients with VLM in seven (6%) we found data for low avidity of specific IgG antibodies, in 5 (4%) intermediate values and in 108 (90%) high avidity. According to the leading clinical symptom with which the patients were

referred for examination, the majority of people with low avidity were with eosinophilia (n = 4, 44.4%), one with allergic manifestations, one with fever and one with renal involvement.

Of the patients with OLM, in two (20%) the avidity was low and in eight (80%) high. Comparative analysis of the data on the relative share of persons with low avidity in ocular and visceral forms of toxocarosis did not establish statistically significant difference (p = 0.173) (Table 2).

We followed the dynamics of avidity values in five of the nine patients with low avidity. In re-testing after 3 months, we found that the avidity increased above 50% (between 54.7% and 62.2%) in three persons, and in the other two it increased up to intermediate values between 40 and 50%.

3.2. Determination of specific IgA antibodies in patients with toxocarosis

All of the 130 serum samples positive for presence of anti-*Toxocara* IgG antibodies were also examined for presence of specific IgA antibodies. We obtained positive results in 34 (26.2%) of them, and six (4.6%) were with borderline values. The remaining 90 (69.2%) were negative for specific IgA (Table 3). The mean value of the obtained index for IgA was 0.905 ± 0.926, with the lowest reported value being 0.10 and the highest being 5.90.

Depending on the sex of the subjects, 18 men (52.9%) and 16 women (47.1%) were positive for specific IgA, and we did not find statistically significant difference between them (p = 0.671). The mean value of the obtained antibody index was slightly higher in females (0.937) than in males (0.873), but there was also no statistically significant difference (p = 0.699). Of the 34 positive patients, eight were children and adolescents (23.5%) and 26 (76.5%) were over 18 years old. We did not find a statistical relationship between the age of the subjects and the IgA positivity (p = 0.317) (Table 4).

In terms of clinical symptoms, the largest number of positive for presence of specific IgA antibodies were patients with VLM - 32 out of 34 (94.1%), while those with ocular involvement were only two (5.9%). The mean value of the obtained IgA antibody index was higher in patients with VLM - 0.929, compared to that found in those with OLM - 0.614, and the difference between them was not statistically significant (p = 0.304) (Table 4). The largest was the relative share of positive patients with eosinophilia as a leading clinical symptom, 14 out of 43 (41.2%) and those with allergies - 8 out of 47 (23.5%). @Monitoring the dynamics of specific anti-*Toxocara* IgA in follow up showed that out of 34 positive 14 maintained almost unchanged titer for this class of antibodies up to 6–9 months after the initial study. After this period, all samples tested for specific IgA were below the diagnostic threshold of 0.440.

Table 2
Avidity index values in patients with toxocarosis by sex, age and clinical form.

	Low avidity (<40%)	Intermediate and high avidity (>40%)	Total	P
Sex				
Males	5 (7.6%)	61 (92.4%)	66 (100%)	0,692
Females	4 (6.3%)	60 (93.6%)	64(100%)	
Age				
<18	4 (9.6%)	37 (90.4%)	41 (100%)	0,383
>18	5 (5.6%)	84(94, 4%)	89 (100%)	
Clinical form				
VLM	7 (5.8%)	113 (94.2%)	120 (100%)	0,046
OLM	2 (20%)	8 (80%)	10 (100%)	

3.3. Comparison of the results of ELISA IgA with the results of the avidity of specific IgG antibodies in patients with toxocarosis (correlation of the two methods)

Comparing the results with respect to the two studied indicators, we found that three of the studied patients had low avidity of the specific IgG antibodies and presence of anti-*Toxocara* IgA (8.8% of the positive for IgA and 33.3% of those with low avidity). The other six (66.7%) of the patients with low avidity had negative values for anti-*Toxocara* IgA antibodies. Of those with borderline avidity (n = 5), two were positive for anti-*Toxocara* IgA. Of 116 patients with high avidity 31 (26%) had a positive result for IgA, six (5%) had a borderline result and 79 (69%) were negative for IgA antibodies. Our results show that in most of the patients with specific IgA antibodies (n = 29), IgG avidity was high. The majority of patients with low and borderline avidity (9 out of 14) were IgA negative. When comparing the mean values of the avidity and IgA antibodies in patients with toxocarosis, we found that in those with low avidity (mean 32.5%) the mean IP value of IgA was 1,241, which is higher than in patients with high avidity - 0.912, although the difference between them is not statistically significant (p = 0.311). @

The data from the follow-up of the change of the two indicators in different periods after diagnosis of the disease showed that specific IgA antibodies were observed for up to 9 months, in contrast to the low and borderline avidity that were observed in the first tests and up to 3 months on re-examination. Specific IgG antibodies persisted in all of the 34 monitored patients for about a year after the initial study.

4. Discussion

Long-term persistence of *Toxocara* larvae in the body is causing chronic disease and often the exact time of infection cannot be determined (Pawlowski, 2001). The main two methods used for diagnosis, ELISA and Western blot, cannot distinguish past from recent infection or reactivation (Glickman et al., 1978; Jacquier et al., 1991). The first synthesized antibodies in the early stages of the disease have low avidity to the antigen, so its determination helps to distinguish acute from chronic infection. Various methods have been developed to find the avidity of the specific IgG antibodies (Hubner et al., 2001; Dziemian et al., 2008). For this purpose, we used a laboratory ELISA with E/S antigens from parasite larvae and denaturing agent 6 M urea. Depending on the clinical data of the patients for the time of the first symptoms and the literature data that the avidity index increases about 50% between 40 and 80 days after infection in mice, we determined for our study a limit of 40% for low, between 40 and 50% for borderline and over 50% for high avidity (Fenoy et al., 2008). Data for avidity index of or below 40% we found in nine (6.9%) of 130 patients positive for toxocarosis in routine tests. Similar results were obtained in other studies: low avidity was determined in 5.09% of the studied persons in the Czech Republic (Hubner et al., 2001). In Rubinsky – Elephant (2004) serological follow-up of 27 children with toxocarosis it was found that, all have high IgG avidity, which is a sign of a chronic stage of infection. Rychlicki (2004) determined specific IgG avidity against *Toxocara* E/S antigens in 212 patients and found low IgG avidity in 6.6% of patients in the acute phase, and high avidity in all chronic phase samples. According to the author, the avidity test for specific IgG antibodies is a useful diagnostic method that can help distinguish early from later stages of *Toxocara* infection (Rychlicki, 2004). In a more recent study, Boldiš et al. (2015) found low IgG avidity in 30.7% of 88 subjects with clinical and epidemiological data of toxocarosis. According to the same authors, information only about the avidity of specific IgG antibodies is not enough to distinguish acute from chronic toxocarosis and additional studies such as the level of total IgE are needed. @

In our study, there were four children and five adults with low avidity, which shows that *Toxocara* infection may occur at all ages, despite reports by a number of authors for more frequent invasion of children, especially those between 2 and 8 years of age (Beaver et al.,

Table 3
Values of specific IgA antibodies in patients with toxocarosis.

IgA	% of the total number	N	IgA IP average value	Lowest value	Highest value	Range
IgA (+)	26.2%	34	2.063	1.12	5.90	4.78
IgA(+/−)	4.6%	6	0.992	0.91	1.10	0.19
IgA (−)	69.2%	90	0461	0.10	0.90	0.80
Total	100.0	130	0905	0.10	5.90	5.80

Table 4
Data from the study of patients with toxocarosis for specific IgA antibodies by sex, age and clinical form of the disease.

Index	ELISA *Toxocara* IgA (+)(%)	ELISA *Toxocara* IgA (±)(%)	ELISA *Toxocara* IgA (−)(%)	ELISA IgA IP mean	P
By sex:					
Males	18(52.9%)	4(66.7%)	44(48.9%)	0.873	0.699
Females	16(47.1%	2(33.3%)	46(51.1%)	0.937	
By age:					
Children	8(23.5%)	1(16.7%)	32 (35.6%)	0.709	0.103
Adults	26(76.5%)	5(83.3%)	58(64.4%)	0.995	
Clinical form:					
VLM	32(94.1%)	6(100%)	82 (91.1%)	0.929	0.304
OLM	2 (5.9%)	0(0%)	8(8.9%)	0.614	

1952; Glickman et al., 1981; Matsumura and Endo, 1983; Wisniewska-Ligier et al., 2012).

Common clinical and laboratory findings among patients with low avidity were allergy, eosinophilia and fever, which indicates that these may be the initial symptoms of the disease. Boldiš et al. (2015) found a similar relationship regarding low IgG avidity and a study group of patients with eosinophilia.

The patients we studied with clinical signs of OLM were relatively small in number and despite the high relative share (20% versus 6% for patients with visceral form) of all persons with low avidity, we cannot draw definite conclusion that low avidity is more common among people with OLM. Dziemian et al. (2008) also reported that in patients with ocular form, low avidity was found in 36.8%, and in those with visceral form, in 22.4%.

In addition to the avidity for determining the stage of the disease, the significance of other specific immunoglobulins has been explored in recent years (Rubinsky – Elephant, 2004). Study by Boldiš et al. (2018) of 52 seropositive for *Toxocara* patients found presence of specific IgA in 32.7% of them, with their titer being the highest in the oldest study group. Rubinsky – Elephant (2004) determined that the sensitivity of ELISA IgA was 47.8% in children with toxocarosis. In our survey, we found anti-*Toxocara* IgA antibodies in 26.2% (34) of the patients with a predominance of persons over 18 years of age and among them, the IP values were higher. Similar results have been described in schistosomiasis by Grzych et al. (1993), who found that the amounts of specific IgA increase with the age of the population and reach maximum values the group over 50 years. Positive for specific IgA antibodies are mainly patients with clinical symptoms of VLM (94.1%) compared to those with ocular form (5.9%) The largest number of positive for specific IgA are patients with a leading clinical symptom of eosinophilia and allergy. The level of *Toxocara* IgA antibodies was monitored in 14 of the 34 patients positive for this class of immunoglobulins, and the presence of specific IgA was detected up to 9 months after the initial study, but after this period, the test results were negative. The value of the avidity index was monitored in five of the nine patients with low avidity, and it increased after the third month from the initial study. The comparison of the two indicators showed differences in time periods. IgA antibodies persist longer and the avidity of the synthesized IgG antibodies increases faster. Individuals with presence of IgA displayed different avidity of specific IgG antibodies. From 34 IgA positive individuals - in three (8.8%) IgG avidity index is below 40%, but the group of patients with IgA and high avidity of IgG predominates. The lack of correlation between the two tests was also observed by Boldiš et al. (2018), who believed that it is probably due to differences in the used methods or the disease stage. However, we believe that determining the avidity of specific IgG antibodies is a better marker for distinguishing acute from chronic toxocarosis because IgA antibodies persist for up to 9 months after infection. It is recommended that the results of serological tests for this parasitic disease be accompanied by examination of other laboratory parameters such as eosinophilia, eosinophil cationic protein, increased total IgE and subclasses of specific IgG antibodies (Kaneva, 2020).

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Author contributions

Conceptualization: EK and IR; Data curation: EK, IR, RH and IK; Original draft: EK and IR; Review & editing: RH, IK.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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