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ORIGINAL ARTICLE

Designing and standardizing *Toxocara* serologic diagnostic kit and determining anti-*Toxocara* antibodies frequency in patients referred to health care centers in Urmia (northwest of Iran)

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Abstract Toxocariasis is a helminthic zoonosis caused by larval stages of the roundworm of dog, *Toxocara canis*, and less frequently by *T. cati*, the roundworm of cats. Eosinophilia in peripheral blood may be indicative of a disease; however, it does not necessarily represent toxocariasis. Therefore, it is necessary to investigate the relationship between eosinophilia and toxocariasis in a region. The aims of this study were the diagnosis of hyperosinophilia patients using traditional ELISA kit and also by a handmade ELISA kit produced by *T. canis* excretory-secretory (TCES) antigens as well as the determination of the abundance of anti-*Toxocara* antibodies among people referred to Urmia, northwest of Iran care centers. Traditional ELISA kit was used to determine anti-TCES-specific IgG antibodies on 180 hypereosinophilic samples. These antibodies were evaluated in 1002 samples, including 180 hypereosinophilic samples and 822 random samples without eosinophilia by a handmade ELISA kit produced by TCES antigens. A Western-blot confirmatory test was performed on ELISA-positive samples. Our results showed a 17.22% prevalence rate of *Toxocara* antibodies among hypereosinophilic samples with traditional ELISA kit, and this rate was 3.89% in the 1002 study population with random sampling (with or without eosinophilia). Also, there was a good match between the results of handmade ELISA with those of traditional kit. The positive results in the ELISA method were confirmed by the Western-blot analysis. Our findings show that although the high eosinophil count is not necessarily a sign of toxocariasis, in Urmia distric, about one-fifth of eosinophilia cases have anti-toxocariasis antibodies. In addition, the abundance of anti-Toxocara antibodies in this area was 3.89%.

Keywords Toxocariasis · Excretory secretory antigen · Eosinophilia · Frequency · ELISA · Western blot

Introduction

Toxocariasis is one of the most common and neglected human parasitic worm infections in the world (Farmer et al. 2017; Mattos et al. 2016). This zoonotic disease spreads through unembryonated eggs, shed by infected dog and cat (as common definitive hosts) and become infectious after 10–14 days (Despommier 2003; Iddawela et al. 2017). If these infective eggs (containing a L2 larva) are ingested by human, in the duodenum, the larva will hatch and find its route to the liver via the portal circulation, then from vascular channels to the lung systemic circulation. Eventually, larva will settle in various organs and tissues, including eye, brain, lungs, liver, and muscles, resulting in two major clinical syndromes of toxocariasis, visceral larva migrans (VLM) and ocular larva migrans (OLM) (Bae et al. 2016; Despommier 2003; Fialho et al. 2016; Fisher 2003). Other recognized clinical manifestations include neurological toxocarisis and occult toxocariasis(Mazur-Melewska et al. 2015; Pawlowski 2001). Various studies have suggested that geographical location, socioeconomical status, contact with soil, dog, and cat, age, sex, pica, and ingestion of raw cow liver or meat are the most important risk factors for acquiring the disease (Campos

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Júnior et al. 2003; Fillaux et al. 2007; Kwon et al. 2017; Kwon et al. 2011). Eosinophilia in peripheral blood occurs in allergic diseases and cancers and is commonly found in tissue-invading parasites (Merdin et al. 2016; Won et al. 2015); VLM is often complicated by blood eosinophilia (Van Den Broucke et al. 2015).

Visceral and ocular toxocariasis are routinely diagnosed with the combination of various strategies such as risk factor assessment, clinical signs, and various laboratory tests. Some laboratory findings that are normally observed in association with toxocariasis comprise eosinophilia, hypergammaglobulinemia (IgG, IgM, and IgE), elevated erythrocyte sedimentation rate, and the increased titers of isohemagglutinin A and B; however, all of them necessarily are not specific for Toxocara infection. Also, a number of these findings are not present in some patients with visceral or ocular toxocariasis based on antibody testing (Woodhall and Fiore 2013).

Eosinophilia does not necessarily represent toxocariasis; therefore, it is required to investigate the connection between eosinophilia and toxocariasis in a region. In the present work, our primary goal was to diagnose the IgG antibodies against *T. canis* in hyperosinophilia patients using traditional ELISA kit, in order to evaluate the association rate between blood eosinophilia and Toxocara seropositivity. Our secondary goal was to assess the abundance of *T. canis* antibodies by means of a handmade ELISA kit produced by *T. canis* excretory-secretory (TCES) antigens among people referred to Urmia health care centers (Urmia, Iran).

Materials and methods

Collection of samples

The study was performed in Urmia City, West Azerbaijan Province, northwest of Iran. Samples used in this work were obtained from 1002 patients attending the hospitals and medical laboratories for various medical problems during the year 2017. According to eosinophil counts, patients' samples were divided into two main groups, non-eosinophilic (n = 822) and eosinophilic groups (n = 180), including patients having < 350/ml and ≥ 350/ml eosinophils, respectively. Serum samples were preserved at − 20 °C.

Culture of *T. canis* egg

After prescribing the praziquantel to female stray dogs to defecate parasites, we obtained stool specimens to collect the adult *T. canis* worms. Fan's method was employed for culturing the eggs harvested from the part of the worm body where uterus of female *Toxocara.* Spp is present (Fu et al. 2014). In brief, the eggs were excised in a 1% sodium hypochlorite solution and left at room temperature for 5 min, then centrifuged at 2000 rpm for 5 min. Subsequently, two wash with distilled water and one wash with 2% formalin were performed on the obtained eggs. These eggs were transferred to an Erlenmeyer flask, and then 2% formalin was added until nearly one centimeter deep liquid layer was made. Afterwards, the flask was kept at room temperature for 8–9 weeks while a gentle agitation was performed once a week. During this period, the eggs contained second-stage larvae and were finally stored at 4 °C until use (Fu et al. 2014).

Preparation of larval TCES antigens

The embryonated eggs were hatched under aseptic conditions when larvae were required. The infectious larval TCES antigens were prepared by de Savingi's method. Briefly, embryonated eggs were washed and centrifuged with sterile phosphate-buffered saline (PBS) at 2000 rpm for 5 min. The washed egg mass re-immersed in 1% sodium hypochlorite solution and then incubated in a 5% CO_2 atmosphere at 37 °C for 30 min under sterile conditions. After several washings with sterile PBS containing some antibiotics such as 100 IU/ml penicillin, 250 µg/ml streptomycin, and 25 µg/ml nystatin, the egg mass was again immersed in 100 ml of sterile RPMI-1640 medium containing the same antibiotics. Motile larvae were collected using a modified Baermann apparatus, which was maintained in an atmosphere containing 5% CO_2, at 37 °C for 12 h. The larvae were transferred to 50-mL tissue culture flasks containing fresh RPMI-1640 medium and antibiotics, yielding 10,000 larvae/ml (calculated with Neubauer lam), and then incubated in an atmosphere of 5% CO_2 at 37 °C. The supernatant (containing the excretory-secretory antigens) was collected weekly and centrifuged until all probable debris was precipitated. The final supernatant was transferred to a sterile dialysis tube following filtration with a 0.2-mm filter (molecular weight cut-off = 6000-8000 D). In the next step, the excretory-secretory antigen was dialyzed against sterile PBS at 4 °C for 12 h until the disappearance of phenol red. The protein content was then measured by the Bradford method (concentration of protein was 1.0 mg/dl, and we diluted it to reach 5 µg/ml), and the resulting antigen was stored at − 70 °C until use (Fu et al. 2014).

ELISA for detecting serum TCES-specific IgG antigens

Handmade ELISA method

ELISA method provided by Jimenez et al. (1997) was utilized to detect the serum TCES-specific IgG antibodies among the 822 non-eosinophilic samples. Briefly, 50 µl of TES antigen (5 µg/ml) (Regis et al. 2011) in carbonate-bicarbonate buffer was used for microtiter plates coating and incubated at 4 °C overnight. Then three washings were performed with 200 µl of 0.05% Tween 20 (PBS-T; washing buffer) to eliminate any unbounded antigens. To avoid non-specific binding, microtiter plates were blocked with BSA 2% in PBS and incubated at 37 °C for 30 min. After five washings with 200 µl of washing buffer, 1:50 diluted serum samples were added to each microtiter well (in duplicate) and incubated at 37 °C for 90 min. In each plate, one positive and one negative control (from IBL TCES IgG ELISA kit) were used. After five washings with 200 µl of washing buffer, 50 µl of (1:1000 diluted) goat anti-human IgG conjugated with horse radish peroxidase and 100 µl of 3,3′,5,5′-tetramethylbenzidine (TMB) were added to each well and incubated in a dark place at room temperature for 60 and 30 min, respectively. The reaction was stopped by adding 5% sulfuric acid to each well. The absorbance of specimens were measured with an ELISA plate reader at 450/630 nm (Kyei et al. 2015).

Commercial ELISA method

Two plates of commercial ELISA kits (IBL *T. canis* IgG ELISA LOT: TCG-056, REF: RE58721, Germany) were applied for detecting TES-specific IgG antibodies among 180 hypereosinophilic samples. In accordance with the manufacturer's instructions, negative, positive, and cut-off controls and 1:100 diluted sera were transferred to test wells. An anti-IgG enzyme conjugate was used to detect antigen–antibody complexes, and these reactions were visualized with a chromogen solution (TMB). The absorbance of specimens was measured with an ELISA plate reader at 450/630 nm within 30 min after adding stop solution. Samples were considered positive if the absorbance value was higher than 10% of the cut-off.

Western-blot analysis

A confirmatory Western-blot test was performed on ELISA-positive samples. The specimen of each serum was separately incubated with a strip coated with *Toxocara* antigen. The anti-*Toxocara* antibodies potentially present in the sample were selectively bounded to the E/S antigens of *T. canis* (present on strips). The alkaline phosphatase-anti human IgG conjugate was then added and bounded to bind anti-*Toxocara* antibodies. Finally, the immune complexes reacted with the substrate. The antigens were recognized by the anti-*Toxocara* antibodies (IgG) present in the samples and were revealed as purple transversal bands.

Statistical analysis

Data were analyzed using SPSS version 16.0 software for Windows. The Chi squared test was used to compare categorical variables. P values < 0.05 were considered statistically significant.

Results

A total of 180 hypereosinophil serum samples, including 92 (51.11%) females and 88 (48.89%) males participated in the study (Table 1).

The seroprevalence of *Toxocara* was 17.2% among all study participants, including 20 females (64.52%) and 11 males (35.48%). In addition, all 1002 (180 eosinophilic and 822 non-eosinophilic) serum samples were analyzed by aforementioned handmade ELISA method for detecting the serum TCES-specific IgG antibodies.

Overall, 39 of 1002 samples (3.89%) were positive by handmade ELISA method. Without calculating 180 hypereosinophil samples, abundance among 822 non-eosinophilic samples was only eight samples (0.973%). The differences between the results of 180 serum samples analyzed by both commercial and handmade ELISA methods were as follows: in commercial method, two cases showed borderline results (9–11 U) with the test result of 10.01 and 10.05 U, while in handmade method, the cases indicated positive result. In one case, the test result with the commercial kit was at the lowest possible level, which was considered as a positive result (11.1 U), but with handmade kit, it was negative. One case also showed positive result by commercial kit but negative by handmade method. Also, five serum samples had positive results among the 822 non-eosinophilic samples.

Comparison of the results of TCES antigen assays by ELISA and Western-blot methods

A Western-blot test was used to confirm the positive results of commercial and handmade ELISA methods. All ELISA-positive serum samples were also positive by Western-blot method; two or more bands were observed in the range of 24 to 35 kDa molecular weight, indicating that both assays have high specificities.

Table 1 Age and sex distribution of patients with hyperosinophilia

Age groups (year)	1–10	11–20	21–30	31–40	41–50	51–60	61–70	71–80	81–90
Female	1	6	19	16	15	14	12	7	2
Male	15	2	15	8	12	11	13	11	1

A statistically significant correlation was evident between serum TCES-specific IgG antibody and soil contact ($P < 0.001$), dog contact ($P < 0.001$), and habitat in the village ($P = 0.006$), while no statistically significant correlation was observed in relation with gender ($P = 0.116$) and allergy history ($P = 0.060$) (Table 2).

Discussion

In this study, the results of the handmade method acceptably matched with the commercial method, as the standard strategy. In the handmade method, 39 serum samples were positive for TES IgG and 963 were negative; however, the commercial ELISA test showed 31 positive cases. On the other hand, *T. canis* antibodies were observed only in eight out of 822 cases, without considering 180 hypereosinophil samples. Therefore, the serum frequency in this condition was very low and equal to 0.973%, while the prevalence rate among 180 hypereosinophil samples was 17.2%; this discrepancy suggests that there is a connection between hypereosinophilia and toxocariasis. To confirm the results, TCES-specific IgG-positive samples were analyzed using Western-blot method, which showed a consistency between the results. Hence, it could be concluded that in Urmia region, about one-fifth of individuals who have hyperosinophilia, they may have toxocariasis; the serum antibodies against this parasite in random sampling were about 0.973%. In a similar study, in urban and rural areas of South Korea, Kim et al. (2014) randomly examined the proportion of toxocariasis among 610 healthy individuals visited for health checkup (with or without eosinophilia) and also assessed the risk factors for positive cases. The serum samples were tested with raw antigen of larval toxocariasis using handmade ELISA kit (produced at Seoul National University, Seoul, South Korea). The Western-blot technique was also used to confirm the positive cases. The overall rate of toxocariasis antibody present among these 610 cases was 8.7% (6.1% in urban areas and 11.3% in rural areas). Their results showed that the prevalence of anti-*T. canis* antibodies increased with the high percentages of blood eosinophils counts (5.9% in the group < 350/μl, 10% in the group 350–500/μl, and 12.4% in the group > 500/μl ($P = 0.028$). In the present study, although there was a relationship between hyporosinophilia and serum antibodies against *T. canis* antigens, contrary to the results of Kim et al. (2014), the number of positive cases did not necessarily elevate with an increase in the number of blood eosinophils. In addition, the most antibody positive cases were found in the eosinophilia ranges of 6% to 9%, which could be the indicative of an agent(s) other than Toxocara species that are likely involved in the increased number of eosinophils in the Urmia region, which is worth considering in the future. In this study, according to a completed questionnaire analysis, alcohol and smoking were identified as the main risk factors for toxocariasis. Their results also showed a correlation between the seroprevalence of antibodies against toxocara and hypereosinophilia. Therefore, they apparently suggested that healthy individuals with eosinophilia should be routinely checked for risk factors associated with Toxocara serology (Kim et al. 2014).

In 2013, Ebrahimi Fard et al. (2015) examined the seroprevalence of *Toxocara* infection and its associated risk factors among adults with more than 10% eosinophilia who referred to Babol (north of Iran) treatment centers (EbrahimiFard et al. 2015). They observed statistically no significant association between age groups and toxocara infection, whereas a meaningful correlation was found between occupation and Toxocara infection so that farmers and housewives were more likely to be infected than freelancers, workers and other occupations. Besides, there was no significant relationship between the history of allergy and Toxocara infection, and the prevalence was

Table 2 The relationship between the positivity of TCES-specific IgG antibody and risk factors

Risk factor	Allergy history	Housing village	Dog touch	Soil contact	Gender
P value	0.060	0.006	< 0.001	< 0.001	0.116
Statistically significant	No	Yes	Yes	Yes	No

higher in boys than in girls, which could be attributed to boys' specific games and behaviors. Outbreaks of this infection in rural areas were 57 (75%) and 19 (25%) residents, with a significant relationship (EbrahimiFard et al. 2015). In our study, despite difference in the number of positive antibodies between women and men, no significant difference ($P = 0.116$) was observed in the prevalence between the genders. However, there was a significant correlation between residency in the village (7 out of 31 positive cases, 22.88% positive) and serum *Toxocara* antibodies ($P = 0.006$). Although the serum antibodies in Urmia district were higher at older age cases (perhaps due to the agronomy of the area and the contact of more aged people with the soil during farming), no significant association was detected between the age and the prevalence of serum antibodies. Karadam et al. (2008) examined the prevalence of IgG antibodies against *Toxocara* species in 700 people (350 with high eosinophils and 350 without eosinophilia) by ELISA method in Turkey (Karadam et al. 2008). According to their results, the prevalence of antibodies against *Toxocara* species in high eosinophilic group was 32.6% (114 cases) and 20.3% in the group without eosinophilia. Comparison of our study with Karadam's study (2008) indicated that in their region, there was a large number of people with positive antibodies among non-eosinophilic subjects. This increased number of individuals is likely due to the difference between the climates of the two regions, their soil moisture, and temperature. In various studies, the prevalence of anti-*Toxocara* antibodies in Turkey has been reported to be 28–51%, which is much higher than that of Iran (15.8% in different regions of Iran), according to the results of a meta-analysis and systematic review study (Abdi et al. 2012). However, in the present study, a high eosinophilic role in increasing the seroprevalence of antibodies against *Toxocara* was clearly consistent with Karadam et al.'s (2008) study.

Conclusion

Our study revealed a significant consistency between the results of handmade ELISA and those of the standard commercial kit. However, more investigations are needed to be carried out toward identifying the immunogenic antigens of common parasites in different regions of the country and also designing and constructing such kits so as to help better disease identification.

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Author's contribution Simin Ashtari and Esmaeil Abasi prepared the proposal, collected samples and did the laboratory workup, Shahram Khademvatan helped with laboratory techniques such as Western blotting and Arash Aminpour designed the work, analyzed the data and prepared the manuscript.

Compliance with ethical standards

Conflict of interest Authors declare that we have no conflict of interest.

Ethical issues The study was approved by the Ethics Committee of Urmia University of Medical Sciences, Urmia, Iran (approval number: 1395-01-43-2603).

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