

# Evaluation of *Toxocara cati* Excretory–Secretory Larval Antigens in Serodiagnosis of Human Toxocariasis

Mohammad Zibaei,<sup>1</sup> Seyed Mahmoud Sadjjadi,<sup>2,3\*</sup> Bahador Sarkari,<sup>2</sup> and Shoji Uga<sup>4</sup>

<sup>1</sup>Department of Parasitology and Mycology, School of Medicine, Alborz University of Medical Sciences, Karaj, Iran

<sup>2</sup>Department of Parasitology and Mycology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

<sup>3</sup>Basic Sciences in Infectious Diseases Research Center, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

<sup>4</sup>Department of Parasitology, Faculty of Health Sciences, Kobe University Graduate School of Health Sciences, Kobe, Japan

---

**Background:** Toxocariasis is the clinical term that is applied to infection in the human host with *Toxocara* species larvae. Serological tests are important tools for the diagnosis of toxocariasis. The aim of this study was to evaluate the excretory–secretory (ES) antigens of *T. cati* larvae using enzyme-linked immunosorbent assay (ELISA) and also Western blotting for serodiagnosis of human toxocariasis. **Method:** The ES antigens were prepared from *T. cati* third-stage larvae. Serum samples were obtained from 33 confirmed cases of toxocariasis, 35 patients infected with other parasitic diseases, and 30 from healthy individuals tested with ELISA and immunoblotting. **Results:** The

ELISA showed appropriate performance in term of specificity (96.7%) and sensitivity (97.0%). Electrophoretic analysis of *T. cati* ES antigens revealed a range of 20- to 150-kDa fractions. The highest sensitivity was achieved with 42- and 50-kDa fractions. **Conclusion:** The ELISA analyses using *T. cati* ES antigens demonstrated good sensitivity and specificity compared to *T. canis* ES as antigens for diagnosis of human toxocariasis. Accordingly, application of Western blotting, based on 42- and 50-kDa fractions of ES antigens, can be recommended for the accurate diagnosis of toxocariasis. J. Clin. Lab. Anal. 30:248–253, 2016. © 2015 Wiley Periodicals, Inc.

**Key words:** *Toxocara cati*; serodiagnosis; excretory–secretory antigens; ELISA; Western blotting

---

## INTRODUCTION

Toxocariasis is a worldwide helminthic zoonosis caused by the ascariid nematode *Toxocara* species. Dogs and cats are the definitive hosts and act as reservoirs for *T. canis* and *T. cati*, respectively (1). Humans are infected by ingestion of embryonated eggs in the soil or through contaminated hands and fomites, and eating the meat of paratenic hosts containing encapsulated larvae (2). The larva hatches in the intestine and migrates into different organs, mainly the liver and lungs. It may occasionally migrate into the kidney, myocardium, and central nervous system (3). There are four clinical forms of toxocariasis including visceral larva migrans (VLM), ocular larva migrans, covert toxocariasis, and neurotoxocariasis (4).

Prevalence of *Toxocara* infestation of dogs and cats and the resulting contamination of the ground is relatively high in many countries all over the world. The presence of *T. canis* infected dogs and *T. cati* infected felines in public spaces, their defecation habits, and their direct contact with humans constitute significant factors that promote the transmission of this zoonosis. A number of

---

\*Correspondence to: Seyed Mahmoud Sadjjadi, Department of Parasitology and Mycology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, P.O. Box 71345-1735, Iran. E-mail: smsadjjadi@sums.ac.ir

Received 15 April 2014; Accepted 23 February 2015

DOI 10.1002/jcla.21844

Published online in Wiley Online Library (wileyonlinelibrary.com).

studies taking place worldwide have described the extent of environmental contamination with this parasite. It has been determined that in Iran 42.6% of stray cats are infected with *T. cati* (5).

There is little finding on the location of *T. cati* larvae in paratenic hosts as causative agents of human toxocariasis (6). The diagnosis of human toxocariasis is mainly based on clinical, epidemiological, and laboratory data, which include imaging exams, blood exams, eosinophilia, total IgE level, and serological tests (1). Enzyme-linked immunosorbent assay (ELISA) and Western blotting are two types of tests that are available for the immunodiagnosis of toxocariasis, both using *Toxocara* excretory–secretory (ES) antigens. The test currently employed for the serodiagnosis of toxocariasis is ELISA using ES antigens from *Toxocara* larvae (7). Due to the greater sensitivity and specificity of the *Toxocara* Western blotting, investigations demonstrate that this method is a better technique than ELISA to investigate patients having visceral or ocular involvement consistent with toxocariasis (7–10). Considering that *T. cati* is one of common helminths in Iran and that it is a potentially preventable disease and information about the diagnosis of patients with *Toxocara* infection by using the *T. cati* ES antigens is scanty (11), we describe the development, sensitivity, and specificity for Western blotting analysis made with ES *T. cati* antigens.

## METHODS

### Patients and sera

Serum samples from 33 patients with toxocariasis diagnosed based on clinical (fever, anorexia, cough, nausea, vomiting), hematological (leukocytosis and eosinophilia), and serological findings were used to identify specific *Toxocara* antibodies. Sera from 30 healthy individuals (with repetitive negative results for intestinal parasites examination) were used as controls.

Cross-reactivity was assessed by selecting sera from 30 healthy subjects and 35 patients with other proven parasitic infections confirmed by blood smear, stool examination, positive specific serological test results: leishmaniasis (five patients), strongyloidiasis (six patients), hydatidiasis (six patients), toxoplasmosis (seven patients), trichostrongyloidiasis (six patients), and ascariasis (five patients).

### Preparation of *T. cati* ES antigens

*Toxocara cati* ES antigens were prepared from third-stage larvae by the method described by de Savigny (12) with some modifications. In brief, *T. cati* adult worms were collected from the intestines of infected stray cats from different areas of Shiraz, Iran. The *T. cati* eggs were isolated from uteri of female worms and were decoated by sodium

hypochlorite (7% w/v) and embryonated in 2.5% formalin/ringer solution while incubated in an atmosphere of 5% CO<sub>2</sub> at 25 °C for 3 weeks (13). The embryonated eggs suspension was washed in sterile distilled water to remove the traces of chlorine. The larvae were collected aseptically in a Baermann's apparatus. The larvae were cultivated in RPMI 1640 (Sigma-Aldrich Chemie GmbH, Germany) medium supplemented with sodium bicarbonate (Merck, Darmstadt, Germany; 8.5% µg/ml), L-glutamine (20 mM), HEPES (Merck) penicillin/streptomycin (100 IU, 100 µg/ml), and 1% glucose (Merck, Darmstadt, Germany). Culture supernatant was exchanged weekly and pooled; then dialyzed overnight against 5 mM acetate buffer (pH 5) at 4 °C, protease inhibitors (phenylmethylsulfonyl fluoride) were added, concentrated, and stored at –20 °C. The protein content (120 µg/ml) was measured by the Bradford protein assay (14). The ES antigens' profile was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

### Enzyme-linked immunosorbent assay

ELISA microplates (Nunc, Roskilde, Denmark) were coated with 5 µg/ml of *T. cati* ES antigens (100 µl/well) in coating buffer (0.05 M carbonate–bicarbonate buffer, pH 9.6) and incubated overnight at 4 °C. Plates were washed five times in phosphate-buffered saline containing 0.05% Tween 20 (PBST). After removing unbound coating antigen, the excess binding sites were blocked with 3% skimmed milk in PBST and plates incubated for 2 h at room temperature. The wells were washed in PBST and 100 µl of diluted (1:100) serum samples were added to the plates and incubated at room temperature for 1 h. The plates were washed again and 100 µl of diluted anti-human IgG horseradish peroxidase conjugated (Sigma-Poole, UK) at a 1,000-fold dilution in PBST (1:1,000) was added to each well and incubated for 1 h at 37 °C. After washing three times, the plates were incubated with chromogen/substrate (100 µg/well of OPD, 0.025% H<sub>2</sub>O<sub>2</sub> in 0.1 M citrate buffer pH 5) and were stopped by addition of 100 µl of 1 M sulfuric acid after 30 min. The optical density (OD) of samples was monitored at a wavelength of 450 nm using a microplate reader. The cut-off point was set as the mean optical density of the negative controls plus two standard deviations.

### SDS-PAGE and Western blotting

*Toxocara cati* ES antigens were diluted in sample buffer and were boiled at 100 °C for 5 min. The prepared antigens along with molecular weight markers (Fermentas, UK) were submitted to electrophoresis in SDS-PAGE, using a Mini-PROTEAN III Electrophoresis Cells (Bio-Rad

**TABLE 1. Frequency of Different Antigenic Fractions Reacting With Sera From Patients With Toxocariasis, Other Parasitic Infection, and Healthy Individuals**

Infection	Sera ( <i>n</i> = 98)	Detection of ES antigen fractions (kDa)						
		20	28	32	42	50	70	150
Trichostrongyliasis	6	0	0	0	0	0	0	0
Strongyloidiasis	6	0	0	0	0	0	0	0
Ascariasis	5	0	0	0	0	0	0	0
Hydatidosis	6	0	0	0	0	0	0	0
Leishmaniasis	5	0	0	0	0	0	0	0
Toxoplasmosis	7	0	0	0	0	0	0	0
Healthy control	30	0	0	0	0	0	0	0
Toxocariasis	33	20 (60.6%)	8 (54.5%)	24 (72.2%)	33 (100%)	33 (100%)	12 (36.4%)	14 (42.4%)

Laboratories, Hercules, CA) and 12.5% polyacrylamide gel in the presence of 10% sodium dodecyl sulfate at 50 mA/gel for 1 h. The antigens were transferred into nitrocellulose membrane (Schleicher & Schuell BioScience, Hamburg, Germany). Efficacy of transfer was checked by staining the membrane with Ponceau S stain (0.001 g/ml) in 3% trichloroacetic acid. The membranes with blotted antigen were cut into strips and blocked with 5% (w/v) of skimmed milk in washing buffer (10 mM Tris, 150 mM NaCl, and 0.05% Tween 20; pH 7.4) for 2 h at 37 °C. The strips were incubated with test sera (1/100 dilution in washing buffer with 1% bovine serum albumin) for 2 h at room temperature. After washing three times (each for 15 min) in PBST, the strips were incubated with horseradish peroxidase conjugated anti-human immunoglobulin (Dako) at a dilution of 1:2,000 for 2 h at room temperature. After three washes as before, bound antigens was developed using diaminobenzidine (DAB) substrate (0.1% H<sub>2</sub>O<sub>2</sub> + DAB in 50 mM Tris-HCl, pH 7.6). Positive reactions were determined by the appearance of clearly defined bands. The relative molecular weight of each recognized band was estimated by comparison with standard marker.

### Ethical Considerations

The study was approved by ethical committee of the Shiraz University of Medical Sciences and informed consent was obtained from the participants prior to data collection.

### Statistical Analysis

Analyses of differences in sensitivity and specificity of ELISA and Western blotting were performed using 2 × 2 tables.

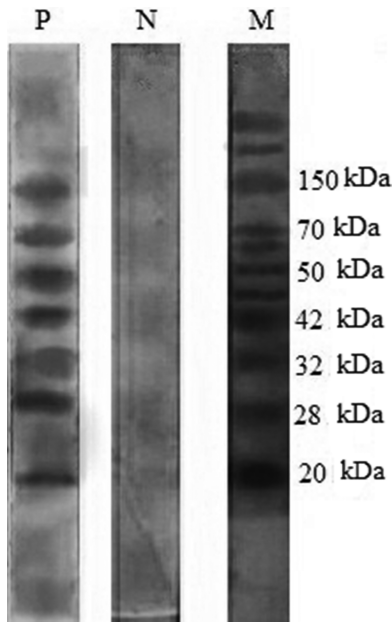
## RESULTS

### ELISA

In this study, ELISA and Western blotting were performed with a panel of 98 sera including 33 serologically confirmed toxocariasis (*Toxocara* infection), 35 patients with parasitic disease other than toxocariasis, and 30 healthy individuals. Of the 33 serum samples from positively diagnosed toxocariasis patients, one serum sample was negative using the ELISA based on *T. cati* antigens. In these patients, the diagnosis of toxocariasis was confirmed by Western blotting using *T. cati* ES antigens. The ELISA yielded one false-positive result with sera from healthy individuals. The sensitivity and specificity of ELISA for diagnosis of human toxocariasis were found to be 97.0% and 96.7%, respectively.

### SDS-PAGE and Western blotting

Using the SDS-PAGE and Western blotting, fractions of 20, 28, 32, 42, 50, 70, and 150 kDa were detected in sera of serologically confirmed patients. Frequencies of different antigenic fractions reacting with the sera from *Toxocara* infection were presented in the bands of 20 (*n* = 20, 60.6%), 28 (*n* = 18, 54.5%), 32 (*n* = 24, 72.7%), 42 (*n* = 33, 100%), 50 (*n* = 33, 100%), 70 (*n* = 12, 36.4%), and 150 kDa (*n* = 14, 42.4%). Samples from patients with leishmaniasis, trichostrongyliasis, hydatidiasis, toxoplasmosis, and ascariasis did not yield any detectable band in the Western blotting (Table 1). The 42- and 50-kDa bands were reactive with all of the sera from 33 toxocariasis patients (Fig. 1). The Western blotting presented good reproducibility. The diagnostic sensitivity and specificity of immunoblotting assay are summarized in Fig. 2. The 42- and 50-kDa fractions have been recognized by all of the patient's sera with toxocariasis, but not by any of sera from nontoxocariasis patients. Accordingly, specificity of the system was found to be 100%.



**Fig. 1.** Western blotting with *T. cati* ES antigens. P, toxocariasis patients; N, toxocariasis-negative sera; M, molecular weights marker. All sera from healthy individuals and from other parasitic diseases did not react with any band.

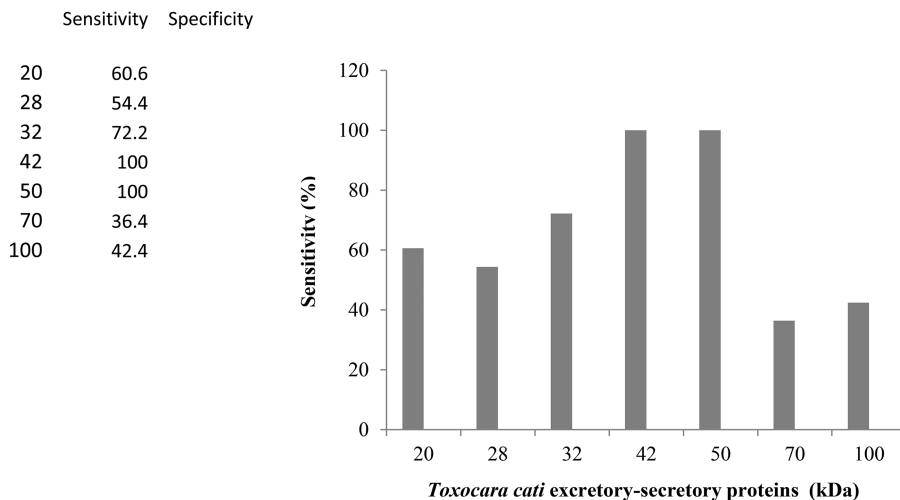
**DISCUSSION**

The development of specific, sensitive, and reliable methods to demonstrate of antibodies in *Toxocara* infection is an important step toward improving diagnosis. Several serodiagnostic techniques, using *Toxocara* antigens, have been developed to improve the diagnosis of toxocariasis. Sensitivity and specificity of each test are different according to the source of antigens (10, 15). The most common assay format is an indirect antibody ELISA using *T. canis* ES antigens secreted from larvae maintained

in culture (16). Serological tests are also useful for seroepidemiological studies of prevalence of human toxocariasis (4).

Variations in the sensitivity and specificity of *Toxocara (canis)* serodiagnostic ELISAs may be due to differences in ES antigens preparation, also disparity in methods between laboratories (15, 16). Glickman et al. (17) reported a sensitivity and specificity of 73% and 92%, respectively, for an indirect ELISA system, using *T. canis* ES antigens. In a relatively similar study by Magnaval et al. (7), a sensitivity of 62.9% has been reported for sera from 66 patients with toxocariasis when tested independently by ELISA using *T. canis* ES antigens. In a study has been demonstrated the development of a highly specific recombinant *T. canis* antigen that yields 100% specificity as compared to the conventional ES antigens (18). Jin et al. (19) reported that the in-house ELISA using crude antigen of *T. canis* larvae is useful for serodiagnosis of human toxocariasis with 92.2% sensitivity and 86.6% specificity. However, these and other similar studies have evaluated the efficacy of ES antigens derived from the third-stage larvae of *T. canis* not *T. cati*.

Using sera from 33 patients with *Toxocara* infection from Iran, where a relatively common of human toxocariasis was reported (4), we evaluated and compared the negative and positive predictive values for ELISA using *T. cati* ES antigens for identifying those individuals that were exposed to infection. The ELISA analyses using *T. cati* ES antigens demonstrated good sensitivity and specificity compared to *T. canis* ES as antigens for diagnosis of human toxocariasis. One of 33 individuals seropositive by employing *T. canis* ES as antigens was seronegative by *T. cati* ES antigens. The ELISA of the current study presented good sensitivity (97%) and specificity (96.7%). In a study by Roldan et al. (10), sensitivity and specificity



**Fig. 2.** Frequency of immunodominant proteins (kDa) of anti-*T. cati* ES antigens recognized by 33 serum samples from toxocariasis.



were 100% and 95.5%, respectively, considering healthy individuals, and 67.9% also considering sera from patients with other helminthiasis. In another study of Roldan et al. (20), sensitivity and specificity were 100% and 90%, respectively, in *Toxocara* infection. This result shows that *T. canis* ES antigens provided higher sensitivity for the immunodiagnosis of human toxocariasis.

Previous studies have reported that Western blotting technique has greatly decreased the risk of cross-reaction in studies carried out in humans with toxocariasis (7, 21). We found a characteristic pattern of five antigenic bands that were recognized by sera from clinically and serologically confirmed human toxocariasis. Our findings revealed that reactivity towards the 42- and 50-kDa was not obtained with sera from human infected with other parasitic diseases, indicating that these bands can be used as specific bands for diagnosis of human toxocariasis.

Kennedy et al. (22) studied the immune responses of mice to surface component and ES glycoprotein of infective larvae of *T. cati* and determined that three bands of 32, 120, and 400 kDa obtained from surface antigens and five bands of 28, 32, 60, 120, and 400 kDa obtained from ES glycoproteins could be used for diagnosis of toxocariasis. Two of these antigens were correlated with the bands present in our study. Magnaval et al. (7) obtained sera from human patients infected with *Toxocara* and performed Western blotting assay following SDS-PAGE with *T. canis* ES antigens in order to evaluate their immunodiagnostic potential. They reported that serum samples with toxocariasis recognized two groups of antigenic polypeptides: the first included bands of 24, 28, 30, and 35 kDa (low molecular weight) and other consisted bands of 132, 147, and 200 kDa (high molecular weight). These authors found that the low molecular weight fractions were specific to *T. canis* infection. Nunes et al. (23) observed numerous bands in patients with signs of VLM, among which five principal components can be distinguished—above 205, around 205, 116–97, 55–50, 35–29 kDa, and agree in that the low molecular weight fractions do not present any cross-reactivity with other ascariid antigens. The authors have found that the 55–50 kDa antigenic band had cross-reactions mainly with sera from patients with ascariasis (*Ascaris suum*).

In summary, we have demonstrated that *T. cati* ES antigens can be used for sensitive and specific diagnosis of toxocariasis using an ELISA. However, the ELISA using *T. canis* ES antigens is more sensitive for diagnosis of human toxocariasis than *T. cati* ES antigens. The work has clearly demonstrated that Western blotting using the 42- and 50-kDa bands is a useful tool and reliable method for confirmatory diagnosis of human toxocariasis.

## ACKNOWLEDGMENTS

We thank the Parasitology Laboratory, Faculty of Health Sciences, Kobe University Graduate School of Health Sciences, Japan, for providing some chemical and instrumental facilities. The authors thank the staff, Parasitology Laboratory, Shiraz University of Medical Sciences for technical assistance and Prof. Fasihi-Harandi from Kerman University of Medical Sciences, Iran, for providing positive serum samples. This study was supported by grant number 2893 from SUMS. The authors declare that there is no conflict of interest statement.

## REFERENCES

1. Despommier D. Toxocariasis: Clinical aspects, epidemiological, medical ecology and molecular aspects. *Clin Microbiol Rev* 2005;16:265–272.
2. Azizi S, Oryan A, Sadjjadi SM, Zibaei M. Histopathologic change and larval recovery of *Toxocara cati* in experimentally infected chickens. *Parasitol Res* 2007;102:47–52.
3. Rubinsky-Elefant G, Hirata CE, Yamamoto JH, Ferreira MU. Human toxocariasis: Diagnosis, worldwide seroprevalences and clinical expression of the systemic and ocular forms. *Ann Trop Med Parasitol* 2010;104:3–23.
4. Sadjjadi SM, Khosravi M, Mehrabani D, Oryan A. Seroprevalence of *Toxocara* infection in school children in Shiraz, Southern Iran. *J Trop Pediatrics* 2000;46:327–330.
5. Zibaei M, Sadjjadi SM, Sarkari B. Prevalence of *Toxocara cati* and other intestinal helminths in stray cats in Shiraz, Iran. *Trop Biomed* 2007;24:39–43.
6. Strube C, Heuer L, Janecek E. *Toxocara* spp. infections in paratenic hosts. *Vet Parasitol* 2013;193:375–389.
7. Magnaval JF, Faber R, Maurieres P, Charlet JP, de Larrad B. Application of the Western blotting procedure for the immunodiagnosis of human toxocariasis. *Parasitol Res* 1991;77:697–702.
8. Ishiyama S, Ono K, Rai SK, Uga S. Method for detecting circulating *Toxocara canis* antigen and its application in human serum samples. *Nepal Med Coll J* 2009;11:9–13.
9. Maraghi S, Rafiei A, Hajihosseini R, Sadjjadi SM. Seroprevalence of toxocariasis in hyper eosinophilic individuals in Ahwaz, south-western Iran. *J Helminthol* 2012;86:241–244.
10. Roldan WH, Espinoza YA. Evaluation of an enzyme-linked immunoelectrotransfer blot test for the confirmatory serodiagnosis of human toxocariasis. *Mem Inst Oswaldo Cruz* 2009;104:411–418.
11. Fillaux U, Magnaval J. Laboratory diagnosis of human toxocariasis. *Vet Parasitol* 2013;193:327–336.
12. de Savigny DH. In vitro maintenance of *Toxocara canis* larvae and a simple method for the production of *Toxocara* ES antigens for use in serodiagnosis tests for visceral larva migrans. *J Parasitol* 1975;61:781–782.
13. Zibaei M, Sadjjadi SM, Jahadi Hosseini SH, Sarkari B. A method for accelerating the maturation of *Toxocara cati* eggs. *Iran J Parasitol* 2007;2:39–42.
14. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248–254.
15. Elefant GR, Shimizu SH, Sanchez MC, Jacob CM. A serological follow-up of toxocariasis patients after chemotherapy based on the detection of IgG, IgA, and IgE antibodies by enzyme-linked immunosorbent assay. *J Clin Lab Anal* 2006;20:164–172.
16. Watthanakulpanich D, Smith HV, Hobbs G, Whalley AJ, Billington D. Application of *Toxocara canis* excretory-secretory antigens

- and IgG subclass antibodies (IgG-4) in serodiagnostic assays of human toxocariasis. *Acta Trop* 2006;106:90–95.
17. Glickman L, Schantz P, Dombroske R, Cypess R. Evaluation of serodiagnostic tests for visceral larva migrans. *Am J Trop Med Hyg* 1978;27:492–498.
  18. Yamasaki H, Araki K, Lim PKC, Zasmy N, Mak JW, Taib R, et al. Development of highly specific recombinant *Toxocara canis* second-stage larvae excretory-secretory antigen for immunodiagnosis of human toxocariasis. *J Clin Microbiol* 2000;38:1409–1413.
  19. Jin Y, Shen C, Huh S, Sohn WM, Choi MH, Hong ST. Serodiagnosis of Toxocariasis by ELISA using crude antigen of *Toxocara canis* larvae. *Korean J Parasitol* 2013;51:433–439.
  20. Roldan WH, Cornejo W, Espinoz YA. Evaluation of the dot-enzyme-linked immunosorbent assay in comparison with standard ELISA for the immunodiagnosis of human toxocariasis. *Mem Inst Oswaldo Cruz* 2006;101:71–74.
  21. Magnaval JF, Glickman LT, Dorchies P, Morassin B. Highlights of human toxocariasis. *Korean J Parasitol* 2001;39:1–11.
  22. Kennedy MW, Maizels RM, Meghji M, Yong L, Qureshi F, Smith HV. Species-specific and common epitopes on the secreted and surface antigens of *Toxocara canis* and *Toxocara cati* infective larvae. *Parasite Immunol* 1987;9:407–420.
  23. Nunes CM, Tundisi RN, Garcia JF, Heinemann MB, Ogassawara S, Richtzenhain LJ. Cross reactions between *Toxocara canis* and *Ascaris suum* in the diagnosis of visceral larva migrans by Western blotting technique. *Rev Ins Med Trop Sao Paulo* 1997;39:253–256.