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Original Article

Evaluation of a 70 kDa Excreted/Secreted Coproantigen Immunoassay for the Detection of *Toxocara canis* in Dogs

Ana Cristina González-Morales [1], Zinnia Judith Molina-Garza [1], Ricardo Gomez-Flores [2], Juan José Zárate-Ramos [3], *Lucio Galaviz-Silva [1]

1. *Department of Invertebrates Zoology, Biological Sciences School, Autonomous University of Nuevo León, San Nicolás de los Garza, Nuevo León, México*
2. *Department of Immunology and Virology, Biological Sciences School, Autonomous University of Nuevo León, San Nicolás de los Garza, Nuevo León, México*
3. *Department of Parasitology, Agricultural Sciences Campus, Veterinary Medicine and Zootechnics School, Autonomous University of Nuevo León, San Nicolás de los Garza, Nuevo León, México*

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***Correspondence Email:**
lucio.galavizsl@uanl.edu.mx

Abstract

Background: We aimed to develop a sandwich ELISA, using polyclonal antibodies against excretory/secretory (E/S) antigens specific to coproantigens present in *Toxocara canis*-positive dogs.

Methods: Antibodies were produced at Biological Sciences School, Autonomous University of Nuevo León, *México* in 2023 by immunization of rabbits with antigenic extracts from in vitro cultures of *T. canis* larvae. Assays were performed on 100 stool samples from pet dogs, measuring sensitivity, specificity, and cross-reactivity against other parasitic infections.

Results: High values of sensitivity (100%), specificity (90.9%), and positive (93.47%) and negative (95.45%) predictive values were obtained, respectively. We obtained an E/S protein with a molecular weight of 70 kDa, which showed high sensitivity and specificity by ELISA, but it presented cross-reactivity against *Ancylostoma caninum* and *Strongyloides stercoralis*.

Conclusion: Additional studies are necessary to increase the specificity percentage since this assay demonstrated significant potential as a useful and inexpensive diagnostic tool for immunodiagnostic in dog feces.

Introduction

Toxocariasis is an important zoonotic helminthiasis caused by the roundworm *Toxocara* spp., particularly *T. canis* in dogs, but *T. cati* may also infect cats (1). Despite the global distribution of *T. canis* in the Americas, Africa, and Asian countries, reports of *T. canis* infection in Mexico are rare (2, 3).

Commonly observed disease signs include visceral (VLM) and ocular (OML) larva migrans, neurotoxocariasis, and hidden or covert toxocariasis (4, 5). Ingestion of infective *Toxocara* eggs, which are found in canid and felid feces, is the main route of human infection.

Traditional diagnosis of toxocariasis involves the detection of eggs in fecal samples of the definitive host or larvae in tissue biopsies in paratenic hosts, including humans, by microscopy, which is time-consuming and requires highly trained personnel (6). ELISA and Western blot are two types of tests available for the immunodiagnosis of *Toxocara* infection, using Toxocariasis-secreted (ES) antigens (7). However, it shows cross-reactivity against other helminths (8).

Application of recombinant antigens for the detection of toxocariasis in various animal species has demonstrated higher sensitivity and specificity, compared with native E/S antigens. Recombinant antigens for serodiagnosis involved the tests *TES*-30 (8) and *TES*-120 (1), but an increased number of such tests are required for validation (9). We aimed to evaluate a 70 kDa excreted/secreted coproantigen from *T. canis* for in vitro diagnosis of animal toxocariasis.

Materials and Methods

Sample collection

Collection of dog feces samples was performed under the informed consent of the owners from suburban (Cities of Allende, Montemorelos, and Linares, México between 2023 and 2024) and urban municipalities (Cities of Apodaca, San Nicolás de los Garza, General Escobedo, Monterrey, Guadalupe, San Pedro Garza García, Santa Catarina, García, and Juárez, located in the northeastern state of Nuevo León, México (25°40'30.3" N 100°19.108' W) and preserved in neutral buffered formalin. They were then transported to the laboratory for analysis.

This study was approved by the Animals Experimentation Ethics Committee of the Autonomous University of Nuevo León, México, with the number CEIBA-2022-002.

Parasitological study as gold standard

Samples were examined by optical microscopy (OM), as the gold standard (10), and by the technique of centrifugation and flotation with zinc sulphate (11).

Adults T. canis collection

Naturally infected canids were dewormed with 15 mg/kg pyrantel pamoate to obtain adult specimens of *T. canis*. Once worms were released, they were washed with physiological saline to eliminate residual fecal material and further identified by visualizing their morphologic characteristics (12).

In vitro spawning of adult specimens

Female adult worms were selected and washed in 0.85% saline solution, followed by a solution of 1% penicillin (10,000 U; Thermo Fisher Scientific, San Nicolás de los Garza, Nuevo León, México), 10 mg of streptomycin (Thermo Fisher Scientific, México), and 25 µg/mL amphotericin B (Sigma-Aldrich, St. Louis, MO, USA). Spawning was induced by hysterectomy. Released eggs were aseptically collected and incubated at 28 °C for 45 d. The development of the embryonation of L2/L3 larvae was daily monitored by OM at 10 × and 40 × (12, 13).

In vitro hatching and maintenance of the larval phase

Larvae were obtained as reported by Zibaei et al. (14) with some modifications. In brief, embryonated eggs were washed with sterile

phosphate-buffered saline (PBS) and centrifuged at 3000 rpm for five minutes. Hatching was stimulated with 5% sodium hypochlorite solution for two hours with stirring at 50 rpm, after which larvae were washed twice with sterile PBS and centrifuged at 3000 rpm and 4 °C for five minutes. Supernatant fluids were discarded and larvae were rinsed with RPMI-1640 medium, and incubated at 28 °C in 10 mL of RPMI-1640 medium with antibiotics solution plus 1% glucose (14, 15).

Antigenic extract

To obtain E/S antigens, we weekly collected seven milliliters of culture medium, which was replaced with 10 mL of fresh culture medium, filtered through a 22 µm-pore size nitrocellulose membrane (Millipore, Watford, UK), dialyzed with a 200,000 Da molecular weight cut-off membrane against 50 volumes of dH_2O for 24 h, and frozen at -40 °C, until lyophilized (12,13).

Protein concentration

The protein concentration of the E/S antigen and the polyclonal anti-E/S antibodies was determined by Bradford´s method (16).

Polyclonal antibodies (pAbs) production

Two-month-old New Zealand rabbits were intramuscularly injected with two milligrams of the E/S antigen mixed with Freund's complete adjuvant (1:1; Sigma-Aldrich), following an administration of two booster doses of 0.5 mg of the antigenic extract, emulsified in an equal volume of incomplete Freund's adjuvant, seven days apart (17). Rabbits were bled 10 d after each booster, and serum was collected and kept at -30 °C. The sodium periodate method was used to conjugate anti-E/S IgG antibodies with horseradish peroxidase (HRP; Sigma-Aldrich) (17-19).

SDS-PAGE

The E/S extract protein profile was determined by sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis (SDS–PAGE), under reducing conditions (20), with 1 M Tris-HCL (pH = 6.8), 20% glycerol, 2% SDS, 0.5% bromophenol blue, 12.5 mM EDTA, 5 mM dithiothreitol, and 5% 2-β-mercaptoethanol, run for one hour at 100 V, and stained with Coomassie blue R-250 overnight to visualize bands (21).

Western blotting (WB)

The E/S antigen separated by SDS-PAGE was then transferred to a nitrocellulose (NC) membrane, after which each lane was cut into strips and blocked with PBS-5% skim milk, followed by incubation with rabbit serum (diluted 1:100 in PBS-5% skimmed milk solution) at 4 ℃ overnight and the addition of secondary HRP-conjugated polyclonal anti-rabbit IgG antibodies. The blot was revealed with AP-1X and NBT-BCIP (Boehringer Mannheim Corp., Germany) (13).

Standardization of the copro-ELISA

Determination of the optimal concentration of the antigenic extract was evaluated at 1, 2, 4, and 6 µg/mL, using 1:1000 to 1:128000 dilutions of native anti-E/S IgG polyclonal antibodies. A batch of antibodies purified by affinity chromatography, in a HiTrapTM Protein G packed column (Cytiva, Sweden AB), was used as the developing antibodies. Protein concentration was quantified by Bradford´s method and antibodies were conjugated with peroxidase (Sigma-Aldrich) and diluted 1:4000 (17). Criteria used were the following: none, little, moderate, and high discrimination between samples.

Copro-ELISA

Sandwich ELISA used in this study was based on the capture and detection of *T. canis* antigens in stool samples, as previously reported (22). Microplate (Immulon 4HBX; Thermo Fisher Scientific, Waltham, MA, USA) wells were coated with 0.8 µg/mL of anti-E/S polyclonal IgG antibodies in carbonate/bicarbonate buffer (0.05 M at pH 9.6) and incubated at 4 °C for 24 h, after which

they were washed five times with 200 µL of 150 mM PBS supplemented with 0.05% (v/v) Tween 20 (Sigma-Aldrich). Blocking was performed with 200 µL of 5% skim milk (Becton Dickinson, Franklin Lake, NJ, USA) in PBS-0.3% Tween for one hour at room temperature. A 50 µL fecal sample was placed in each well and incubated for one hour. Detection was developed using 0.9 µg/mL anti-E/S polyclonal IgG antibodies conjugated with peroxidase and incubating for one hour, followed by incubation for 20 min with substrate (100 µL of the 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid; Sigma-Aldrich) mixed in citrate buffer pH 7.0. Next, optical densities (OD) were read at 450 nm in an Asys UVM 340 ELISA reader (Biochrome Ltd., Cambridge, UK) (19, 22).

Controls

Ancylostoma caninum, Dipylidium caninum, Strongyloides stercoralis, Trichuris vulpis, and *Giardia lamblia* were used to evaluate the sensitivity and specificity of the native anti- E/S antibodies from the Copro-ELISA-E/S. In addition, *T. canis*-positive feces were donated by the Parasitology Laboratory in Veterinary Medicine and Zootechnics School at Autonomous University of Nuevo León, México (FMVZ-UANL).

Statistics

The cut-off point was determined from the positive and negative controls, using the formula $\bar{X}$ + 3 SD, and the diagnostic indices of sensitivity (S), specificity (E), and negative, (NPV) and positive (PPV) predictive values of the E/S-native ELISA test. A chi-square test (X^2) was used to determine significant differences ($P < 0.05$) between the ES-native ELISA and the coprological technique, as the gold standard. All statistical analyzes were processed in the SPSS ver.21 program (IBM Corp., Armonk, NY, USA).

Results

Stool samples

We analyzed 100 stool samples, including 70 positives, of which 46 (66%) presented eggs and 24 (34%) adults, and 30 negative samples.

Identification of T. canis eggs and adults

Worms' length ranged from 4 cm to 12 cm, possessed whitish colors and a ringed cuticle, and the cephalic region had two lateral cuticular extensions. Specimens were sexed based on males and females' distinct morphology. In this regard, females measured up to 12 cm in length, whereas males were not larger than five centimeters. In addition, females had a blunt and slightly thinner tip-type termination at their caudal end and males had a spicule at the caudal end. The distinctive morphological characteristics of this genus and the structural differentiation between male and female specimens allowed the identification of the recovered specimens as *T. canis* (Fig. 1).

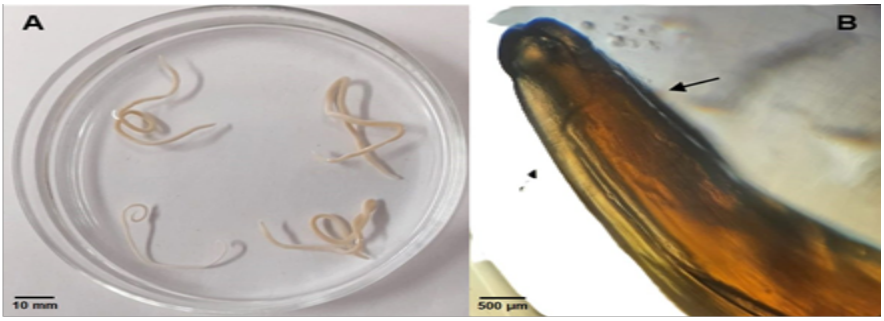

Fig. 1: A) Male and female specimens of *Toxocara canis* collected from canid feces. B) Cephalic part of an adult specimen, showing cervical wings (arrows) (Original picture)

Larvae culture and hatching

Eggs that were extracted after 30 d showed up to 80% embryonation in each batch, of which 70% developed the L2/L3 larval phase (Fig. 2).

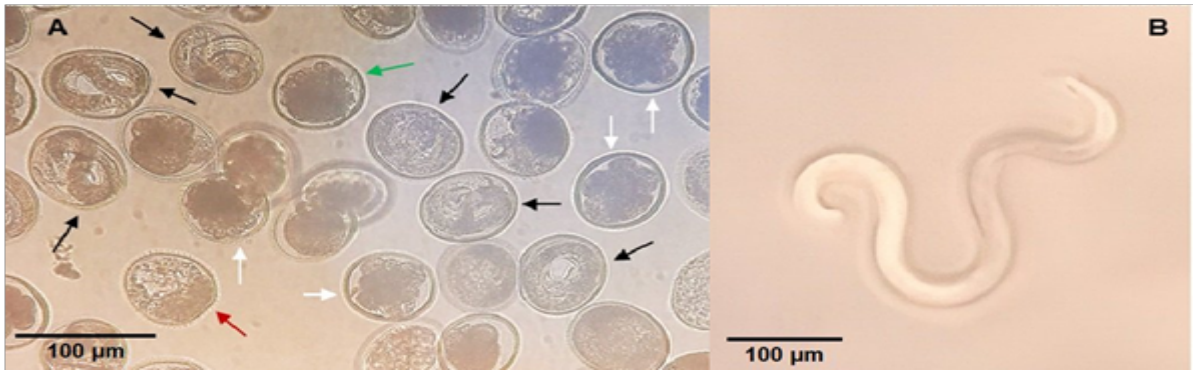

Fig. 2: A) Eggs obtained in vitro at different stages of development. White arrows indicate embryonated eggs with 14 to 16 blastomeres; green arrow shows an embryonated egg in the morula phase; red arrow shows a non-fertile egg; and black arrows shows eggs with at the second larval stage (L2). B) *T. canis* second-third juvenile stage (L2/L3) (Original picture)

Antigen extract and protein profile

We obtained three batches containing 1.4, 1.7, and 2 mg/mL antigen respectively, in a final volume of 80 mL. E/S antigen dialysates of *T. canis* larvae showed a molecular weight band of 70 kDa by SDS-PAGE, which was confirmed by WB (Figs. 3 and 4).

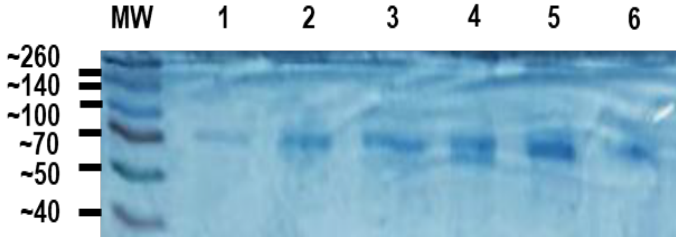

Fig. 3: Electrophoresis of different fractions obtained from the L2 larval culture of *T. canis*. MW, molecular weight markers; lanes 1 to 6 show larval culture fractions obtained on weeks 1 through 6

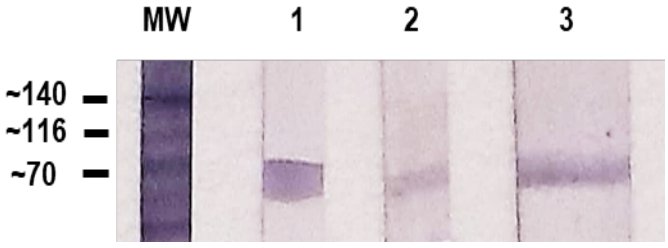

Fig. 4: Western blot of three different batches of the antigens extract obtained from the culture of the second-larval stage of *T. canis*, using rabbit anti-ES IgG antibodies. MW, Molecular weight markers; lanes 1 to 3 show *T. canis* TES antigen preparation

Copro-ELISA technique

The optimal concentration of the TES antigen was 1 µg/mL for the capture antibodies and the optimal dilution evaluated was 1:8000, whereas for the conjugated antibodies, optimal dilution was 1:4000. These concentrations presented the highest difference between positive and negative controls (Table 1).

Table 1: Discrimination between positive and negative samples from *T. canis* and other parasitic infections.

AntiTES-Ig	*Positive samples*	*Negative samples*	*Samples from other parasites*
1:1000	-	+	+
1:2000	+	-	++
1:4000	++[a]	-	+
1:8000	++[b]	-	-
1:16000	+++	-	-

[a] Conjugated polyclonal antibodies [b] Capture polyclonal antibodies. -, No discrimination between samples; +, limited discrimination between samples; ++, moderate discrimination between samples; and +++, high discrimination between samples

Once the optimal conditions were obtained, the cut-off value was determined at an OD = 0.206. Results of the OM of 100 samples showed 40 positive samples for *T. canis*, 30 for other parasites (five of *Trichuris vulpis*, seven of *Dipylidium caninum*, seven of *Ancylostoma caninum*, four of *Strongyloides* spp., and seven of *Giardia* spp.), and 30 negative samples (Fig. 5). The 40 positive samples analyzed showed reactivity in the Copro-ELISA over the cut-off value, thus a sensitivity of 100% was obtained. Cross-reactivity was observed in three samples (false positives) out of the 30 samples positive for other parasitic infections, obtaining a specificity of 90.9%. Samples that presented cross-reactivity were positive for *A. caninum* and *S. stercoralis*. We did not show reactivity in the 30 negative samples. The negative and positive predictive values were respectively 95.45% and 93.47%. Since all positive samples showed an OD over the cut-off value, only four samples produced uncertain values, which were considered false positive results.

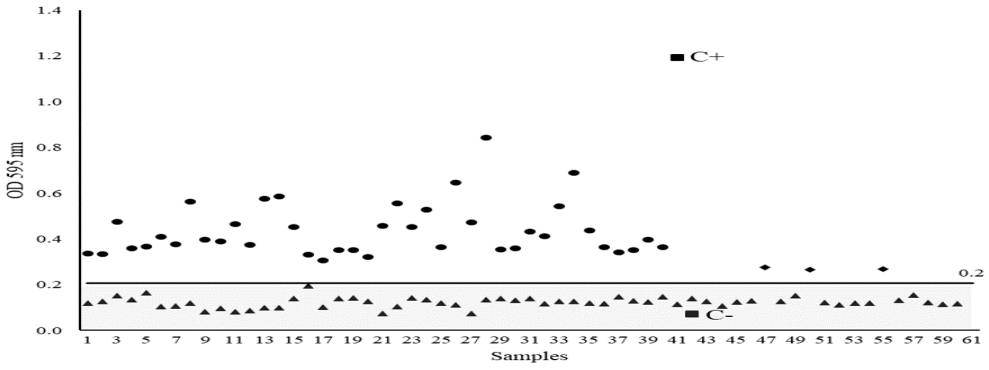

Fig. 5: Detection of *T. canis* in dogs by the ELISA technique. The cut-off value was established at an OD = 0.206, which corresponds to 1 µg/mL of the parasite extract. OD values were obtained at 595 nm (n = 100). Negative samples are shown below the cut line in the shaded area (triangles) and positive samples are shown as circles. Controls (C+ and C-) are shown as squares and only three cases with cross-reactivity (diamonds) to anti- native E/S antibodies are observed; two of them corresponded to *A. caninum* and *S. stercoralis*

The contingency table showed $X^2 = 000004$, df = 1, and $P < 0.05$. Therefore, the proportion of samples positive to the combined positive score (CPS) is different from the samples that were positive to the ELISA developed in the present study (Table 2).

Table 2: Contingency table between the techniques used and results of the detection of *T. canis* in dog stool samples

Results	***Techniques***		
	ELISA	OM	Total
Positive	37 (63.8%)	21 (36.2 %)	58
Negative	6 (14.3 %)	36 (85.7 %)	42
Total	43	57	100

$X^2 = 0.000004$; $P < 0.05$

Discussion

Toxocariasis is a zoonotic parasitosis with a worldwide distribution, which is underdiagnosed due to its non-specific signs of disease. It is considered one of the five neglected parasitic diseases (22). This reason prompted the development of our improved Copro-ELISA test for stool samples, focusing on standardizing an immunological, rapid, and sensitive technique for the diagnosis of this parasite.

We showed that the prevalence of infected dogs in Nuevo León was higher than expected (70%), as compared with a previous report from the Mexican city Puerto Escondido, Oaxaca (47.78% positive dogs) and similar to that reported in Tulancingo, Hidalgo (71.11% positive dogs) (23,24). One of the main problems of epidemiology in Mexico is the unknown prevalence of this disease in most states, including Nuevo León (23, 24).

The traditional diagnosis of toxocariasis in dogs is determined by examination of feces and the observation of eggs or adult worms, although the results are sometimes unsatisfactory and in the case of human infection, it may misdiagnose patients if trained personnel is not available. Due to the problems in diagnosis, the use of serological methods such as ELISA has been replaced (25).

A limited number of Copro-ELISA tests based on the detection of E/S antigens have been developed for *T. canis*, which have shown distinct sensitivities and specificities. Our present results demonstrated higher sensitivity and faster results for its detection compared to the classic deworming method with arecoline (26). A commercial and expensive ELISA kit for diagnosis in dogs is currently available (US$ 572.75/96 tests; https://www.abbexa.com/dog-toxocara-canis-igg-elisa-kit). Hence the importance of the improved Copro-ELISA test developed in the present study, which may be considered an effective alternative for diagnosis. Its main characteristic is the use of specific anti-native ES antibodies that facilitate professionals to provide a timely diagnosis and obtain a reliable epidemiological profile as a short-term goal of this disease.

The strategy for standardization of our technique consisted of obtaining the E/S antigen from *T. canis* by a modified method (12,27). We first administered two doses of pyrantel pamoate for a complete deworming since a single dose is not effective probably due to the amount of dewormer administered or the degree of parasitosis of the infected animal. Implementing a deworming schedule was necessary to avoid euthanasia, as previously reported (12, 20). We then included a step to improve the percentage of eggs that were obtained in the embryonic phase, which was the highest (up to 70% in 45 d) in a short-

er period than that reported in previous studies. In addition, we obtained 80 mL of supernatant fluid from the larval culture, which resulted in a better efficiency but in a shorter period (12), as compared with other researchers, who obtained 200 mL because they kept larva cultures for six months. Furthermore, we lyophilized the antigens extract and dialyzed it for purification, which improved the detection level of the Copro-ELISA, unlike other procedures where the extract was not purified. In this regard, we produced antigen concentrations of 1.4, 1.7, and 2 mg/mL in three different batches (10, 26, 29).

In the present study, we demonstrated a distinct 70 kDa coproantigen. It may be related to C-type lectins, which are the most abundant components in the secretion products of *T. canis* and have homology to TES-32/TES-70 and mammalian proteins, as well as CD23 and the mannose receptor in macrophages. Glycans play a key role in modulating the immune response, in addition to having the potential to interfere with Toll-like receptor (TLR) and C-type lectin receptor activation in dendritic cells, thus evading the immune response (28).

A series of proteins considered to be of low molecular weight (24 kDa to 70 kDa) have been reported in previous studies and proposed as candidates for the diagnosis of toxocariasis, since they are highly specific to *T. canis* and present low or no reactivity against other helminths. However, proteins between 55 kDa and 66 kDa have shown cross-reactions with *Ascaris lumbricoides*. Some authors recommended developing research aimed at increasing the detection level of these antigens to improve the specificity and sensitivity of the ELISA technique and avoid cross-reactions with other helminths (7, 18, 29).

According to our results in the standardization of the Copro-ELISA, it is recommended to use at least 1 µg/mL of E/S antigen to be detected by polyclonal antibodies, unlike other studies that reported concentrations between 2 µg/mL to 50 µg/mL. We also suggested a 1:8000 dilution as capture antibodies and 1:4000 for conjugated antibodies, unlike other researchers that used antibody dilutions from 1:100 to 1:24,000. Although the dilutions are higher, the number of diluted antibodies necessary to perform the technique is still small (12, 13).

Our developed Copro-ELISA improved the optimal sensitivity up to 100%, which was higher than that previously reported by others (12, 13), although the specificity was 90.9%, which was lower than a previous study (30). Copro-ELISAs have been also used for the diagnosis of other helminthiases, such as the one caused by *Echinococcus granulosus* (16). However, in the present study, we developed one of the first Copro-ELISA based on native anti-*TES* antibodies implemented for the diagnosis of the *T. canis* nematode in canids.

In addition, we observed three false positive samples. However, cross-reactions have been reported against other helminths such as *A. lumbricoides* and in our study we showed cross-reactions with *A. caninum* and *S. stercoralis*. The parasite load and the time post-infection with *T. canis* may influence the results of the test, due to the number of parasites that are present and their low protein concentration. Therefore, we recommend developing a comprehensive post-infection study (31).

Conclusion

The present study reported one of the first Copro-ELISA for the detection of polyclonal anti-native E/S antibodies to *T. canis*. Our test showed 100% sensitivity and 90.9% specificity, as compared with the ELISA designed for the detection of human toxocariasis. Additional studies should then be aimed to increase the specificity percentage. However, our test resulted in an effective and inexpensive diagnostic tool for immunodiagnosis of toxocariasis in dog feces.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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