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Development of a Highly Specific Recombinant *Toxocara canis* Second-Stage Larva Excretory-Secretory Antigen for Immunodiagnosis of Human Toxocariasis

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The specificity of the recombinant *Toxocara canis* antigen developed for the immunodiagnosis of human toxocariasis was compared with that of the excretory-secretory antigen from *T. canis* second-stage larvae (TES) by enzyme-linked immunosorbent assay. A total of 153 human serum samples from patients infected with 20 different helminths, including 11 cases of toxocariasis, were examined. No false-negative reactions were observed for the toxocariasis cases. When the TES was used at concentrations of 0.5 and 0.125 μg/ml, cross-reactions were observed in 79 (55.6%) and 61 (43.0%) of 142 cases, respectively. In contrast, when the recombinant antigen was tested at a concentration of 0.5 μg/ml, cross-reactions were observed in 19 (13.4%) of 142 cases. At a concentration of 0.125 μg/ml, however, the cross-reaction rate decreased sharply to only 2.1%, corresponding to 3 of 142 cases. The cross-reactions occurred with one case each of gnathostomiasis, paragonimiasis with *Paragonimus miyazakii*, and spirometriasis, in which high antibody titers were detected. In addition, the recombinant antigen showed negative reactions with serum samples from patients infected with *Ascaris* and hookworms, which are the most common parasites in the world. These findings are also supported by experiments with animals infected with *Ascaris* and hookworm. From these results, the recombinant antigen is highly specific for toxocariasis and may provide more reliable diagnostic results than other methods.

Toxocariasis is an important zoonosis caused by the infection of humans with ascarid nematode larvae of *Toxocara canis* from dogs and *T. cati* from cats (10, 24). Once the embryonated *Toxocara* eggs are accidentally ingested by the host animal, the larvae hatch in the small intestine and migrate through the somatic organs. In humans, two types of larva migrans syndromes have been identified: visceral larva migrans (4) and ocular larva migrans (20). Recent studies suggest that the disease frequently assumes the features of a syndrome comprising chronic weakness, abdominal pain, various signs of allergy, and hypereosinophilia (9, 29). Toxocariasis has been proposed as a possible etiology in various neurologic syndromes (15, 28).

The diagnosis of human toxocariasis currently depends on immunological examinations because it is extremely difficult to detect an infective *Toxocara* larva(e) in biopsy samples. In most immunological tests, excretory-secretory antigens from *T. canis* second-stage larvae (TES) have been used conventionally (7, 11). Western blotting (14) and, more recently, ToxocaraCHEK (1) have been used, both of which detect immunoglobulin G against TES. However, since cross-reactivities have been reported for these procedures when the TES for some helminthic infections are used (8, 11, 13, 14), the development of an antigen more specific than TES has been attempted. Recently, we developed a recombinant *T. canis* second-stage larva antigen corresponding to the 30-kDa protein of the TES secreted by infective larvae and tested its specificity as an antigen with limited numbers of serum samples from helminthiasis patients (30). In the present study, the specificity of the *T. canis* recombinant antigen has been evaluated by comparing it with TES in an enzyme-linked immunosorbent assay (ELISA) using serum samples from patients infected with a wide variety of helminths.

MATERIALS AND METHODS

Preparations of TES and recombinant antigen. For the preparation of TES, the protocol of de Savigny was modified (6). The culture medium (RPMI 1640) for *T. canis* second-stage larvae was collected every 3 to 4 days, pooled, and centrifuged to precipitate all debris. The resulting supernatant was filtered through a 0.2-μm Supor Acrodisc 32 syringe filter (Gelman Sciences) into a Spectrapor dialysis tube (molecular weight cutoff, 6,000 to 8,000; Spectrum Medical Industries Inc.). The solution was dialyzed against 250 volumes of chilled sterile distilled water at 4°C until the phenol red disappeared. After dialysis, the supernatant was concentrated with a vacuum concentrator, reconstituted with sterile distilled water, and then kept in aliquots at −20°C. The recombinant *T. canis* antigen was prepared by the protocol described previously (30). Briefly, expression of the recombinant antigen in bacteria was induced by adding isopropyl-β-D-thiogalactopyranoside at a final concentration of 0.4 mM at 37°C for 3 h. The induced cells were disrupted by sonication in 20 mM Tris-HCl, pH 8.0, containing 100 mM NaCl and 1% Triton X-100. The insoluble recombinant protein was solubilized in 8 M urea in 20 mM Tris-HCl, pH 8.0, containing 100 mM NaCl and then purified on TALON metal affinity resin (Clontech). The recombinant antigen was eluted with 50 mM imidazole and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12). Protein content was measured by the Bradford method with a protein assay kit (Bio-Rad). The antigen was kept at −80°C until use.

Human serum samples. A total of 153 serum samples from patients with confirmed helminthiasis proven parasitologically and/or clinically were examined. Most samples were also serologically positive for homologous parasite antigens except in two cases of capillariasis. Since the antigen from *Capillaria philippinensis* was not available, TES had to be used in these cases. Serum samples from patients infected with roundworm (*Ascaris lumbricoides*), hookworm (*Ancylostoma duodenale* and/or *Necator americanus*), and fish tapeworm (*Diphyllobothrium nihonkaiense*) were serologically negative for homologous parasite antigens. Of nine patients with suspected toxocariasis with *T. canis*, six with

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the visceral type showed marked eosinophilia and hepatic lesions on ultrasound, and two of them have been reported previously (21). The remaining three patients had the ocular type, with unilateral uveitis, protuberant exudative lesions, and vitreous opacity. All patients with toxocariasis were negative for toxoplasmosis. Toxocariasis with *T. cati*, dirofilariasis with *Dirofilaria immitis*, and brugiasis with *Brugia malayi* have been described previously (30). One patient each with infection with African eye worm, *Loa loa* (22), and a filarial worm, *Mansonella perstans*, were also examined. In six cases of creeping eruption caused by larval *Gnathostoma* spp., marked eosinophilia was observed, and the patients were seropositive for somatic antigens from *Gnathostoma doloresi* adult worms. Five cases of tropical eosinophilia were diagnosed on the basis of the following criteria: persistent hypereosinophilia in the peripheral blood (>10%); respiratory manifestations such as cough and asthma; high titers of anti-*Dirofilaria immitis* antibodies, travel to tropical areas such as Africa or Southeast Asia; rapid improvement of clinical symptoms on treatment with diethyl carbamazine; and a decrease in antibody titers against *D. immitis* antigen after treatment. Of more than 200 Japanese anisakiasis patients, 20 whose sera showed a high optical density (OD) when they were tested against larval *Anisakis* antigen in the ELISA were selected. Nine cases of schistosomiasis japonica were from an area where it is endemic in Leyte Island, Philippines. Twenty-four Japanese patients with lung fluke diseases (13 cases of *Paragonimus miyazakii* infection and 11 cases of *Paragonimus westermani* infection) showed clinical symptoms such as eosinophilia, massive pleural effusion, and pulmonary consolidation seen in chest radiographs. Cases of fascioliasis caused by infection with liver fluke were diagnosed based on clinical findings such as marked eosinophila, liver abscess, fever, and abdominal pain. In most patients with sparganosis, plerocercoids of *Spirometra erinaceieuropaei* were recovered upon surgery. Four cases infected with adult *S. erinaceieuropaei* worms were also examined. Of three patients with echinococciasis or hydatid disease, one was infected with *Echinococcus granulosus* and the others were infected with *Echinococcus multilocularis*. Serum samples from 40 healthy Japanese subjects without infections were used to estimate the mean and standard deviation (SD), and the pooled serum samples from the 40 individuals were used as a negative control in further experiments.

Serum samples from experimental animals infected with either *Ancylostoma caninum*, *Ascaris suum*, or *T. canis*. Since we were unable to obtain human serum samples positive for *Ascaris* and hookworm antigens, animal serum samples positive for either *Ascaris suum* or *Ancylostoma caninum* antigens were used to examine the cross-reactivities of TES and the recombinant antigen. BALB/c mice and Wistar rats were experimentally fed 1,000 embryonated *Ascaris suum* eggs, and blood samples were collected 3 to 4 weeks after infection. Five serum samples from rabbits infected with *Ancylostoma caninum*, which had been stored, were used. Production of anti-*Ascaris* or anti-*Ancylostoma* antibodies was confirmed by ELISA before use. Two serum samples each from mice and rabbits infected with *T. canis* larvae were used as positive controls.

ELISA. ELISA was performed as reported by Matsuda et al. (18) with slight modifications. Briefly, 96-well microtiter plates (Dynatech; M-129A) were sensitized with either TES or recombinant antigen at a concentration of 0.5 or 0.125 μg of proteins per ml in 0.05 M bicarbonate buffer, pH 9.6 (100 μl/well), for 2 h at 37°C. Although the recombinant antigen was resolved in 20 mM Tris-HCl, pH 8.0, containing 100 mM NaCl and 8 M urea, the urea concentration was neglible and had no effect on the ELISA after dilution. The microtiter plates were washed three times with 0.15 M phosphate-buffered saline–0.05% Tween 20 (PBS/T) and then probed with a 1:200-diluted human serum sample (100 μl/well) in PBS/T containing 1% bovine serum albumin for 40 min at 37°C. After a wash, 100 μl of diluted rabbit anti-human immunoglobulin G conjugated with 1:10,000-diluted horseradish peroxidase (Cappel) was incubated for 35 min at 37°C. For color development, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) was added to each well as a substrate (0.3 mg/ml, 100 μl/well), and the reaction was terminated after 7 min by adding 50 μl of 1.25% sodium fluoride per well. Absorbance at 414 nm was monitored with a Multiscan Plus plate reader (Titertek). The cutoff point was set at three times the OD for the negative pooled serum samples from 40 healthy persons. This value corresponds to more than the mean plus 4 SD of the values for the 40 healthy individuals, as described below.

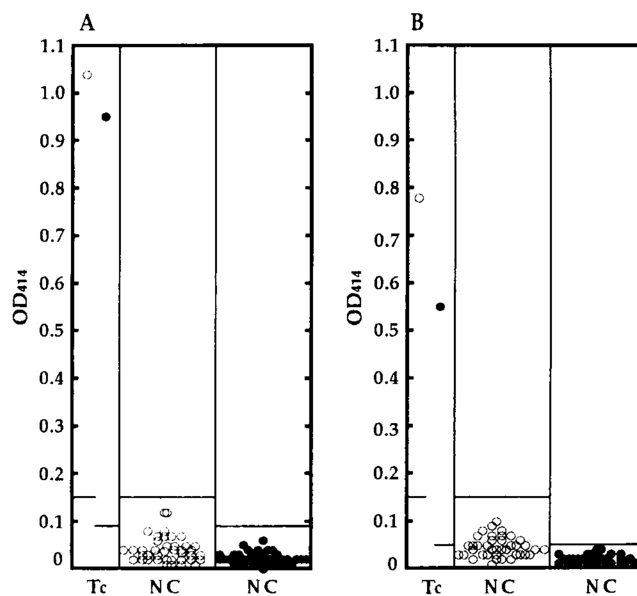

FIG. 1. Reactivities in ELISA of TES (○) and the recombinant antigen (●) against serum samples from 40 healthy individuals. Each antigen was sensitized at concentrations of 0.5 μg/ml (A) and 0.125 μg/ml (B). Bars are the cutoff points calculated on the basis of three times the OD value for the negative pooled serum. Tc, one patient with toxocariasis as a positive control; NC, individual healthy person.

RESULTS

Figure 1 shows the reactivities of the TES and recombinant antigen against serum samples from 40 healthy persons. The means and SDs obtained were 0.036 ± 0.022 and 0.021 ± 0.011 OD_{414} units for TES and recombinant antigen, respectively, at a concentration of 0.5 μg/ml. Similarly, values of 0.038 ± 0.020 and 0.017 ± 0.007 were obtained with TES and recombinant antigen, respectively, at 0.125 μg/ml. Based on these values, the means plus 4 SDs were 0.124 for TES and 0.065 for recombinant antigen at a concentration of 0.5 μg/ml. At 0.125 μg/ml, the means plus 4 SDs were 0.098 and 0.038 for TES and recombinant antigen, respectively. The OD_{414} values for the pooled sample from 40 healthy individuals were 0.052 for TES and 0.031 for recombinant antigen at a concentration of 0.5 μg/ml. At a concentration of 0.125 μg/ml, the OD_{414} values were 0.055 and 0.018 for TES and recombinant antigen, respectively. Since three times the OD_{414} value for the pooled human serum was larger than the means plus 4 SDs, the pooled serum sample was used as a negative control in further experiments.

Figure 2 illustrates the reactivities of TES and the recombinant antigen against various helminthic infections at a concentration of 0.5 μg/ml. Table 1 summarizes the data obtained at concentrations of 0.5 and 0.125 μg/ml. The serum samples from nine *T. canis*-infected and two *T. cati*-infected patients reacted intensely with both TES and recombinant antigen at concentrations of 0.5 and 0.125 μg/ml, but two ocular toxocariasis samples gave lower OD values than visceral toxocariasis samples (Fig. 2). At a concentration of 0.125 μg/ml, all toxocariasis samples showed positive reactions against both antigens (Table 1). Regarding the cross-reactivities against other helminth infections at a concentration of 0.5 μg/ml, TES reacted with the serum samples from 39 (59.1%) of 66 patients with nematode infections. Notably, whereas 14 of 20 anisakiasis samples were positive for TES at a concentration of 0.5 μg/ml, none of the samples was positive with the recombinant antigen even at a concentration of 0.5 μg/ml. TES also cross-reacted with several serum samples from ascariasis and/or ancylostomiasis patients (Fig. 2 and Table 1). Among trematode and cestode infections, 29 of 50 and 11 of 26 samples, respectively, cross-reacted with TES at a concentration of 0.5 μg/ml, yielding cross-reactivities of 58.0 and 42.3%, respectively (Fig. 2 and Table 1). With TES, the cross-reactivity rates were still high (34.8 to 56.0%) even when the concentration was reduced to 0.125 μg/ml. In contrast, when the recombinant antigen was tested at a concentration of 0.5 μg/ml, one case each of *B. malayi* infection and creeping eruption caused by a larval *Gnathostoma* sp. was positive, and the cross-reactivity rate was only 3.0% in nematode infections. However, when the antigen con-

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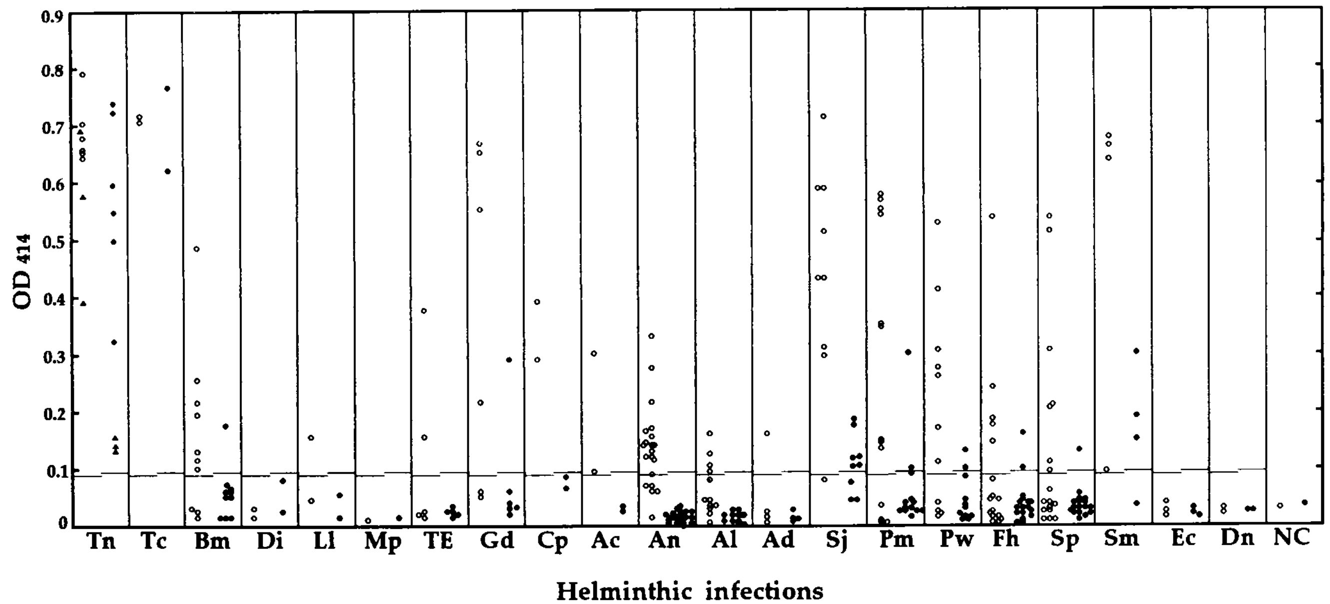

FIG. 2. Comparison of reactivities in ELISA of TES (○) and the recombinant antigen (●) against serum samples from patients infected with different helminths. For cases of *T. canis* infection, circles and triangles indicate visceral and ocular toxocariasis, respectively. The concentration of antigens tested was 0.5 μg/ml. Bars denote cutoff points. Abbreviations: Tn, toxocariasis with *T. canis*; Tc, toxocariasis with *T. cati*; Bm, brugiasis (*B. malayi*); Di, dirofilariasis (*D. immitis*); L1, loiasis; Mp, mansonelliasis (*Mansonella perstans*); TE, tropical eosinophilia; Gd, gnathostomiasis; Cp, intestinal capillariasis; Ac, angiostrongylosis (*Angiostrongylus cantonensis*); An, anisakiasis; Al, ascariasis; Ad, ancylostomiasis; Sj, schistosomiasis japonica; Pm, paragonimiasis (*P. miyazakii*); Pw, paragonimiasis (*P. westermani*); Fh, fascioliasis; Sp, sparganosis; Sm, spirometriasis; Ec, echinococciasis; Dn, diphyllobothriasis; NC, pooled negative serum.

centration was reduced to 0.125 μg/ml, only one sample from a case of creeping eruption produced a positive reaction. The cross-reactivity rate of 1.5% was very low, compared with 34.8% for TES. Although the recombinant antigen was tested at a concentration of 0.5 μg/ml, 13 (26.0%) of 50 samples from trematode infections and 4 (16.7%) of 26 samples from cestode infections were positive, and only one sample each from cases of *P. miyazakii* infection and spirometriasis was positive at a concentration of 0.125 μg/ml. In total, the cross-reactivity (2.1%) of the recombinant antigen was very low compared with that (43.0%) for TES at a concentration of 0.125 μg/ml (Table 1).

Several serum samples from patients with ascariasis and/or ancylostomiasis infections reacted with TES but not with the recombinant antigen (Table 1). Therefore, in order to clarify whether the recombinant antigen reacts with serum samples positive for either *Ascaris* or hookworm antigens, we used animal models. As shown in Table 2, all serum samples from rabbits with hookworm infections reacted with TES at concentrations of 0.5 and 0.125 μg/ml but not with the recombinant antigen at either concentration. In contrast, neither serum sample from mice infected with *Ascanis suum* reacted with the TES or recombinant antigen. Similar results were obtained with *Ascaris*-infected rats (data not shown). All serum samples from *T. canis*-infected mice and rabbits as positive controls reacted with TES and the recombinant antigen (Table 2).

DISCUSSION

Recently we cloned a cDNA encoding a component of the excretory-secretory antigens from *T. canis* second-stage larvae and applied the recombinant protein to the immunodiagnosis of human toxocariasis (30). In the present study, we evaluated the usefulness of the recombinant antigen as an immunodiagnostic antigen by comparing its specificity with that of TES on a total of 153 serum samples from patients with 20 different helminthic infections. When TES was used at a concentration of 0.125 μg/ml, cross-reactions were observed in 61 cases, corresponding to 43.0% of the helminthic infections examined. In contrast, the recombinant antigen at the same concentration hardly cross-reacted at all with serum samples from patients with helminthic infections, demonstrating that the recombinant antigen is highly specific for toxocariasis compared with TES. One explanation for the high specificity of the recombinant antigen is that it is a single molecule with a molecular mass of 41 kDa, whereas TES consists of multiple components with a wide range of molecular masses (2, 3, 16). Second, in contrast to glycosylated TES (17, 19, 24), the recombinant antigen produced in bacteria is not glycosylated. This may also lead to a decrease in cross-reactivity with antibodies that recognize the sugar moieties of the TES produced in *T. canis* larvae.

It has been reported that the sensitivity of ELISA for ocular toxocariasis is lower than that for visceral toxocariasis (25, 26, 27). Samples from two patients with ocular toxocariasis examined in the present study showed lower OD_{414} values than those from patients with visceral disease. This might be due to differences in the immune responses in the eye and liver and may also be related to the number of *T. canis* larvae trapped in the eye or to the longer period between the onset of illness and serologic testing (28). Thus, in cases of ocular toxocariasis, it is recommended that the recombinant antigen be used at a concentration of 0.5 μg/ml.

Some routine assay kits that use TES for the immunodiagnosis of human toxocariasis have been developed (1, 11). As demonstrated here, however, TES cross-reacts with serum from patients infected with various helminths, as noted by some researchers (11, 13, 14). In the cases of cross-reacting samples from patients with paragonimiasis, gnathostomiasis, and spirometriasis, the reactions are considered nonspecific

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TABLE 1. Specificity of the recombinant antigen and TES in ELISA

Infection group	No. of serum samples examined	No. positive in ELISA				Reactivity[a] (%)			
		Recombinant		TES		Recombinant		TES	
		0.5 μg/ml	0.125 μg/ml	0.5 μg/ml	0.125 μg/ml	0.5 μg/ml	0.125 μg/ml	0.5 μg/ml	0.125 μg/ml
Nematodes									
Toxocariasis (*T. canis*)	9	9	9	9	9				
Toxocariasis (*T. cati*)	2	2	2	2	2				
Subtotal	11	11	11	11	11	100.0	100.0	100.0	100.0
Other nematodes									
Filariasis malayi	10	1	0	7	4				
Dirofilariasis (*D. immitis*)	2	0	0	0	0				
Loiasis	2	0	0	1	0				
Mansonelliasis (*M. perstans*)	1	0	0	0	0				
Tropical eosinophilia	5	0	0	2	1				
Gnathostomiasis	6	1	1	4	3				
Capillariasis (*C. philippinensis*)	2	0	0	2	2				
Angiostrongylosis (*A. cantonensis*)	2	0	0	2	1				
Anisakiasis	20	0	0	14	6				
Ascariasis	12	0	0	6	5				
Ancylostomiasis	4	0	0	1	1				
Subtotal	66	2	1	39	23	3.0	1.5	59.1	34.8
Trematodes									
Schistosomiasis japonica	9	6	0	8	8				
Paragonimiasis (*P. miyazakii*)	13	3	1	9	9				
Paragonimiasis (*P. westermani*)	11	2	0	7	6				
Fascioliasis	17	2	0	5	5				
Subtotal	50	13	1	29	13	26.0	2.0	58.0	56.0
Cestodes									
Sparganosis	17	1	0	7	6				
Spirometriasis	4	3	1	4	4				
Echinococciasis	3	0	0	0	0				
Diphyllobothriasis	2	0	0	0	0				
Subtotal	26	4	1	11	10	15.4	3.8	42.3	38.5
Total	142[b]	19	3	79	61	13.4	2.1	55.6	43.0

[a] Number of positive cases/subtotal.
[b] The 11 cases of toxocariasis are excluded.

reactions due to the high antibody titers (>6,400) rather than to concurrent infection with *Toxocara* larvae. However, as concurrent infection with *Toxocara* larvae cannot be ruled out, it may be necessary to consider the possibility of infection by computed tomographic scan or ultrasonic examination. In fact, such cross-reactivity would not cause any major problems because of different clinical symptoms.

Although the numbers of patients with *Ascaris* and/or hookworms examined in this study were quite limited, the possibility that the recombinant antigen cross-reacts with samples positive

TABLE 2. Reactivities of TES and the recombinant antigen against serum samples from animals experimentally infected with nematode parasites

Antigen[a]	Concn (μg/ml)	Reactivity (mean OD_{414} ± SD) with serum from:					
		Rabbit			Mouse		
		Noninfected (pooled)	Infected with *A. caninum* ($n = 5$)	Infected with *T. canis* ($n = 2$)	Noninfected (pooled)	Infected with *A. suum* ($n = 8$)	Infected with *T. canis* ($n = 2$)
TES	0.5	0.017	0.318 ± 0.011	0.558 ± 0.013	0.005	0.020 ± 0.008	0.632 ± 0.062
	0.125	0.013	0.092 ± 0.004	0.194 ± 0.001	0.003	0.011 ± 0.006	0.138 ± 0.018
rTc	0.5	0.011	0.011 ± 0.001	0.252 ± 0.010	0.005	0.012 ± 0.015	0.836 ± 0.069
	0.125	0.008	0.007 ± 0.002	0.103 ± 0.003	0.002	0.002 ± 0.001	0.107 ± 0.013
Ac	5.0	0.040	0.255 ± 0.045	0.294 ± 0.040	ND[b]	ND	ND
As	5.0	ND	ND	ND	0.020	0.240 ± 0.023	0.027 ± 0.011

[a] rTc, recombinant *T. canis* antigen; Ac, *Ancylostoma caninum* adult worm antigen; As, *Ascaris suum* adult worm antigen.
[b] ND, not done.

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for ascariasis and/or ancylostomiasis is probably low. This is supported by the results obtained with animal models. However, in areas where *Ascaris* and hookworm infections are endemic, special attention should be given to cross-reactivity or false-positive results if only TES is used (5). In Japan, anisakiasis is prevalent because the Japanese people frequently eat sashimi (sliced raw fish) and many people possess anti-*Anisakis* antibodies. In the United States, a sudden increase in cases of anisakiasis has been seen, corresponding with the increasing popularity of such Japanese foods since 1972 (23). Thus, the use of the recombinant antigen may provide more reliable diagnostic results because it does not cross-react with samples containing anisakiasis antibodies.

In conclusion, it has been demonstrated that the recombinant *T. canis* antigen is useful for the immunodiagnosis of human toxocariasis and will provide more reliable results not only for routine diagnosis but also for epidemiological surveys of human toxocariasis.

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