

Molecular characterization of a cDNA encoding an excretory–secretory antigen from *Toxocara canis* second stage larvae and its application to the immunodiagnosis of human toxocariasis¹

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Abstract

The cDNA encoding an excretory–secretory antigen from the second stage larvae of *Toxocara canis* has been characterized. Sequence analysis revealed an open reading frame encoding a protein of 226 amino acid residues ($M_r = 24398$). Sequence database searches showed similarities to regions corresponding to epidermal growth factor-like and lectin-like domains of the core proteins of vertebrate chondroitin sulfate proteoglycans, which are major components of the extracellular matrix. The *T. canis* core protein was expressed as a fusion protein with thioredoxin A using an *Escherichia coli* expression system, and then affinity purified on a metal affinity resin in the presence of 8 M urea. When the purified recombinant *T. canis* protein was used as an antigen, immunoblot analysis

Abbreviations: TES, *Toxocara canis* L₂ excretory–secretory antigens; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; IPTG, isopropyl- β -D-thiogalactopyranoside; PCR, polymerase chain reaction; CNBr, cyanogen bromide; CSPG, chondroitin sulfate proteoglycan; PG, proteoglycan

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revealed the protein specifically reacted with sera from toxocariasis patients. The antigenic protein did not react with sera from patients with *Brugia malayi* infection, dirofilariasis, or ascariasis. In some cases of anisakiasis, cross-reactions were observed; however, the cross-reacting bands disappeared when anisakiasis sera preabsorbed with *Anisakis* antigen were used, indicating that the recombinant *T. canis* protein is very promising for use as an immunodiagnostic antigen for human toxocariasis. © 1998 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: *Toxocara canis*; Second stage larvae; Excretory–secretory antigen; Proteoglycan core protein; Recombinant antigen; Human toxocariasis; Immunodiagnosis

1. Introduction

Toxocariasis canis is an important parasitic disease in humans caused by infection with the second stage larvae (L₂) of the ascarid parasitic nematode in canines, *Toxocara canis*. Toxocariasis canis is now recognized worldwide [1–3], including in Malaysia [4], as a human infection. The infection is acquired through the ingestion of embryonated eggs in sand pits and playgrounds contaminated with dog feces. Upon hatching in the small intestine, the larvae migrate through somatic organs causing clinical symptoms known as visceral larva migrans (VLM). These are characterized by fever, hepatomegaly and persistent eosinophilia. Rare fatal cases have resulted from larval migration through the myocardium and central nervous system [3]. Ocular larva migrans (OLM) occurs in children and young adults when migrating *T. canis* larvae become trapped in the eye, resulting in damage to the uveitis and optic papillitis [5]. In extreme cases, OLM may lead to the sudden loss of vision.

Definitive diagnosis of toxocariasis can be provided by patho-histological examination for *Toxocara* larva(e) in biopsy materials. However, it is very difficult to detect fine larva(e) in tissues, and even when detectable, the diagnosis is time-consuming. Tissue biopsy is not usually recommended. Therefore the diagnosis of the disease currently depends on immunological examinations. Using *T. canis* L₂ excretory–secretory antigens (TES), sensitive ELISA tests and western blot analyses to detect antibodies directed against the TES have been commonly used for the immunodiagnosis of human toxocariasis [6–9]. However, these assays give false-positive reactions due

to cross-reactions with other parasitic helminths commonly found in tropical areas [10–12]. It is also laborious to obtain large amounts of excretory–secretory antigens by in vitro culture of *T. canis* L₂. In the present paper we report the development of an immunodiagnostic antigen for human toxocariasis by recombinant DNA technology. The molecular properties of the antigen are also characterized.

2. Materials and methods

2.1. Parasites

Adult females of *T. canis* were collected from puppies in Kuala Lumpur, Malaysia. Eggs were obtained from the uteri of gravid females and cultured in vitro as reported by de Savigny [6] and Hakim et al. [13]. Excretory–secretory antigens from *T. canis* L₂ and adult worms were prepared by concentrating the culture medium.

2.2. Cloning procedures

Toxocara canis L₂, developed for 2 months, were washed with chilled PBS and immediately frozen in liquid nitrogen until use. mRNA was purified using a QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech, Sweden); 1.5 µg of poly(A)⁺ RNA was obtained from approx. 0.1 mg of *T. canis* L₂. One microgram of mRNA was used for cDNA synthesis. A cDNA library was constructed using λZAPII-cDNA Synthesis/Gigapak II Gold Kit (Stratagene, USA). The cDNA library was then immunoscreened using sera from suspected toxocariasis patients and an

anti-TES polyclonal antibody raised in rabbits. Sera for screening were preabsorbed with *Escherichia coli* lysate.

2.3. Expression and purification of recombinant *T. canis* antigenic protein

Some of the 11 positive cDNA clones obtained from the cDNA library were converted to a phagemid (pBluescriptSK-carrying cDNAs) by *in vivo* excision, and each phagemid was propagated in SOLR cells. Only pBluescriptSK-clone 2 among some phagemids produced the recombinant protein stably in the β -galactosidase expression system; thus, this clone was chosen for further experiments. The *Bam*HI and *Xho*I restriction fragment from clone 2 was extracted from the agarose gel and subcloned into the pET-32b(+) expression vector (Novagen, USA). The recombinant plasmid was transformed into *E. coli* BL21(DE3)-pLysS, and the expression of a thioredoxin-*T. canis* fusion protein was induced by adding IPTG at a final concentration of 0.4 mM at 37°C for 2–3 h. The induced cells were harvested and disrupted by sonication in 20 mM Tris-HCl, pH 8.0, containing 100 mM NaCl and 1% Triton X-100. Fusion proteins expressed as inclusion bodies were solubilized completely with 8 M urea in 20 mM Tris-HCl, pH 8.0, and 100 mM NaCl, and then purified using a TALON™ Metal Affinity Resin Column (Clontech, USA) according to manufacturer's instructions. Proteins expressed in *E. coli* were analyzed by SDS-PAGE [14].

2.4. Nucleotide sequence analysis

The sequences of the 5'- and 3'-ends of the 11 positive clones were determined. In order to determine definitely the nucleotide sequence of the central region of a cDNA fragment, the region was amplified by PCR using *Tth* DNA polymerase (TOYOBO, Japan) and clone 2 as a template. The following oligonucleotide primers were synthesized on the basis of nucleotide sequences determined partially; 5'-TTAAAGCTTGCCAC-CCAATTGGACGCCGTTCA-3' (P1 in Fig. 1) as sense primer and 5'-ACACTCGAGCTAGGC-CTCCAGTTCGAAAATATG-3' (P2 in Fig. 1) as

antisense primer. Restriction sites (*Hind*III for P1 and *Xho*I for P2) are underlined. PCR was performed at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min for 30 cycles. The PCR-amplified fragment (300 bp, data not shown) was subcloned into pCR™ II plasmid vector (Invitrogen, USA). The nucleotide sequences were determined using fluorescence-labeled M13 primers in an automated DNA sequencer (ABI, Model 373A).

Sequence data were analyzed using Genetix-Mac, and EMBL/Swiss-Plot database searches were performed with the FASTA program of Pearson and Lipman [16] using a BLAST e-mail server.

2.5. Sequencing of the recombinant protein and TES

The NH₂-terminal sequence of the recombinant *T. canis* protein was determined according to the following procedure; the purified fusion protein was dialyzed against PBS to remove the urea, and then cleaved with enterokinase (Novagen, USA) at room temperature overnight. The target protein was electrophoresed on a 10–20% gradient polyacrylamide gel containing SDS, and electroblotted onto a polyvinylidene difluoride (PVDF)-type membrane (Millipore, USA) in CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) buffer system at 50 V for 30 min [15]. The membrane was then stained with 0.1% Coomassie Brilliant Blue-R250 in 10% acetic acid/40% methanol for 1–2 min, and destained with 50% methanol. The target band was cut out and applied to a Protein Sequencer (Hewlett Packard, Model G1005A). In order to clarify the relationship between the protein encoded by the cDNA reported here and TES components, TES were also run on SDS-PAGE for western blot analysis and protein sequencing.

2.6. Human sera used for immunological analysis

Of 32 patients suspected toxocariasis canis, 30 were Malaysians. Two Japanese cases have been reported previously to have toxocariasis canis [17]. Eosinophilia were seen in all cases. In addition, most patients exhibited either hepatomegaly, posterior uveitis or blurred vision. They were also

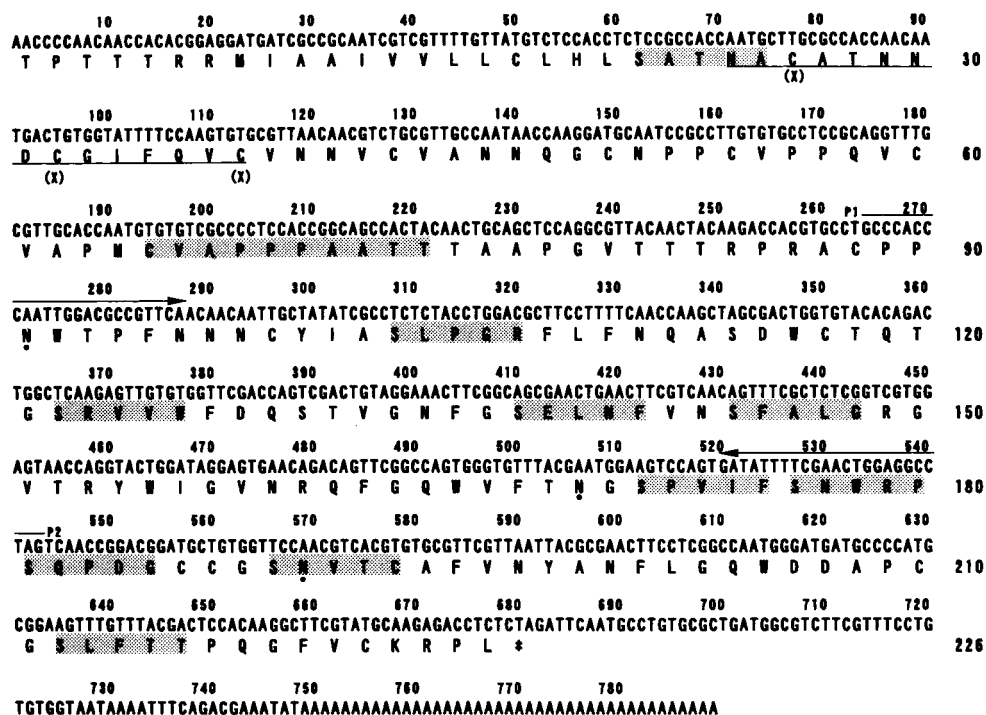


Fig. 1. Nucleotide and deduced amino acid sequences of the *T. canis* PG core protein. The nucleotide sequence is based on the sequences of three overlapping clones (clones 2 and 15, and the PCR product). The NH₂-terminal sequence of the recombinant protein derived from clone 2 is underlined. An X in parentheses under the peptide sequence indicates an amino acid residue that could not be determined. Three potential N-linked glycosylation sites are marked by dots. The regions of primers used for PCR are indicated by long arrows. The polyadenylation signal is denoted by dotted underlines. The asterisk denotes the translation termination codon. Numbers on the right-hand side indicate the numbers of amino acid residues. Peptide sequences identified by chemical treatment of TES-30 are shaded. One peptide (Cys-65 to Thr-74) was obtained by CNBr cleavage.

serologically positive by ELISA using TES. Nineteen of the cases (60%) were in teenagers. Two cases of suspected toxocariasis cati were Japanese who exhibited high eosinophilia (51 and 77%) and hepatic lesions on ultrasound. Their sera reacted more strongly with the *T. cati* antigen than the *T. canis* antigen in ELISA. Thirty serum samples from patients infected with *Brugia malayi* were collected from an endemic area of the disease in Perak, Malaysia. Microfilariae were detected in all cases. All patient sera were also seropositive by ELISA using *B. malayi* excretory-secretory antigens. Almost all patients with ages ranging from 16 to 64 years old were aborigines, Orang Asli, who live in tropical forests and have a lifestyle closely associated with dogs. Five

Japanese cases of dirofilariasis immitis were definitively diagnosed by characteristic lung X-ray findings and the presence of *Dirofilaria immitis* worms in lung biopsy tissues. They were also seropositive by ELISA using *D. immitis* antigen. Nine Japanese cases of anisakiasis were parasitologically diagnosed and were also seropositive by ELISA using *Anisakis* larval antigen. One case of gnathostomiasis, in which larval *Gnathostoma spinigerum* was detected, and a seropositive case of angiostrongyliasis cantonensis was also used for comparison. Three sera from patients infected with the *Ascaris lumbricoides* used were seronegative by ELISA using *A. lumbricoides* adult antigen. Ten sera from non-infectious individuals were used as negative controls.

2.7. Immunoblot analysis using recombinant *T. canis* antigen

Immunoblot analysis was performed using purified recombinant *T. canis* fusion protein. After electrophoresis on a 12.5% polyacrylamide gel at 150 V, the separated protein was transblotted onto PVDF-type membranes at 80 V for 75 min, blocked overnight in 3% gelatin in TBST (10 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 0.05% Tween 20), and then incubated with 1:2000 dilutions of human sera preabsorbed with *E. coli* lysate (3.2 mg/ml) at a final concentration of 1:10. The membranes were then probed with 1:5000 dilutions of alkaline phosphatase-conjugated anti-human IgG (Kirkegaard and Perry Laboratories, USA) and finally incubated in nitroblue tetrazolium and 5-bromo-4-chloro 3-indoryl phosphate. PBS-extracted somatic antigens from *Anisakis* larvae and *T. canis* and *T. cati* adult worms were used for absorption of patient sera. Protein concentrations of the PBS-extracts

were 2.4 mg/ml, 2.3 mg/ml and 3.0 mg/ml for *Anisakis*, *T. canis* and *T. cati*, respectively.

3. Results

3.1. Molecular characterization of cDNA encoding the *T. canis* proteoglycan core protein

DNA sequencing showed that nine out of 11 positive clones overlapped each other. Fig. 1 shows the nucleotide sequence of three overlapping clones and the deduced amino acid sequence. The clone consisted of 789 bp including a 3'-untranslated region and a poly(A)⁺ tail. The sequence predicted an open reading frame encoding a protein of 226 amino acid residues with a molecular mass of 24389, but the translation initiation site could not be determined for the reasons to be described later. Database searching revealed more than 20 core protein sequences of CSPGs showing similarities to the predicted protein. In Fig. 2, four representative CSPG core

<i>T. canis</i> PG	1	-----TPTTTRMIAAIWLLCLHLS	21
Rat brevican	516	GPTVETLQPPGEGSLTS-TP-D--GAREVGGETGSPELSGVPREREAGSSSLEDGPSLL	571
Bovine brevican	538	GPPTKTLPTREGNLASPPSTLVGAREIEETGGPELSGAPRGSEETGSS-EDAPSEL	596
Chick PG	3180	GQQEITTISSNIATNNTAPGNPYSNEQSTISSELLNTIELVTSSF-SLPEVTNGSDFLI	3238
Human PG	281	GELQ-AAWRNGFDQCDYGLSDASVHRPVTVARAQCGG-GLLGVR--TLYRFENQTGF--	334
<i>T. canis</i> PG	22	ATNACATNNDGGIFQVCVNVVCVA-NNQCCNPPCVPPQVC-VAPMCVAPPPAATTTAAPG	79
Rat brevican	572	PETWAPVGTREVETPSEE-KSGRT-VLTGTSVQAQPVLP-TDSASRGGVAVAPSSGDCIP	628
Bovine brevican	597	PATRAPGDTROLETPEE-MSRRT-VPAGTSVRAQPVLP-TDSASRGGVAVAPSSGDCVP	653
Chick PG	3239	GTSVGSVEGTAVQIPGQDPCKSNPCLNGGTCYPRGSFYICTCLPGFNGEQCELDIDECQS	3298
Human PG	335	PPDSRFDAYCFKRP--DRCKMNPCLNGGTCYPTETSYVCTCYPGYSGDQCELDIDECQS	392
<i>T. canis</i> PG	80	VTTTRPRACPPNWTPENNNC---YIASLPGRFLFNQASDW-----GTQT-GSRVW-FD	128
Rat brevican	629	SPCHNGGTCLEEKEGFRCLCPVPGGDLCDVGLHFCSPGNEPFQACYKHFSRRSNEEA	688
Bovine brevican	654	SPCHNGGTCLEEKEGVRCLCLPGYGGDLCDVGLHFCSPGNDAFQACYKHFSARRSNEEA	713
Chick PG	3299	NPCRNGATCIDGLNTETCLCLPSYIGALCEQDTETCDYGNHFKFQGCYKYFAHRRRTWDA	3358
Human PG	393	NPCRNGATCDVGFNTERCLCLPSYVGAICEQDTETCDYGNHFKFQGCYKYFAHRRRTWDA	452
<i>T. canis</i> PG	129	QSTYGNFGSELNFVNSF---AL-GRGVTRY-WIGVNRQFGQWVF--TNGSPVIFSNNRPS	181
Rat brevican	689	ESQCRALGAHETSICTPEEQDFVNDRYREYQWIGLNDRTIEGDFLWSDGPPLLYENWNP	748
Bovine brevican	714	ENKCRMYGAHETASISTPEEQDFINNRYREYQWIGLNDRTIEGDFLWSDGPPLLYENWNP	773
Chick PG	3359	ERECRLQGAHETSILSHEEQVFVNRIGHDYQWIGLNDKMFERDFRNTDGSPLQYENWRPN	3418
Human PG	453	ERECRLQGAHETSILSHEEQMFVNRVGHDTQWIGLNDKMFERDFRNTDGSPLQYENWRPN	512
<i>T. canis</i> PG	182	QPDGCGGNNVTCAFVNYANFLGQWDBAPGG-SL-FITPQGFV-C-KRPL-----	226
Rat brevican	749	QPDYFLSGENGCVVMVW-HDQGGNSDVPCNYHLSYTCMKGLVSCGPPPLPLAQIFGRPR	807
Bovine brevican	774	QPDYFLSGENGCVVMVW-HDQGGNSDVPCNYHLSYTCMKGLVSCGPPPELPLAEVFGRR	832
Chick PG	3419	QPDSSFSAGEDGVVLIW-HENGQWNVPCNYHLSYTCCKGTVACGQPPVVENAKTFGKMK	3477
Human PG	513	QPDSSFSAGEDGVVLIW-HENGQWNVPCNYHLSYTCCKGTVACGQPPVVENAKTFGKMK	571

Fig. 2. Sequence alignment of the *T. canis* PG core protein and four homologs. Residues identical to the *T. canis* PG core protein are shaded. Rat brevican [18]; chick proteoglycan [19]; bovine brevican [20]; and human proteoglycan [21]. Numbers refer to the amino acid positions in each protein.

protein sequences are aligned, rat brain CSPG, brevican [18], chicken limb CSPG [19], bovine brain CSPG, brevican [20] and human CSPG [21]. Amino acid sequence homologies were 30.1, 21.7, 28.7 and 21.2% for rat, chicken, bovine and human CSPGs, respectively. The deduced protein seems to be a COOH-terminal region of the core protein corresponding to epidermal growth factor (EGF)-like and lectin-like domains (e.g. to Gly-3337 for EGF-like and from Try-3338 to Gly-3457 for lectin-like domains in chicken PG; in bovine brevican, to Val-26 for EGF-like and from Gly-685 to Val-814 for lectin-like domains). Thus, the *T. canis* protein is hereafter designated *T. canis* PG core protein. The region corresponding to a complement regulatory domain (e.g. after Thr-3458 and Ser-815 in chicken PG and bovine brevican, respectively) at the COOH-terminus of the CSPG core protein is too short in the *T. canis* PG core protein. Three potential glycosylation sites for *N*-linked oligosaccharides are present as shown in dots (Fig. 1), whereas no *O*-linked glycosylation site consensus tetrapeptide Ser-Gly-Xaa-Gly [22] were found in this region.

3.2. Expression of the *T. canis* PG core protein in *E. coli* and its affinity purification

In order to examine the specificity and availability of the *T. canis* PG core protein as an immunodiagnostic antigen, the recombinant protein was produced in an *E. coli* expression system. As shown in Fig. 3, the 38-kDa recombinant fusion protein was much more highly expressed as inclusion bodies in induced cells (lane 4). To confirm whether the expressed protein is the same as the protein deduced from its DNA sequence, it was separated from its fusion partner by cleavage with enterokinase. Subsequently, the NH₂-terminal sequence of the cleaved product containing the *T. canis* PG core protein was Ala-Met-Ala-Ile-Ser-Asp-Pro-Pro-Gly-Xaa-Arg-Asn-Ser-Ala-Arg-Ala-Asn-Ala-Xaa-Ala-Thr-Asn-Asn-Asp-Xaa-Gly-Ile-Phe-Gln-Val-Xaa (the *T. canis* protein is underlined), the same as the deduced protein. The fusion protein was purified using TALONTM Metal Affinity Resin (Fig.

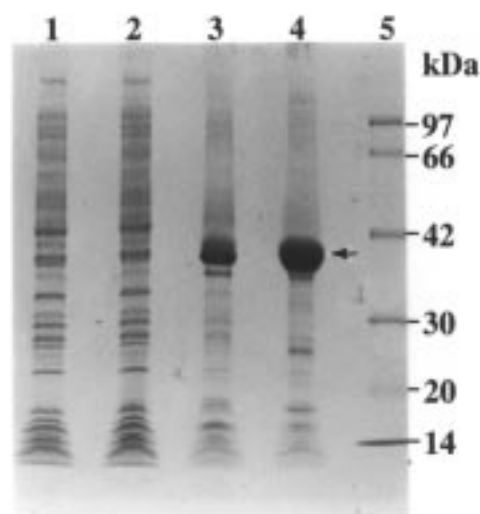


Fig. 3. SDS-PAGE analysis of proteins produced in non-induced and induced cells. 7.5–10- μ l aliquots were electrophoresed on a 10–20% gradient polyacrylamide gel at 150 V. Lanes 1–2 are supernatants and lanes 3–4 are precipitants of cell lysates. Odd lanes are from non-induced and even lanes from induced cells. The *T. canis* PG core protein fused with thioredoxin is indicated by an arrow.

4). The purified protein that eluted with 50 mM imidazole was used for immunological assays.

3.3. Relationship between the recombinant *T. canis* PG core protein and TES components

To clarify the relationship between the PG core protein encoded by the cDNA and TES components, western blotting was carried out using mouse serum immunized with the purified recombinant protein. As shown in Fig. 5, the antibody reacted specifically with a 30-kDa protein among TES components (lanes 5–6, hereafter TES-30). Although the NH₂-terminus of the TES-30 was chemically modified, 11 peptides identical to those deduced from the nucleotide sequence were identified by either CNBr or trifluoroacetic acid treatments of TES-30 (shaded boxes in Fig. 1). It was shown that the protein encoded by the cDNA is a TES-30. In addition, the protein was not detected in excretory–secretory antigens (lanes 8–9) or PBS-extracted somatic antigens (data not shown) from adult *T. canis* worms, indicating that the

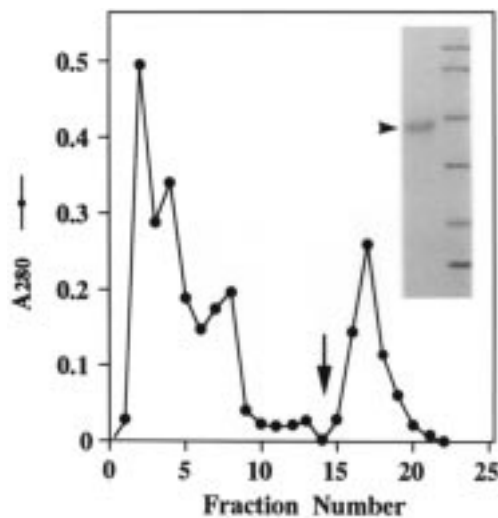


Fig. 4. Elution profile of thioredoxin-*T. canis* fusion protein from a TALON™ Metal Affinity Resin Column and SDS-PAGE analysis of the purified protein. The urea-solubilized fusion protein was applied to the column (2 ml bed volume). Contaminating proteins were washed out with 20 mM Tris-HCl, pH 8.0, containing 100 mM NaCl, 8 M urea, and 12.5 mM imidazole, and then the fusion protein was eluted with the same buffer containing 50 mM imidazole (indicated by an arrow). One ml fractions were collected. The purity of each fraction was monitored by SDS-PAGE. A 38-kDa purified fusion protein is shown by the arrowhead. Molecular markers in the right lane are same as in Fig. 3.

protein expressed in stage-specific manner. This finding was supported by northern blot analysis (data not shown).

3.4. Immunoblot analysis of human toxocariasis using the recombinant *T. canis* PG core protein as an antigen

Fig. 6 shows the specificity of the recombinant protein as an antigen in infectious parasitic diseases in humans. In toxocariasis canis, patient sera strongly recognized the 38-kDa protein, although only 14 representative cases are shown. Two serum samples from toxocariasis cati patients also reacted intensely with the recombinant protein. In the case of Malayan filariasis, 25 out of 30 patient serum samples showed no reaction with the recombinant antigen, with 12 representative

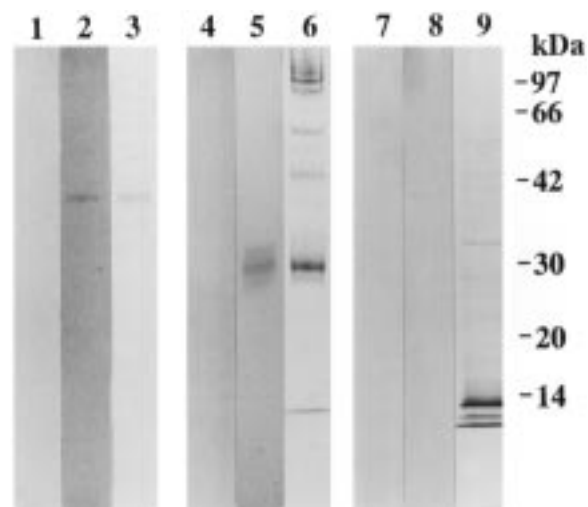


Fig. 5. Identification of a TES component using mouse serum against the recombinant *T. canis* antigen. The purified recombinant antigen (lanes 1–3, 1 µg/lane), TES (lanes 4–6, 5 µg/lane), and excretory–secretory antigens from adult *T. canis* worms (lanes 7–9, 5 µg/lane) were electrophoresed and stained with Coomassie Brilliant Blue (lanes 3, 6 and 9). Lanes 1, 4 and 7 show reactions with preimmune mouse serum (1:1000) as controls. Lanes 2, 5 and 8 are reactions with immune mouse serum (1:1000). Alkaline phosphatase-conjugated mouse IgG (BioRad, USA, 1:5000 in dilution) was used.

cases shown. However, the remaining five cases (three shown in lanes 5, 6 and 9) that reacted with the *T. canis* antigen probably represent concurrent infection with *Toxocara* parasites rather than cross-reaction with the *B. malayi* antigen [13]. Serum from rabbits experimentally infected with *T. canis* larvae recognizes the 38-kDa recombinant antigen in ELISA (data not shown). No serum sample from five dirofilariasis immitis patients, three ascariasis patients, or seven non-infectious individuals as negative controls reacted with the 38-kDa *T. canis* antigen. Serum samples from single cases of gnathostomiasis and angiostrongyliasis showed no cross-reactions (data not shown). For anisakiasis, six out of nine serum samples cross-reacted slightly with the recombinant *T. canis* fusion protein (lanes 1–6). However, no homologous antigenic protein has been found in *Anisakis* worms. As demonstrated in

Fig. 7, however, the cross-reaction (lane 1 in panel C) completely disappeared when anisakiasis serum samples preabsorbed with *Anisakis* antigen were used (lanes 2–4 in panel C). In contrast with anisakiasis, when toxocariasis serum samples preabsorbed with *Anisakis* antigen were used (lanes 5, 8 and 11 in panel A, lanes 3 and 6 in panel B), the 38-kDa band did not disappear. When toxocariasis serum preabsorbed with either *T. canis* (lanes 2, 6 and 10 in panel A) or *T. cati* antigen (lanes 3 and 7 in panel A, lanes 2 and 5 in panel B) was used, the 38-kDa band either weakly reacted or disappeared (lane 2 in panel A). This indicates that it is easy to distinguish between toxocariasis and anisakiasis if anisakiasis sera preabsorbed with *Anisakis* antigen are used. However, it appears difficult to discriminate immunologically between toxocariasis *canis* and toxocariasis *cati* using the recombinant *T. canis* PG core protein.

4. Discussion

We report here the molecular characterization of the *T. canis* PG core protein and its availability as an immunodiagnostic antigen for human toxocariasis. The translation initiation site for the PG core protein encoded by the cDNA could not be determined definitely. First, since the TES-30 mRNA was not detected in RNAs from adult *T. canis* worms, it was impossible to estimate the size of the transcript. Secondly, the chemical modification of the NH₂-terminus of TES-30 made it impossible to determine it. Considering the domain structures of the PG core protein [23] and the nucleotide sequence in the vicinity of a translation initiation codon in eukaryotes [24], Met-8 in Fig. 1 may not be the NH₂-terminus. As indicated in Fig. 1, although a peptide sequence after Met-64 was identified by CNBr cleavage of

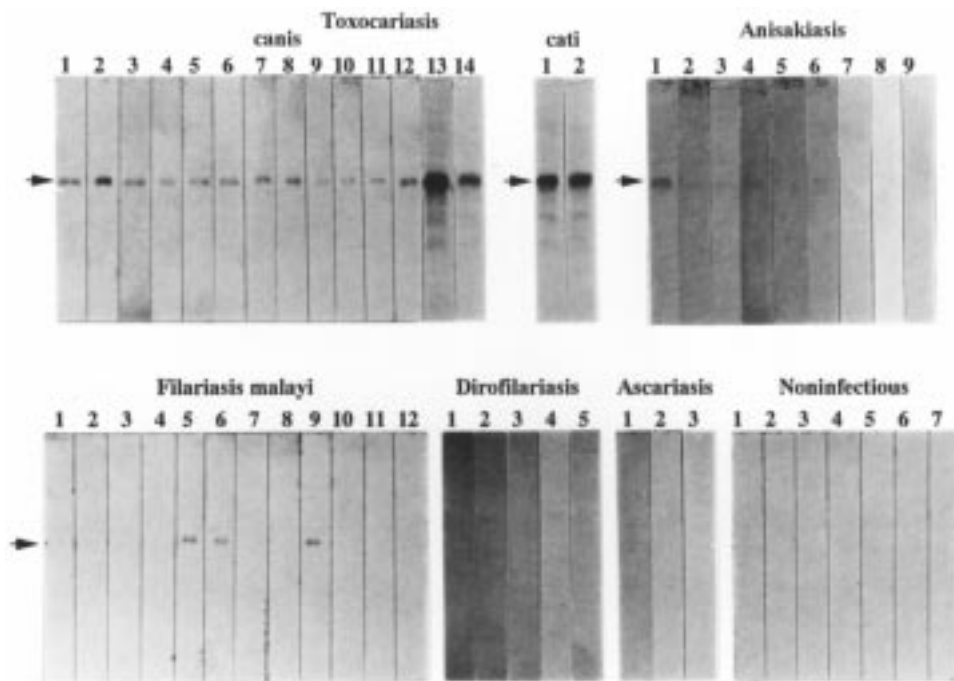


Fig. 6. Immunoblot analysis for various infectious parasitic diseases using the recombinant *T. canis* protein as an antigen. One μ g of the recombinant protein was electrophoresed on 12.5% polyacryamide gels, transblotted, and incubated with sera from parasite-infected patients as indicated above the strips. Arrows indicate the position of the 38-kDa recombinant protein. Human sera used were preabsorbed with *E. coli* lysate.

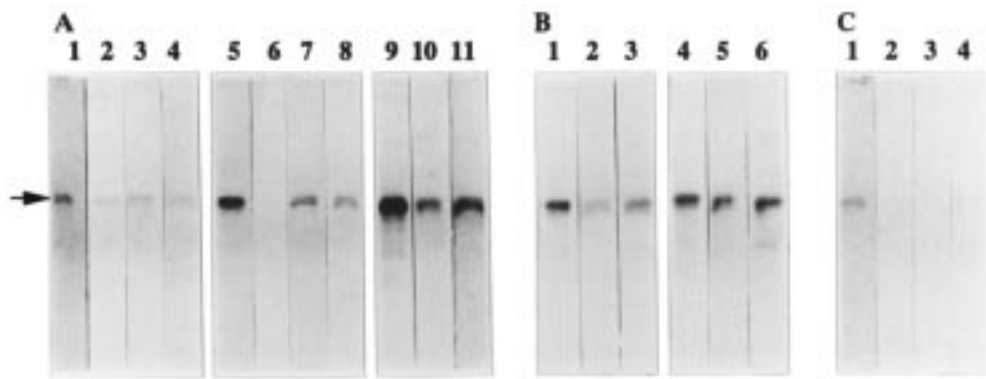


Fig. 7. Distinguishable diagnosis of toxocariasis and anisakiasis using preabsorbed sera. Panel A: three cases (lanes 1–4, 5–8, 9–11) of *toxocariasis canis*; panel B: two cases (lanes 1–3, 4–6) of *toxocariasis cati*; panel C: three cases of anisakiasis (lanes 1–2 are from same patient). Selected sera toxocariasis were preabsorbed at 37°C for 30 min with either *T. canis* antigen (lanes 2, 6, 10 in panel A), *T. cati* antigen (lanes 3 and 7 in panel A; lanes 2 and 5 in panel B) or *Anisakis* antigen (lanes 4, 8, 11 in panel A; lanes 3 and 6 in panel B), before incubation with the 38-kDa protein. Sera from three anisakiasis patients were preabsorbed with *Anisakis* antigen by the same procedure (lanes 2–4 in panel C). Arrows indicate the position of the 38-kDa band.

TES-30, the other peptide after Met-8 could not be detected. Therefore it may be considered that TES-30 is a post-translationally processed form. However, the possibility that chemically modified Met-8 is the first methionine can not rule out. In any event, based on sequence homologies, the deduced protein is probably a COOH-terminal region of a CSPG core protein belonging to the aggrecan/versican family in vertebrates [18–21,23]. In addition, no *O*-linked glycosylation site is found in the predicted protein that could be due to the presence of a glycosaminoglycan (GAG) attachment domain containing *O*-linked glycosylation sites is situated in the central region of the core protein [24]. This may be the reason why the consensus tetrapeptides for GAG attachment were not found in the predicted protein.

Although the recombinant protein is found to be same as TES-30, whether TES-30 is same as TES-32 [25,26], reported as a predominant antigen in *T. canis* L₂, is not yet clear. Furthermore, TES-400 proteoglycan as the largest TES component has been reported [25], but it is unclear at present whether the *T. canis* PG core protein is a part of TES-400 or not. The *T. canis* PG core protein is quite different from TES-26 [27] and TES-120 [28]. So far, there is no sequence homology between the cDNA reported here and

other reported DNA clones derived from *T. canis*. In invertebrates, heparan sulfate proteoglycans in *Caenorhabditis elegans* muscle cells [29] and in *Drosophila* Kc cells and embryos [30] have also been characterized, but the *T. canis* PG core protein differs from core proteins in these invertebrate proteoglycans. When these data are taken together, the *T. canis* protein reported here may be a novel homolog of CSPG core protein from vertebrates.

Excretory–secretory antigens derived from *T. canis* L₂ maintained in defined medium in vitro have been commonly used for the immunodiagnosis of human toxocariasis [7–9,13]. However, not all TES are species or genus specific. Some serum samples from patients with ascariasis, filariasis and strongyloidiasis show cross-reactivities with TES by immunoassay [9,12,25], and infections with protozoan, *Trichinella* and *Fasciola* species [8], although some workers have reported the excellent serologic specificity of an ELISA using TES [7]. The recombinant *T. canis* PG core protein reported here specifically reacts with sera from toxocariasis patients, although it is not possible to distinguish between toxocariasis *canis* and toxocariasis *cati*. The recombinant antigen did not react with filariasis, ascariasis, angiostrongyliasis and gnathostomiasis serum samples. The cross-re-

actions observed in five cases of Malayan filariasis may represent concurrent infections with *Toxocara* worms, considering the aboriginal lifestyles of patients that are closely associated with dogs [13]. Serum from rabbits experimentally infected with *T. canis* larvae recognized the recombinant antigen, suggesting that toxocariasis cases examined in the present study reflect current infection with *T. canis* larvae. Cross-reactions observed in some cases of anisakiasis can be completely removed by using anisakiasis sera preabsorbed with *Anisakis* antigen. When serum samples from toxocariasis patients preabsorbed with *Anisakis* antigen, the band did not disappear. This could be because both the *T. canis* and *Anisakis* parasites possess immunologically common and specific antigens. Thus, antibodies present in the sera of anisakiasis patients could be absorbed with homologous *Anisakis* antigen, but antibodies specific for *T. canis* present in the sera of toxocariasis patients are not absorbed with heterologous *Anisakis* antigen, resulting in the reaction with the specific antibodies recognizing the recombinant *T. canis* antigen. Finally, although its specificity as an antigen must be evaluated using human serum infected with various parasitic helminths, the *T. canis* PG core protein is very promising as an antigen for immunodiagnosis of human toxocariasis and also may provide more reliable diagnostic results.

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