Production of *Toxocara cati* TES-120 Recombinant Antigen and Comparison with its *T. canis* Homolog for Serodiagnosis of Toxocariasis

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Abstract. Toxocariasis is a cosmopolitan zoonotic disease caused by the infective larvae of *Toxocara canis* and *T. cati*. Diagnosis in humans is usually based on clinical symptoms and serology. Immunoglobulin G (IgG)-enzyme-linked immunosorbent assay kits using *T. canis* excretory–secretory (TES) larval antigens are commonly used for serodiagnosis. Differences in the antigens of the two *Toxocara* species may influence the diagnostic sensitivity of the test. In this study, *T. cati* recombinant TES-120 (rTES-120) was cloned, expressed, and compared with its *T. canis* homolog in an IgG4-western blot. The diagnostic sensitivity and specificity of *T. cati* rTES-120 were 70% (33/47) and 100% (39/39), respectively. *T. canis* rTES-120 showed 57.4% sensitivity and 94.4% specificity. When the results of assays using rTES-120 of both species were considered, the diagnostic sensitivity was 76%. This study shows that using antigens from both *Toxocara* species may improve the serodiagnosis of toxocariasis.

INTRODUCTION

Toxocariasis is a global zoonotic parasitic disease caused by the infective larva of *Toxocara canis* and *T. cati*.¹ Humans are infected by accidental ingestion of soil/dirt contaminated with embryonated eggs and/or consumption of paratenic hosts harboring the larvae. The hatched larvae penetrate the human gut wall and migrate to the liver, lungs, and other organs.^{1,2} They cannot develop further and remain trapped in the body tissue, causing a spectrum of clinical illness.

Most cases of human toxocariasis have been attributed to *T. canis*, and there are few reports on the role of *T. cati* in causing the disease.^{3,4} Because cats are a very common pet and the cat population is high, humans can also be expected to be infected with *T. cati* through similar routes as *T. canis* infection.⁵ Furthermore, sandboxes and garden soil are more often polluted with *T. cati* than *T. canis* eggs, which could further the role of *T. cati* in causing toxocariasis.⁶

Clinical manifestations of toxocariasis in humans vary according to the number of larvae and the affected organs; they include visceral larva migrans, ocular larva migrans (OLM), neurological larva migrans, and covert toxocariasis.^{5,7,8} Diagnosis of toxocariasis is often difficult, and is primarily based on clinical signs and symptoms and serodiagnosis. Imaging techniques are also helpful in some cases.⁹ The patient's history, including asthma, travel to tropical areas, contact with domestic animals, and consumption of undercooked meat or liver should also be considered.¹⁰

Serodiagnosis of toxocariasis is often performed using commercial immunoglobulin G-enzyme-linked immunosorbent assay (IgG-ELISA) kits (IBL International GMBH, Hamburg, Germany) that use *T. canis* excretory–secretory (TES) antigens of *T. canis* second-stage (L2) larvae. Production of native TES antigen is a laborious time-consuming technique and the yield is limited. Furthermore, cross-reactivity is an issue in

countries with prevalent soil-transmitted helminths.⁴ Thus, the use of specific recombinant antigens with high diagnostic sensitivity and specificity is preferable.¹¹

Despite many similarities in the antigens of *T. canis* and *T. cati*, some differences in antigenic molecules are expected; thus, it is likely that some cases of *T. cati* infection may be missed by tests targeting *T. canis*, and more research on *T. cati* antigens for serodiagnosis of toxocariasis is needed.^{2,3} The aim of this study was to clone and express a *T. cati* recombinant antigen, *T. cati* rTES-120, and compare its seroreactivity with the *T. canis* homolog.

MATERIALS AND METHODS

Collection of *T. cati* second-stage larvae. Adult female *T. cati* were collected from the intestines of stray cats and kittens, and the mature worms were washed with phosphate-buffered saline (PBS), pH 7.2. The uteri of gravid worms were dissected and the fertile eggs were placed in a 2.5% formalin ringer. This was incubated at 28–30°C for 30 days to allow for embryonation of the larvae.¹² Larvae were hatched and processed according to methods by Alcantara-Neves and others¹³ and Mohamad and others¹⁴ In brief, the formaldehyde was removed by washing five times with sterile PBS. An equal volume of 7–14% sodium hydrochloride (Sigma-Aldrich, St. Louis, MO) was added, and the solution was placed on a shaker at room temperature for 15 minutes until the eggs lost their external layer. Since sodium hydrochloride is very toxic to the larvae, the decoated eggs were then washed 10 times with sterile PBS. Approximately 10 mL of RPMI-1640 medium (Sigma-Aldrich) containing 100 IU/mL penicillin (Sigma-Aldrich), 100 µg/mL streptomycin (Sigma-Aldrich), and 2.5 µg/mL amphotericin B (Sigma-Aldrich) was added to the eggs, followed by incubation at 37°C with continuous bubbling of a 5% CO₂ gas mixture in 95% nitrogen for 1 hour. The suspension was transferred to a Baermann apparatus. The collected larvae were washed two times with cold sterile RPMI-1640 medium and transferred into a microcentrifuge tube. The number of larvae in each microcentrifuge tube was recorded. Finally, 10 times volume of an RNA stabilization reagent (RNAlater®, Qiagen, Hilden, Germany) was added to the tube,

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placed at 4°C for 2 hours, and stored at -80°C. The use of the animals and the study protocol was approved by the Animal Research Ethics Committee of Shiraz University of Medical Sciences.

Human serum samples. A total of 86 serum samples from Iran and Malaysia were used. This comprised 47 serum samples from patients diagnosed with toxocariasis based on clinical symptoms and serology and 39 control serum samples. The latter were from 20 healthy individuals and 19 patients with other diseases, including ascariasis ($N = 2$), strongyloidiasis ($N = 1$), taeniasis ($N = 2$), hydatidosis ($N = 4$), hymenolepiasis ($N = 2$), fascioliasis ($N = 2$), leishmaniasis ($N = 3$), malaria ($N = 2$), and toxoplasmosis ($N = 1$). All serum samples were tested with a commercial *T. canis* IgG-ELISA Kit to confirm that the toxocariasis serum samples were positive and the control samples were negative for the anti-*Toxocara* IgG antibody. The use of the aforementioned stored serum samples was approved by the human research ethics committees of the institutions involved in this study. The Research Ethics Committee at Shiraz University of Medical Sciences reviewed the proposal and approved the collection and use of the patients' samples (ref. no, 2015-258). The Human Research Ethics Committee at Universiti Sains Malaysia permitted the use of previously banked serum samples at Institute for Research in Molecular Medicine (INFORMM) for diagnostic test sensitivity and specificity determination.

RNA extraction. Total RNA was extracted from *T. cati* larvae using an RNeasy® Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Briefly, the larvae in RNAlater solution were removed from the -80°C freezer and centrifuged for 10 minutes at $16,000 \times g$. The pelleted larvae were washed with $1 \times$ PBS to remove any remaining RNAlater solution, lysis buffer was added, and the suspension was passed 10 times through a 20-gauge (0.9 mm) needle attached to a 1 mL sterile plastic syringe to obtain a homogeneous lysate. This was followed by the addition of ethanol to the lysate, and the mixture was applied to the RNeasy Mini spin column. Total RNA bound to the membrane in the column and was eluted in RNase-free water. The yield and purity of the total RNA were assessed using a NanoPhotometer™ (Implen GMBH, München, Germany) and stored at -80°C.

Complementary DNA synthesis and polymerase chain reaction amplification. Conventional reverse transcriptase polymerase chain reaction (RT-PCR) was performed using Easy-A one tube RT-PCR System (Agilent Technologies, Santa Clara, CA), according to the manufacturer's instructions. The TES-120 forward and reverse primers were the same as that used previously¹⁴ and were designed on the basis of the coding sequence of the *T. canis* TES-120 gene (GenBank: U39815), namely: TES-120 F, 5'-ATGCACGTCCTTACCGTCGCT-3' and TES-120 R, 5'-ACAGAAGCCGCACGTCAGTGG-3'. The RT reaction mix was prepared in a 1.5-mL tube by adding 6.2 µL of RNase-free water, 0.8 µL of $10 \times$ RT-PCR buffer, and 1 µL RT enzyme. A Master Mix was prepared by adding 19.25 µL of RNase-free water, 2.5 µL of $10 \times$ RT-PCR buffer, 0.5 µL of each forward and reverse primer, and 0.5 µL dNTP (40 mM) mix. Then, 0.5 µL of the RT mix, 0.25 µL of Easy-A High Fidelity PCR cloning enzyme, and 1.5 µL of RNA (20 ng) template were added to the Master Mix. PCR was performed as follows: first-strand synthesis at 45°C for 15 minutes; denaturation at 95°C for 1 minute; 40 cycles of denaturing at 95°C for 1 minute, template-primer annealing at 65°C for 1 minute, and extension at 68°C for 2 minutes; and final extension at 68°C for 10 minutes. Analysis of the PCR-amplified product was

conducted using 1% (w/v) agarose gel electrophoresis run at 100 V for 1 hour. The expected band of ~528 bp was excised using a sterile cutter, and DNA from the gel band was extracted using the QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions.

Cloning of the *T. cati* TES-120 gene. A-tailing of the purified PCR product was performed by incubating 100 ng of PCR product with $1 \times$ polymerase buffer supplemented with 25 mM MgCl₂, 1 µL of dATP (25 µM), and 0.15 U of Taq polymerase (Thermo Scientific, Rockford, IL) at 72°C for 30 minutes. Ligation of the A-tailed product to pCR® 2.1-TOPO® TA vector (Invitrogen, Carlsbad, CA) was performed according to the manufacturer's protocol. A volume of 1 µL of the ligated product was transformed into *Escherichia coli* TOP10 cells (Invitrogen) by electroporation. The cells were resuspended with 1 mL of Luria Bertani (LB) broth and incubated for 1 hour at 37°C. The culture was then plated on an LB agar plate supplemented with ampicillin (100 µg/mL) and X-gal (20 mg/mL) at 37°C overnight. Plasmid preparations from overnight culture of five white colonies were sent for DNA sequencing using vector-specific (M13) and gene-specific (TES-120) primers.

Sequence analysis. Sequence analysis was performed using BioEdit software (version 7.2.5; Ibis Biosciences, Carlsbad, CA). All sequencing results were manually aligned to produce a consensus sequence. The consensus sequence was then compared with the reported sequence of the *T. canis* TES-120 gene (GenBank: U39815).

Subcloning of the *T. cati* TES-120 gene into an expression vector. Subcloning of the *T. cati* TES-120 gene into the pGEX-4T-1 expression vector (GE Healthcare, Little Chalfont, United Kingdom) was outsourced to Epoch Life Science Inc. (Missouri City, TX). The gene was codon optimized for expression in *E. coli* before it was synthesized, and then it was cloned into the expression vector. Upon receipt of the recombinant plasmid (*T. cati* rTES-120/pGEX-4T-1), it was transformed into *E. coli* BL21(DE3) (New England Biolabs Inc., Ipswich, MA).

Expression and purification of *T. cati* TES-120 recombinant antigen. A single colony of the recombinant bacteria was grown overnight in 250 mL of Terrific Broth (TB) supplemented with 100 µg/mL of ampicillin in a shaking incubator at 37°C. A volume of 50 mL of the culture was inoculated into 4 L of TB-ampicillin broth and cultured in a shaking incubator at 37°C until the optical density (OD₆₀₀) reached 0.4–0.6. Protein expression was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Thermo Scientific, Waltham, MA) for 3 hours at 28°C in a shaking incubator.

The recombinant bacterial cells were harvested by centrifugation ($10,000 \times g$, 30 minutes, 4°C) and the pellet was resuspended in 4 mL of ice-cold 1X GST Bind/Wash Buffer provided in GST.Bind™ Kit (Novagen, Darmstadt, Germany) per 100 mL culture. Lysozyme (Amresco, Solon, OH) at 0.5 mg/mL and a cocktail of protease inhibitors (Roche Diagnostics, Deutschland GMBH, Mannheim, Germany) at 14.8 µg/mL were added to the suspension and incubated on ice for 30 minutes. The cell lysate was sonicated (Misonix Sonicator 3000, Newtown, CT) on ice for 2 minutes. After centrifugation, the supernatant was treated with DNase1 (Amresco) at a final concentration of 0.5 mg/mL and incubated at 4°C for 30 minutes, followed by centrifugation at $10,000 \times g$ for 30 minutes to remove the debris. The supernatant (lysate) was then clarified by filtration through a 0.45-µm filter membrane (Millipore, Billerica, MA).

Affinity purification of the recombinant protein was performed using GST.Bind Kit (Novagen). The lysate was incubated with the resin (pre-equilibrated with 5 volumes of $1 \times$ GST Bind/Wash Buffer) at room temperature for 30 minutes with gentle shaking, and the resin mixture was transferred into a chromatography column. The column was washed with 10 volumes of $1 \times$ GST Bind/Wash Buffer and the bound proteins were eluted with 3 volumes of $1 \times$ GST Elution Buffer. Fractions were collected at 500 μ L/tube for 10–15 fractions. The purity of the protein in each fraction was assessed by running sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Selected fractions were pooled and concentrated using a VivaspinTM column with a 30-kDa cut-off (GE Healthcare). The gel band with the expected molecular mass was sent for analysis using MALDI-TOF-TOFTM 5800 System (AB SCIEX, Framingham, MA). It was first analyzed using Swissprot database, then re-analyzed using a small database called "InfrommDB," which contained 88 sequences of 15 recombinant proteins produced at INFROMM, keratin, *E. coli* histidine-rich proteins, bovine serum albumin, randomly selected proteins, *T. canis* TES-120, and *T. cati* TES-120 protein deduced in this study. The protein concentration was determined using RC DCTM Protein Assay (Bio-Rad, Hercules, CA), and the protein was stored at -80°C .

Determination of the antigenicity of the recombinant antigen by western blotting. The purified recombinant antigen (*T. cati* rTES-120) was loaded at 10 μ g per well onto a 10% resolving gel, and SDS-PAGE was performed at 100 V. The gel was transferred onto a 0.45- μ m nitrocellulose membrane (Bio-Rad) in alkaline transfer buffer (25 mM Tris-base, 190 mM glycine, 10% v/v methanol) using the Trans-Blot[®] SD system (Bio-Rad). The membrane was blocked with $1 \times$ DetectorTM Block solution (KPL, Gaithersburg, MD) for 2 hours at room temperature and was washed in Tris-buffered saline with Tween-20 (TBST, 10 mM Tris-HCl; pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 5, 10, and finally for 15 minutes. The nitrocellulose membrane was then cut into strips, and each strip was incubated with serum samples at a 1:100 dilution in TBST overnight at 4°C . The strips were incubated with monoclonal anti-human IgG4-HRP (Invitrogen) at 1:1000 for 1 hour, washed again to remove the unbound secondary antibody, and developed on CL-XPosure film (Thermo Scientific) using an enhanced chemiluminescence substrate system (Thermo Scientific).

Western blot analysis was also performed with *T. canis* rTES-120 using the same primary and secondary antibodies. The recombinant protein was previously produced using *T. canis* TES-120 sequence¹⁴ in pGEX-5X-1 expression vector (GE Healthcare).

Determination of diagnostic sensitivity and specificity. The diagnostic sensitivity and specificity of both recombinant antigens were calculated as follows: sensitivity = $[\text{TP}/(\text{TP} + \text{FN})] \times 100$; specificity = $[\text{TN}/(\text{TN} + \text{FP})] \times 100$, whereby TP denotes true positive, FP is false positive, TN is true negative, and FN is false negative. True positives were the 47 serum samples from patients diagnosed clinically and serologically as toxocariasis, while true negatives were the 39 control serum samples from healthy patients and those with other infections.

RESULTS

The total amount of *T. cati* RNA isolated was 0.7 μ g, with a purity ($\text{OD}_{260}/\text{OD}_{280}$ ratio) of 2.02 by NanoPhotometerTM

FIGURE 1. Amplified reverse transcriptase polymerase chain reaction (RT-PCR) product of *T. cati* recombinant TES-120 (rTES-120), electrophoresed on a 1% agarose gel. Lane 1: 100 bp DNA Ladder H3 RTU (GeneDireX, Taiwan ROC); Lane 2: negative control; Lane 3: amplified product of *T. cati* rTES-120 gene coding sequence (531 bp).

(Implen GMBH). Gel electrophoresis analysis of the amplified RT-PCR product showed a single band between 500 and 600 bp (Figure 1). Using the sequence data of the five recombinant plasmids, a *T. cati* rTES-120 consensus sequence of 528 bp was obtained, and this sequence has been deposited into Genbank (accession number: KP717078). Figure 2 shows the *T. cati* TES-120 sequence aligned with that of *T. canis* TES-120, which has 531 bp nucleotides. The deduced amino acid sequence of the *T. cati* TES-120 gene was also compared with that of *T. canis* TES-120 (Figure 3), which showed that the *T. cati* TES-120 protein was 77% identical to the *T. canis* TES-120 protein.

SDS-PAGE analysis of the purified GST-fused *T. cati* rTES-120 protein showed that the target band was ~ 44 kDa (Figure 4), which corresponded with its predicted size. The initial matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF-TOF) analysis of the excised band using Swissprot database did not produce any significant score. When re-analyzed using "InfrommDB" it showed good identity with a peptide of 31 residues (SCNVCNCRDSANDCANFV SVCLNPTYQPVLRL) from *T. cati* TES-120, with 17% coverage and protein score of 55 (cut-off score 32). Within the first eight residues of the peptide in the *T. canis* homolog, there are four residues that are different.

Representative IgG4 blots of *T. cati* rTES-120 and *T. canis* rTES-120 probed with human serum samples are shown in Figures 5 and 6, respectively. Table 1 shows the diagnostic

T. cati	TES120	ATGCACGTCCTTACCGTCGCTCTCCTCGTAGCGCTAATTTCGGTTGGTA-	49
T. canis	TES120	ATGCACGTCCTTACCGTCGCTCTCCTCGTAGTCTAATCTGCGTTGGTAC	50
T. cati	TES120	-----AACCGCAGGACGATAGTTCCTCGTGTCTCTGCACATCATCCTCAT	94
T. canis	TES120	ACCACAAATCATGTCAGTTCCTCTTCATCGTCTCCGTCACATCCTCAT	100
T. cati	TES120	CGTCTCGTCATCATCTCATCGTCTCGTGTGCATCATCTCTCGTCTCG	144
T. canis	TES120	CGTCACGCTCAACATCCTCATCGTCCCGTCAACATCTCT---ATCGTCC	147
T. cati	TES120	TCGTCATCATCTCTCAACGCCCTCTCTCAACATCTCTCAACGACCGGTCAAC	194
T. canis	TES120	CGCTCAACATCTCTCAACGCTCTCTCAACATCTCTCATGGCCGCGTCAAC	197
T. cati	TES120	ATCCTCAAGCCCTCTGTCAACATCTCTCAACGACCGGTCAACATCTCTGT	244
T. canis	TES120	ATCCTCATCTGTCCGCGTCAACAGCTCAATGGCGGATCTACATCCACAG	247
T. cati	TES120	CATCTGGGTCAACAGCAGCTAGTGGATCGACAACAAGTTCTGGTCCG	294
T. canis	TES120	CACCTGGGCTCACATCCAGCTCTCTGTCTTCCACACAATCTCCAGCGGT	297
T. cati	TES120	ATTTCAACGACGCCAGCGTGCATTGACACCGCTAATGACTGCCAGCTCTT	344
T. canis	TES120	ATGCAACGACGCCAGCGGTGCATGACACCGCTAACGACTGCCAGCTGTT	347
T. cati	TES120	CATCCCACTCTGTTTCGTTTACGCCATACAGCAGACGATACAGGGAAGAT	394
T. canis	TES120	CATGCCACTCTCTTCTGTTTACGCCATACAGCAGACATACAGGGAAGAT	397
T. cati	TES120	GCGCAGCGTCTGTCAACGTTTGCAACTGTCGGGACAGTGCCAACGACTGT	444
T. canis	TES120	GCGCAGGACGTGCAACATTTGCACTGTGAGGACAGTGCCAACGACTGT	447
T. cati	TES120	GCAAACTTTGTTTTCAGTCTGCTTGAACCCGACCTATCAACAGTGCTTTG	494
T. canis	TES120	GCAAACTTTGTTTTCAGTCTGCTTGAACCCGACCTATCAACAGTGCTTTG	497
T. cati	TES120	ATCGAGATGCCCACTGACGTGGCGCTTCTGTATA	528
T. canis	TES120	ATCGAGATGCCCACTGACGTGGCGCTTCTGTATA	531

FIGURE 2. Nucleotides sequence comparison between the *T. cati* TES-120 and *T. canis* TES-120. The consensus sequence of *T. cati* TES-120 (GenBank: KP717078) was compared with the *T. canis* TES-120 sequence (GenBank: U39815). The shadowed regions indicate the same nucleotides in both sequences.

sensitivity and specificity of *T. cati* rTES-120, and Table 2 shows results for *T. canis* rTES-120. The diagnostic sensitivity of *T. cati* was 70.2% (33/47) and of *T. canis* was 57.4% (27/47). If a serum was considered positive if it reacted with either the *T. cati* or *T. canis* antigen, the assay diagnostic sensitivity was 76.6% (36/47). The diagnostic specificity of both antigens was high, but *T. cati* rTES-120 showed a higher specificity.

DISCUSSION

Toxocariasis is caused by infection with *T. canis* or *T. cati*, but the importance of the latter as an etiologic agent of the disease has been underestimated.^{2,15} It is generally accepted that toxocariasis is mainly caused by *T. canis*, but the contribution of *T. cati* in causing toxocariasis may be substantial in

T. cati	TES120	MHVLTVALIVALICVAKPDDSSSSSSST-SSSSSSSSSSSSSSSSSSSS	49
T. canis	TES120	MHVLTVALVAVLICVATPQMSSSSSSSPSSSSSSSSSSSSSSSSSSA	50
T. cati	TES120	SSSSTPSSSTSTTASTSSSPSSSTSTTGTSTSSSGSTSSSGSTTSSGPI	99
T. canis	TES120	STSSSSSTSSSFATSSSSSATSSMAGSTSTAACTSSSSVSTSTPAVM	100
T. cati	TES120	STTFACIDTANDCQLEFPLCFVQPYSFRAIQGRCRRSNCVNCNCRDSANDCA	149
T. canis	TES120	TTTFACIDTANDCQLEFPLCFVQPYSFRAIQGRCRRNCNCSCCDSANDCA	150
T. cati	TES120	NFVSVCLNPTYQFVLRSCRPLTCGFC	175
T. canis	TES120	NFVSVCLNPTYQFVLRSCRPLTCGFC	176

FIGURE 3. Comparison of the deduced amino acids sequence of *T. cati* TES-120 and *T. canis* TES-120. The deduced amino acids sequence of *T. cati* TES-120 was compared with the *T. canis* TES-120 (GenBank: AAB05820). The shadowed regions indicate the same amino acids in both sequences.

FIGURE 4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of the purified *T. cati* recombinant TES-120 (rTES-120)-GST protein. Lane M: molecular weight marker; Lane 1: purified *T. cati* rTES-120 protein. Arrow indicates the *T. cati* rTES-120 protein band.

some populations.^{2,14} For example, in countries with pre-dominant Muslim populations where cats are more favored than dogs as household pets, more cases of toxocariasis may originate from infected cats.⁴ Human infections by *T. cati* have been identified in several studies. One report, by Petithory and others,¹⁶ used a mini-Ouchterlony test to show that three of 10 OLM patients showed a greater reaction to *T. cati* antigen than *T. canis* antigen. Other studies have also

FIGURE 5. Representative strips from IgG4-western blots of *T. cati* recombinant TES-120 (rTES-120) antigen (~44 kDa) incubated with human serum samples. Lane M: molecular weight markers; Lanes 1–4: individual serum samples from toxocariasis patients; Lanes 5–6: serum samples from healthy individuals; Lanes 7–11: individual serum samples from patients with other parasitic infections (ascariasis, hydatidosis, leishmaniasis, hymenolepiasis, and fascioliasis, respectively).

FIGURE 6. Representative strips from IgG4-western blots of *T. canis* recombinant TES-120 (rTES-120) antigen (~44 kDa) incubated with human serum samples. Lane M: molecular weight marker; Lanes 1–4: individual serum samples from toxocariasis patients; Lanes 5–6: serum samples from healthy individuals; Lanes 7–11: individual serum samples from patients with other parasitic infections (ascariasis, hydatidosis, leishmaniasis, hymenolepiasis, and fascioliasis, respectively).

reported OLM patients who strongly reacted to *T. cati* antigen and weakly to *T. canis* antigen in ELISAs.^{15,17} In another report, the liver biopsy of a patient with visceral larva migrans showed the presence of larvae, and the Ouchterlony test was strongly positive for *T. cati*.¹⁸

Native TES is widely used for diagnosis and seroepidemiological studies.¹⁹ It is obtained from *in vitro* larval culture and contains a mixture of highly immunogenic glycoproteins.²⁰ Meanwhile, *T. cati* TES is rarely used for *Toxocara* serodiagnosis. This may be because of the greater difficulty in its production, and because the *T. canis* TES is generally thought to be able to detect infections by both species.²¹ Kennedy and others²² compared the excretory–secretory antigens of *T. cati* and *T. canis*, and reported considerable similarities between their secreted antigens. However, one of eight monoclonal antibodies produced (Tcb2) was found to be species specific for a carbohydrate determinant, and this determinant is widespread on *T. canis* glycoproteins. This implies that serology using *T. canis* antigens may not detect some *T. cati* infections. In a recently published paper, *T. canis* and *T. cati* excretory–secretory antigens were analyzed using a panel of sera from pigs experimentally mono-infected with the two species. The results showed a heterogenic protein pattern between individual hosts, in particular with *T. cati*-infected pigs, and that there was no protein

TABLE 1
IgG4-western blot reactivity of *T. cati* rTES-120 with three categories of serum samples

Type of serum sample	Number of samples	Number of positive results	Percentage of positive results
Toxocariasis	47	33	70.2
Healthy people	20	0	0
Other diseases*	19	0	0

rTES-120 = recombinant TES-120. Diagnostic sensitivity: 70.2% (33/47). Diagnostic specificity: 100% (39/39).

*Comprised the following diseases: ascariasis (*N* = 2), strongyloidiasis (*N* = 1), hydatidosis (*N* = 4), hymenolepiasis (*N* = 2), taeniasis (*N* = 2), fascioliasis (*N* = 2), leishmaniasis (*N* = 3), malaria (*N* = 2), and toxoplasmosis (*N* = 1).

TABLE 2
IgG4-western blot reactivity of *T. canis* rTES-120 with three categories of serum samples

Sera	Number of samples	Number of positive cases	Percentage of positive cases
Toxocariasis	47	27	57.4
Healthy people	20	0	0
Other diseases*	34	3†	8.8

rTES-120 = recombinant TES-120. Diagnostic sensitivity: 57.4% (27/47). Diagnostic specificity: 94.4% (51/54).

*Comprised the following diseases: ascariasis (*N* = 2), strongyloidiasis (*N* = 1), hydatidosis (*N* = 4), hymenolepiasis (*N* = 3), taeniasis (*N* = 2), fascioliasis (*N* = 2), leishmaniasis (*N* = 12), malaria (*N* = 2), and toxoplasmosis (*N* = 6).

†Leishmaniasis serum samples.

candidate of potential diagnostic value to discriminate between infections with the two species, thus it supported the view that existing serological methods are unable to distinguish between *T. canis* and *T. cati* infections.²³

Commercial diagnostic kits are known to have issues with specificity when used in countries endemic with soil-transmitted helminthiasis, which is because of the non-specific nature of components of native TES antigens that cross-react with other helminth antigens.^{4,24} Serological diagnostic tests based on standardized recombinant antigens will enable higher diagnostic specificity to be achieved.¹¹ Recombinant antigens that have been used for toxocariasis detection have included rTES-120, rTES32, rTES30, and rTES26.^{11,14,20,25} Previous studies showed good diagnostic potential of *T. canis* rTES-120.^{14,25,26} Thus, in this study, we produced rTES-120 from *T. cati* and compared its diagnostic value with the *T. canis* homolog.

The *T. cati* rTES-120 sequence was elucidated by performing RT-PCR on *T. cati* RNA using primers based on the *T. canis* sequence. The length of the *T. cati* TES-120 sequence was 528 bp, that of *T. canis* TES-120 was 531 bp, and there was 84% similarity between the two. The *T. cati* TES-120 sequence was codon optimized to *E. coli*-preferred codons, custom cloned into the pGEX-4T-1 expression vector, and transformed into *E. coli* BL21(DE3). The molecular mass of the expressed GST-fused protein was approximately 44 kDa and its seroreactivity with a panel of serum samples from toxocariasis patients and controls was determined. As a comparison, previously produced *T. canis* rTES-120 was also tested with the serum samples. Detection of the specific IgG4 antibody was used in this study because it has been shown to display high diagnostic specificity for serodiagnosis of toxocariasis.^{4,14,27}

We found that *T. cati* rTES-120 showed good diagnostic value, because its diagnostic sensitivity (70%) was higher than *T. canis* rTES-120 (57%). A total of 24 of 47 (51%) *Toxocara* serum samples were reactive with both recombinant antigens, nine samples were reactive only with *T. cati* rTES-120, and three serum samples were reactive with only *T. canis* rTES-120. If a positive sample by either *T. canis* or *T. cati* rTES-120 was taken to be a positive result, the assay diagnostic sensitivity was 76%. These results emphasize the importance of using recombinant antigens of both species to achieve improved diagnostic sensitivity. Another notable point is that 11 (23%) serum samples were not reactive with either recombinant antigen. Thus, this shows that more than one type of recombinant antigens were probably needed to achieve even higher diagnostic sensitivity. This supported the findings of our previous report that showed the combination

of *T. canis* rTES30 and rTES-120 was required to achieve 100% diagnostic sensitivity.¹⁴

The diagnostic specificity of both recombinant antigens was high, but that of *T. cati* rTES-120 (100%) was slightly higher than *T. canis* rTES-120 (94%). The reduced specificity of the latter was due to false-positive reactions of three of 12 serum samples from leishmaniasis cases. Because a minority (25%) of the serum samples of the leishmaniasis patients were cross-reactive, this may be attributed to other non-specific factors, and increasing the purity of the *T. canis* rTES-120 may eliminate the cross-reactivity.

In a previous study from Malaysia, two *T. canis* rTES-120 antigens were produced in bacterial and yeast host cells and evaluated in IgG-ELISAs.^{24,25} The results showed that all *Toxocara* patients serum samples ($N = 22$) reacted with both recombinant antigens. In our previous study, *T. canis* rTES-120 was tested with an IgG4-ELISA and showed a high sensitivity of 93%.¹⁴ In this study, almost 40% of the toxocariasis samples were from Iran, and the rest from Malaysia (which were different from the serum samples used in our earlier study). Thus, the lower diagnostic sensitivity of *T. canis* rTES-120 in this study may be attributed to the use of a different set of serum samples.

In conclusion, we have produced *T. cati* rTES-120 that showed potential diagnostic value and had higher diagnostic sensitivity than *T. canis* rTES-120. When combined with its *T. canis* recombinant homolog, the sensitivity for toxocariasis serodiagnosis was enhanced. Thus, the results of this study add to the growing body of evidence that reliance on antigens from one *Toxocara* species may not be adequate for the serodiagnosis of toxocariasis.

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REFERENCES

- Magnaval JF, Glickman LT, Dorchie P, Morassin B, 2001. Highlights of human toxocariasis. *Korean J Parasitol* 39: 1–11.
- Smith H, Holland C, Taylor M, Magnaval JF, Schantz P, Maizels R, 2009. How common is human toxocariasis? Towards standardizing our knowledge. *Trends Parasitol* 25: 182–188.
- Fisher M, 2003. *Toxocara cati*: an underestimated zoonotic agent. *Trends Parasitol* 19: 167–170.
- Smith H, Noordin R, 2006. Diagnostic limitations and future trends in the serodiagnosis of human toxocariasis. Holland CV, Smith HV, eds. *Toxocara: The Enigmatic Parasite*. Oxfordshire, United Kingdom: CABI Publishing, 89–112.
- Overgaauw PAM, Nederland V, 1997. Aspects of *Toxocara* epidemiology: human toxocarosis. *Crit Rev Microbiol* 23: 215–231.
- Jansen J, Knapen FV, 1993. *Toxocara* eggs in public parks and sand-boxes in Utrecht. *Tijdschr Diergeneesk* 118: 611–614.
- Overgaauw PAM, Knapen FV, 2008. Toxocarosis, an important zoonosis. *European J Companion Animal Practice* 18: 259–266.
- Wilkins PP, 2014. Immunodiagnosis of human toxocariasis and prospects for improved diagnostics. *Curr Trop Med Rep* 1: 44–51.
- Xinou E, Lefkopoulou A, Gelagoti M, Drevelegas A, Diakou A, Milonas I, Dimitriadis AS, 2003. CT and MR imaging findings in cerebral toxocaral disease. *AJNR Am J Neuroradiol* 24: 714–718.
- Lee AC, Schantz PM, Kazacos KR, Montgomery SP, Bowman DD, 2010. Epidemiologic and zoonotic aspects of ascarid infections in dogs and cats. *Trends Parasitol* 26: 155–161.
- Yamasaki H, Araki K, Lim PK, Zasmy N, Mak JW, Taib R, Aoki T, 2000. Development of a highly specific recombinant *Toxocara canis* second-stage larva excretory-secretory antigen for immunodiagnosis of human toxocariasis. *J Clin Microbiol* 38: 1409–1413.
- Zibaei M, Sadjjadi SM, Sarkari B, 2007. Prevalence of *Toxocara cati* and other intestinal helminths in stray cats in Shiraz, Iran. *Trop Biomed* 24: 39–43.
- Alcântara-Neves NM, dos Santos AB, Mendonça LR, Figueiredo CA, Pontes-de-Carvalho L, 2008. An improved method to obtain antigen-excreting *Toxocara canis* larvae. *Exp Parasitol* 119: 349–351.
- Mohamad S, Azmi NC, Noordin R, 2009. Development and evaluation of a sensitive and specific assay for diagnosis of human toxocariasis by use of three recombinant antigens (TES-26, TES-30USM, and TES-120). *J Clin Microbiol* 47: 1712–1717.
- Zibaei M, Sadjjadi SM, Jahadi-Hosseini SH, 2014. *Toxocara cati* larvae in the eye of a child: a case report. *Asian Pac J Trop Biomed* 4: S53–S55.
- Petithory JC, Chaumeil C, Liotet S, Rosseau M, Bisognani C, 1993. Immunological studies on ocular larva migrans. Lewis JW, Maizels RM, eds. *Toxocara and Toxocariasis: Clinical, Epidemiological and Molecular Perspectives*. London, United Kingdom: The British Society for Parasitology with the Institute of Biology, 181–189.
- Sakai R, Kawashima H, Shibui H, Kamata K, Kambara C, Matsuoka H, 1998. *Toxocara cati*-induced ocular toxocariasis. *Arch Ophthalmol* 116: 1686–1687.
- Shimokawa H, 1982. Visceral larva migrans by *Toxocara cati*. *Fukuoka Igaku Zasshi* 73: 64–69.
- Roldan WH, Espinoza YA, 2009. Evaluation of an enzyme-linked immunoelectrotransfer blot test for the confirmatory serodiagnosis of human toxocariasis. *Mem Inst Oswaldo Cruz* 104: 411–418.
- Maizels RM, 2013. *Toxocara canis*: molecular basis of immune recognition and evasion. *Vet Parasitol* 193: 365–374.
- Hotez PJ, Wilkins PP, 2009. Toxocariasis: America's most common neglected infection of poverty and a helminthiasis of global importance? *PLoS Negl Trop Dis* 3: e400.
- Kennedy MW, Maizels RM, Meghji M, Young L, Qureshi F, Smith HV, 1987. Species-specific and common epitopes on the secreted and surface antigens of *Toxocara cati* and *Toxocara canis* infective larvae. *Parasite Immunol* 9: 407–420.
- Poulsen CS, Skov A, Yoshida A, Skallerup P, Maruyama H, Thamsborg SM, Nejsum P, 2015. Differential serodiagnostics of *Toxocara canis* and *T. cati*—is it possible? *Parasite Immunol*. doi:10.1111/pim.12181.
- Speiser F, Gottstein B, 1984. A collaborative study on larval excretory/secretory antigens of *Toxocara canis* for the

- immunodiagnosis of human toxocariasis with ELISA. *Acta Trop* 41: 361–372.
25. Fong MY, Lau YL, Init I, Jamaiah I, Anuar AK, Noordin R, 2003. Recombinant expression of *Toxocara canis* excretory-secretory antigen TES-120 in *Escherichia coli*. *Southeast Asian J Trop Med Public Health* 34: 723–726.
26. Fong MY, Lau YL, 2004. Recombinant expression of the larval excretory-secretory antigen TES-120 of *Toxocara canis* in the methylotrophic yeast *Pichia pastoris*. *Parasitol Res* 92: 173–176.
27. Noordin R, Smith HV, Mohamad S, Maizels RM, Fong MY, 2005. Comparison of IgG-ELISA and IgG4-ELISA for *Toxocara* serodiagnosis. *Acta Trop* 93: 57–62.