RESEARCH NOTE

RECOMBINANT EXPRESSION OF *TOXOCARA CANIS* EXCRETORY-SECRETORY ANTIGEN TES-120 IN *ESCHERICHIA COLI*

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Abstract. The gene encoding the excretory-secretory antigen TES-120 of dog ascarid worm *Toxocara canis* was cloned into the bacterium *Escherichia coli*. The specificity of the recombinant TES-120 antigen produced by the bacterium was investigated. A total of 45 human serum samples from patients infected with different helminthes and protozoa, including 8 cases of toxocariasis, were tested against the recombinant antigens in immunoblot assays. The results from the assays revealed that the recombinant TES-120 antigen reacted with sera from toxocariasis patients only. This highly specific recombinant TES-120 antigen can potentially be used for the development of an inexpensive serodiagnostic assay for human toxocariasis.

Human toxocariasis, which has a worldwide distribution, is caused by infection with the larvae of the dog ascarid worm *Toxocara canis*. Puppies infected with adult *T. canis* release in the feces large numbers of infectious eggs into the environment. Eggs accidentally ingested by a patient, yield larvae that penetrate the intestinal wall and are carried by the circulation to various organs (Glickman and Schantz, 1981). While the larvae do not undergo any further development in the patient, the larvae can cause local immunological reactions which are the basis of toxocariasis. During their somatic migration through the organs, the larvae shed huge amounts of immunogenic glycoproteins known as *Toxocara* excretory-secretory (TES) antigens. The shedding of prodigious quantities of TES antigens is believed to be a strategy of the larvae to escape the immune attack of the host (Gems and Maizels, 1996).

Most human infections with *T. canis* larvae are asymptomatic. In instances of heavy infection, clinical presentations may be seen. The two main clinical presentations are visceral larva migrans (VLM) and ocular larva migrans (OLM) (Shields, 1984; Schantz, 1989). Diagnosis by identification of the larvae in the tissues is rarely done. Antibody detection is the more common means of confirmation of toxocariasis. The most common serologic test is enzyme-linked immunoassay (ELISA), which uses larval stage antigens extracted from embryonated eggs or TES antigens released by *in vitro* cultured larvae (de Savigny *et al*, 1979; Jaquier *et al*, 1991). Assays using such antigens, have drawbacks. First, the assays may yield false positive results because of cross-reactivity with other parasitic nematodes. Second, *in vitro* culture of *T. canis* larvae and harvest of TES antigens are laborious and time consuming. Hence, there have been attempts to produce specific recombinant antigens for use in toxocariasis seroassays (Yamasaki *et al*, 1998; 2000).

TES-120 glycoproteins of *T. canis* larvae are closely related mucins which form the major constituents of the larval surface coat and ES antigens. Four of the genes encoding these glycopro-

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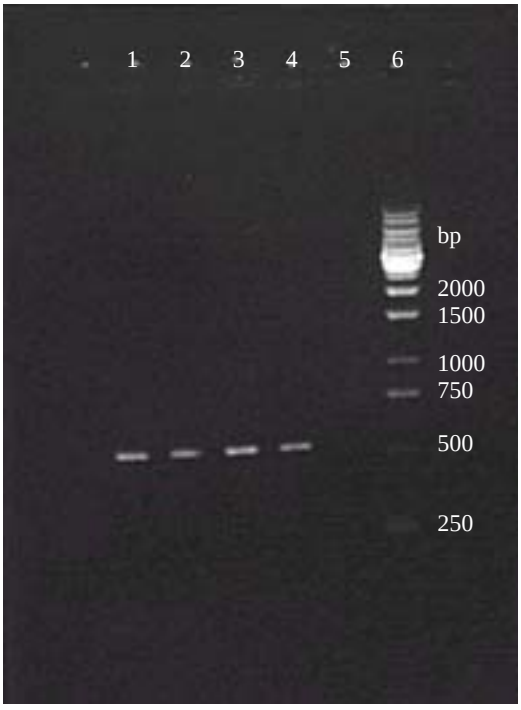

Fig 1–Agarose gel electrophoresis of TES-120 PCR products after RT-PCR. RT-PCR was performed using a oligo(dT)$_{24}$ and TES-120 specific primers. The amplified DNA fragments were 528 bp (lanes 1-4) in size. Lane 5 was loaded with the negative control, in which water was used to replace RNA in the RT-PCR. Lane 6 is the 1 kb ladder with the molecular weight (bp) stated beside.

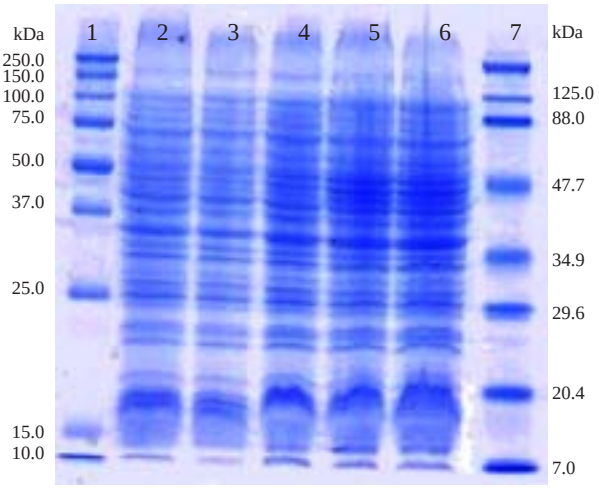

Fig 2–Time point study of protein expression for recombinant pTrcHisC carrying the TES-120 gene in. At zero time point (lane 2) and after an hour of induction (lane 3), no recombinant protein was observed. Recombinant protein started to appear after two hours (lane 4) and continued for the next two hours (lanes 5 and 6). Time point two hours was taken as the starting point of gene expression. Two types of protein markers were used to estimate recombinant protein size. Lane 1 shows the Precision protein standards and lane 7 shows the Prestained SDS-PAGE standards. Estimated size of the recombinant protein is 20.1 kDa.

teins, designated *Tc-muc-1* to *-4*, have been cloned and characterized (Loukas *et al*, 2000). Among the genes, *Tc-muc-1* has been shown to be abundantly expressed and its encoded product is highly antigenic (Gem and Maizels, 1996; Tetteh *et al*, 1999). We postulated that that TES-120 encoded by *Tc-muc-1* would be a suitable candidate as a highly specific recombinant antigen for use in toxocariasis seroassays. In our study, we expressed the mature TES-120 peptide in the bacterium *Escherichia coli*, and evaluated the recombinant TES-120 antigen in immunoblot assays.

Molecular cloning and expression of the *Tc-muc-1* gene were carried out using conventional molecular biology approach. First, an *in vitro* culture of *T. canis* stage 2 larva (L2) was established using the method described by Maizels *et al* (1984). Total RNA was extracted from 200,000 larvae using TRI Reagent (Molecular Research Center, Inc, USA). Reverse-transcription of RNA into cDNA was primed using oligo(dT)$_{24}$ primer, and proceeded with Superscript™ II reverse transcriptase (GibcoBRL®, Life Technologies Inc, USA). PCR amplification on the resulting cDNA was carried out using a primer pair (TES-120F 5´-AGCAGCCGCGGTAATT-3´, and TES-120R 5´-AATCTCTAGTCGGCAT-3´), designed according to the sequence published by Gems and Maizels (1996). The TES-120 PCR fragment of 528 base pairs (Fig 1) was ligated into the intermediate plasmid vector pCR®2.1-TOPO (Invitrogen Corp, USA) and transformed into competent *E. coli* TOP10 cells. The TES-120 fragment in the recombinant plasmid was then excised with *Eco*RI and spliced 'in-frame' into the *Eco*RI cloning site of plasmid expression vector

pTrcHis2C (Invitrogen Corp, USA). Positive recombinant clones harboring the TES-120 fragment were verified via nucleotide sequencing (data not shown).

The expression of the TES-120 antigen in *E. coli* was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) 3 hours after initiation of culture. Protein was harvested at hourly intervals from pelleted *E. coli* cells, and analyzed in denaturing SDS-polyacrylamide (SDS-PAGE) gels (Laemmli, 1970) stained with Coomassie blue. Gel analysis showed the expression of recombinant TES-120 antigen, with the expected size of 20.1 kDa (17.6 kDa of TES-120 polypeptide plus 2.5 kDa of tag sequence of the pTrcHis2C vector), 2 hours after induction (Fig 2). This time interval was used as guide when growth and expression was done in a larger volume of culture. The recombinant TES-120 antigen extracted from a large volume culture was purified using the Xpress System™ (Invitrogen Corp, USA). The purification system involved the use of a denaturing agent (urea), and a dialysis step was carried out to remove these agents. SDS-PAGE analysis on the dialyzed recombinant TES-120 antigen showed a major single band (Fig 3). Purified antigen was electroblotted to nitrocellulose membranes and tested with serum samples of patients suffering helminthic and protozoal infections.

Eight of the serum samples were from patients who were clinically and serologically (using a commercial immunoassay kit) confirmed as having toxocariasis. Twenty-two samples were from individuals who were serologically positive for parasitic infections such as cysticercosis (5 samples), filariasis (5), malaria (2), amebiasis (5) and toxoplasmosis (5). In addition, sera from individuals with soil transmitted helminthiasis (STH) were also tested. Five serum samples were from individuals with *Ascaris lumbricoides* and *Trichuris trichiura* co-infection, 3 with *T. trichiura* infection, and 1 each from cases of *A. lumbricoides* and hookworm infection. In these STH cases, the individuals were confirmed positive by the finding of helminth eggs in their feces. As a control, 5 sera from normal healthy individuals were included in the immunoblot assays. Results from the immunoblot assays showed

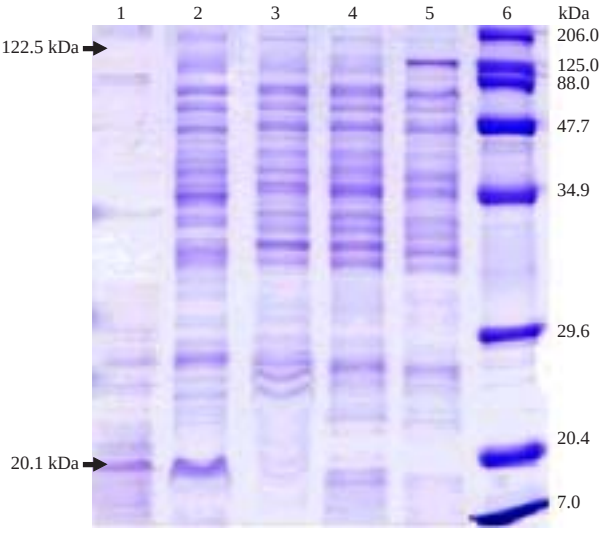

Fig 3–Large-scale protein expression and purification of recombinant TES-120 antigen. *E. coli* cells were grown for 3 hours before 1 mM IPTG was added, and the culture was allowed to grow for a further 2 hours. The recombinant TES-120 antigen expressed was as the estimated size, 20.1 kDa (lane 2). This protein was purified using Xpress system and dialyzed (lane 1). Positive expression control (pTrcHis2/*lacZ*, provided by the manufacturer of the expression system) yields the expected 122.5 kDa β-galactosidase fusion protein (lane 5). Negative controls such as cells harboring non-recombinant pTrcHis2C plasmid and non-induced cells were also loaded into the gel (lanes 3 and 4, respectively).

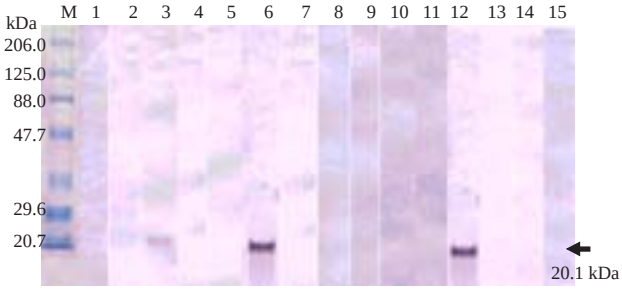

Fig 4–A sample immunoblot assay is shown above. Recombinant TES-120 antigen produced in *E. coli* was electroblotted onto nitrocellulose membrane strips. Each strip was tested with a serum sample. Strips 3, 6 and 12 show bands indicating reaction between sera from three toxocariasis patients and the recombinant antigen (20.1 kDa). Other strips tested with sera from a healthy individual (strip 1) and those infected with other parasites show no reaction bands. Strip M is the protein size standards (kDa).

that the recombinant TES-120 antigen was detected in all the toxocariasis patients' sera but none in the normal or non-*Toxocara* parasitic infection sera (a sample blot is presented in Fig 4). The results indicate that the recombinant antigen was specific for anti-*Toxocara* antibody. In addition, the recombinant TES-120 antigen did not cross-react with antibodies from patients with other helminthic infections.

Most of the commercial toxocariasis seroassay kits use total TES antigens derived from *in vitro* larva culture. These antigens are heterogeneous in their composition, and this may increase the risk of cross-reaction with non-*Toxocara* helminth antibodies. Using a single or homogenous species of *T. canis* larval antigen, such as those produced by recombinant DNA technology, should be more reliable and specific. One of the main explanations for cross-reactivity among helminth protein antigens is the occurrence of common carbohydrate (glycosyl) moieties in their peptides. Prokaryotic expression systems such as *E. coli* produce non-glycosylated proteins, therefore, the use of such proteins in seroassays would reduce the possibility of cross-reactivity. Another advantage of utilizing *E. coli* to produce recombinant antigens is that the technology for large scale culture or fermentation of this bacterium is well established. Furthermore, it is simpler and less expensive to grow *E. coli* than any other organism *in vitro*, because *E. coli* is not a fastidious organism and can be grown in simple and inexpensive growth media.

The findings of our study have laid the foundation for our further endeavor in producing a highly specific recombinant antigen that can be used for the development of an inexpensive seroassay kit for human toxocariasis.

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REFERENCES

de Savigny DH, Voller A, Woodruff AW. Toxocariasis: serodiagnosis by enzyme immuno-assay. *J Clin Pathol* 1979; 46: 284-8.

Gems D, Maizels RM. An abundantly expressed mucin-like protein from *Toxocara canis* infective larvae: the precursor of the larval surface coat glycoproteins. *Proc Natl Acad Sci USA* 1996; 93: 1665-70.

Glickman LT, Schantz PM. Epidemiology and pathogenesis of zoonotic toxocariasis. *Epidemiol Rev* 1981; 3: 230-50.

Jaquier P, Gottstein B, Stingelin Y, Eckert J. Immunodiagnosis of toxocariasis in humans: evaluation of a new enzyme-linked immunosorbent assay kit. *J Clin Microbiol* 1991; 29: 1831-5.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227: 680-5.

Loukas A, Hintz M, Linder D, *et al*. A family of secreted mucins from parasitic nematode *Toxocara canis* bears diverse mucin domains but shares similar flanking six-cysteine repeat motifs. *J Biol Chem* 2000; 275: 39600-7.

Maizels RM, de Savigny DH, Ogilvie BM. Characterization of surface and excretory-secretory antigens of *Toxocara canis* infective larvae. *Parasite Immunol* 1984; 6: 23-37.

Schantz PM. *Toxocara* larva migrans now. *Am J Trop Med Hyg* 1989; 41: 21-34.

Shields DA. Ocular toxocariasis. A review. *Surv Ophthalmol* 1984; 28: 361-81.

Tetteh KK, Loukas A, Tripp C, Maizels RM. Identification of abundantly expressed novel and conserved genes from the infective larval stage of *Toxocara canis* by an expressed sequence tag strategy. *Infect Immun* 1999; 67: 4771-9.

Yamasaki H, Araki K, Lim PKC, *et al*. Development of a highly specific recombinant Toxocara canis second-stage larva excretory-secretory antigen for immunodiagnosis of human toxocariasis. *J Clin Microbiol* 2000; 38: 1409-13.

Yamasaki H, Radzan T, Watanabe Y, *et al*. Molecular characterization of a cDNA encoding an excretory-secretory antigen from *Toxocara canis* second stage larvae and its application to the immunodiagnosis of human toxocariasis. *Parasitol Int* 1998; 47: 171-81.