Parasitol Res (2004) 92: 173–176
DOI 10.1007/s00436-003-1020-5

SHORT COMMUNICATION

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Recombinant expression of the larval excretory-secretory antigen TES-120 of *Toxocara canis* in the methylotrophic yeast *Pichia pastoris*

Received: 2 July 2003 / Accepted: 10 September 2003 / Published online: 4 December 2003

Abstract A gene encoding the larval excretory-secretory antigen TES-120 of the dog ascarid worm *Toxocara canis* was cloned into the methylotrophic yeast *Pichia pastoris*. Specificity of the recombinant TES-120 antigen produced by the yeast was investigated. Forty-five human serum samples from patients infected with differentparasitic organisms, including 8 cases of toxocariasis, were tested against the recombinant antigen in immunoblot assays. Results from the assays showed 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 is caused by infection with the larvae of the dog ascarid worm *Toxocara canis*. Puppies infected with adult *T. canis* shed in their feces large number of infectious eggs into the environment. Larvae hatched from *T. canis* eggs that are accidentally ingested by a patient penetrate the intestinal wall and are carried by the circulatory system 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. 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 large 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).

Human infections with *T. canis* larvae are usually asymptomatic. However, in cases 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 tissues is rarely done. Antibody detection is the more commonly used method of confirmation of toxocariasis. The most common serologic test is enzyme-linked immunosorbent assay (ELISA), which uses larval-stage antigens extracted from embryonated eggs or TES antigens released by larvae cultured in vitro (de Savigny et al. 1979; Jaquier et al. 1991). Assays using such antigens, however, have disadvantages. Firstly, the assays may yield false positive results because of cross-reactivity with other parasitic nematodes. Secondly, in vitro culture of *T. canis* larvae and harvesting 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).

The TES-120 glycoproteins of *T. canis* larvae are a group of closely related mucins, which form the major components of larval surface coat and ES antigens. Four of the genes encoding these glycoproteins, designated *Tc-muc-1* to *Tc-muc-4*, have been cloned and characterized (Loukas et al. 2000). Among these genes, *Tc-muc-1* is abundantly expressed and its encoded product is highly antigenic (Gems and Maizels 1996; Tetteh et al. 1999). We postulate 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. Hence, in our study, we expressed the TES-120 protein in the *Pichia pastoris* yeast expression system, which is well known for its ability to produce high levels of recombinant proteins. The suitability of this recombinant TES-120 as antigen was evaluated in immunoblot assays.

Molecular cloning and expression of the *Tc-muc-1* gene were carried out using conventional molecular biology approaches. Firstly, an in vitro culture of *T. canis* stage 2 larvae (L2) was established using the method described by Maizels et al. (1984). Total RNA

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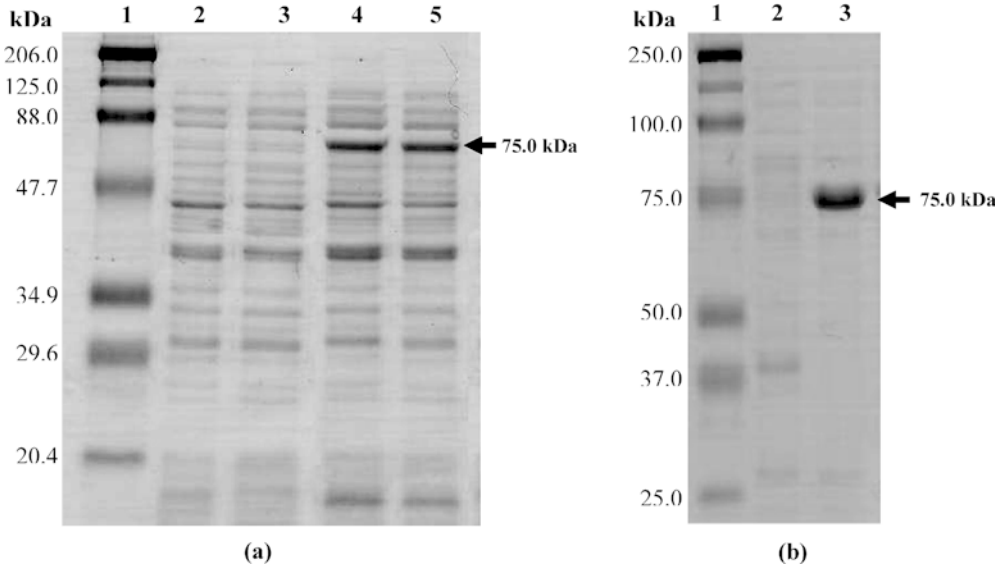

Fig. 1a, b SDS-PAGE analysis of recombinant TES-120 antigen produced in *Pichia pastoris*. **a** Time course study of recombinant expression. Methanol (0.5%) was added to a recombinant *P. pastoris* culture every 24 h to maintain induction of gene expression, and samples (pelleted cells) were collected every 12 h. *Lane 1* is the protein molecular weight standard. *Lane 2* was loaded with sample from time zero (before induction). *Lanes 3–5* were loaded with samples collected 12, 24 and 36 h after induction, respectively. The 75-kDa recombinant TES-120 protein (*arrow*) appeared 24–36 h after induction. **b** Total protein from recombinant *P. pastoris* cells were loaded onto the Xpress System purification column. Eluted material from the column contained mostly the 75-kDa recombinant TES-120 protein (*lane 3*). *Lane 2* is the eluate from a column loaded with total protein from a non-recombinant *P. pastoris* cell (negative control). *Lane 1* is the protein molecular weight standard

was extracted from 200,000 larvae using TRI reagent (Molecular Research Center, USA). Reverse transcription of RNA into cDNA was primed using oligo$(dT)_{24}$ primer, and carried out with Superscript II reverse transcriptase (GibcoBRL, Life Technologies, USA). PCR amplification of the resulting cDNA was carried out using a primer pair consisting of the sense primer 5′-CTAGGTGAATTCACCATGCACGTCCTTACC-3′ and the antisense primer 5′-TTCGGCTGTGAATTCACAGAAGCCGCACGT-3′, which flanked the open reading frame of the mature TES-120 peptide. The primer sequences were based on the sequence published by Gems and Maizels (1996). The PCR product of 545 base pairs was ligated into intermediate plasmid vector pCR2.1-TOPO (Invitrogen, USA), and transformed into competent *Escherichia coli* TOP10 cells. The TES-120 fragment in the recombinant plasmid was excised with restriction endonuclease *Eco*RI, and then spliced 'in-frame' into the *Eco*RI cloning site of plasmid expression vector pPICZ A (Invitrogen, USA). Positive recombinant pPICZ A clones harboring the TES-120 fragment were verified via nucleotide sequencing (data not shown). The recombinant plasmid was then linearized with *Sac*I, and transformed into competent *P. pastoris* cells using chemicals and instructions provided in the EasySelect *Pichia* expression kit (Invitrogen, USA).

Expression of TES-120 antigen in the recombinant *P. pastoris* was induced with 0.5% methanol. Protein was harvested at 12-h intervals from pelleted *P. pastoris* cells, and analyzed in denaturing sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels (Laemmli 1970) stained with Coomassie blue. Gel analysis showed expression of recombinant TES-120 antigen, with an estimated size of 75 kDa, 24–36 h after induction (Fig. 1a). This time interval was used as a guide when growth and expression were done in a larger volume of culture. Recombinant TES-120 antigen extracted from large-volume culture was purified using the Xpress System (Invitrogen, USA). The purification system involved the use of denaturing agents, and a dialysis step was carried out to remove these agents. SDS-PAGE analysis of the dialyzed recombinant TES-120 antigen showed a major single band of 75 kDa (Fig. 1b). The purified recombinant antigen was electroblotted to nitrocellulose membrane strips and tested with serum samples of patients suffering helminthic and protozoal infections.

Of the serum samples, 8 were from patients who were serologically confirmed as having toxocariasis antibody; 22 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; 5 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 finding of helminth eggs in their feces. As control, 5 sera from normal healthy individuals were included in the immunoblot assays. Results from the immunoblot assays showed that the recombinant TES-120 antigen reacted with all the toxocariasis patients' sera but none with the

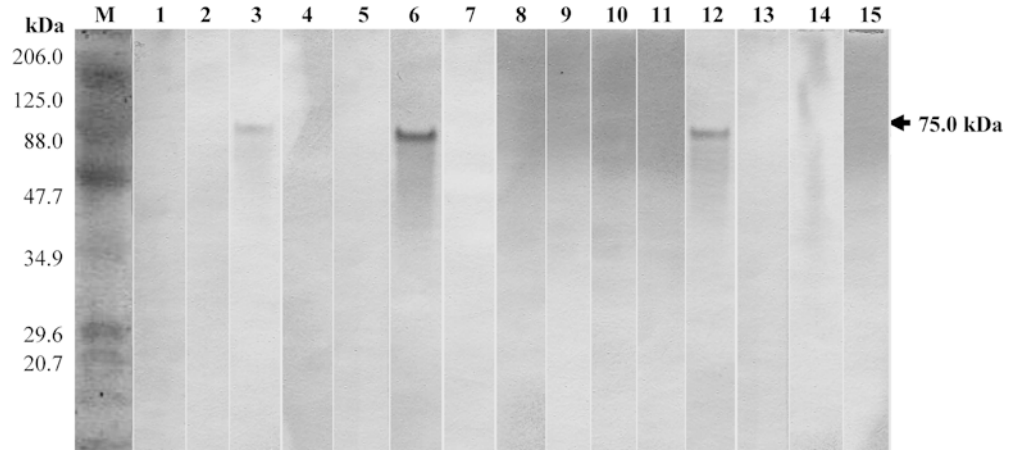

Fig. 2 A sample immunoblot assay. Recombinant TES-120 antigen produced in *P. pastoris* was electroblotted onto nitrocellulose membrane strips. Each strip was tested with a serum sample. *Strips 3*, *6* and *12* show bands indicating reaction between sera from three toxocariasis patients and the recombinant antigen (75.0 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 molecular weight standard

normal sera or sera from patients with non-*Toxocara* parasitic infection (a sample blot is presented in Fig. 2). The results thus indicate that the recombinant antigen was specific for anti-*Toxocara* antibody. More crucially, 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 larva culture in vitro. These antigens are heterogeneous in their composition, and may increase the possibility of cross-reaction with non-*Toxocara* helminth antibodies. However, using a single or homogenous species of *Toxocara canis* larval antigen, such as those produced by recombinant DNA technology, should be more reliable and specific. In our study, we chose the *Pichia* expression system because it offers several advantages over any other expression systems. In addition to its ability to produce high levels of recombinant proteins, up to grams-per-liter culture (Clare et al. 1991), the *Pichia* system has eukaryotic post-translational modification mechanisms which can glycosylate and fold recombinant proteins into forms that are similar to those of the native proteins (Cregg et al. 1993). The recombinant proteins can be selectively secreted by the *P. pastoris* into the growth medium, and this makes harvesting and purification of the proteins much easier. Furthermore, this methylotrophic yeast is not a fastidious organism, and is capable of utilizing methanol as its sole carbon source. Thus, it can be grown in simple and inexpensive growth medium. Finally, growing large-culture *P. pastoris* to produce recombinant antigens would not be difficult as that the technology for large-scale culture or fermentation of yeast is very established.

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

Acknowledgement This study was supported by IRPA Research Grant (06-02-03-0628) of the Ministry of Science, Technology and the Environment, Malaysia.

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