

MOLECULAR CLONING AND SEQUENCE ANALYSIS OF TES-120 GENE OF *Toxocara canis* EXCRETORY-SECRETORY ANTIGEN

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ABSTRACT

The gene encoding the excretory-secretory antigen of TES-120 from larvae of *Toxocara canis* was cloned and the sequence was analyzed for utilization as a diagnostic molecule. The total RNA was isolated from *T. canis* infective larvae and reverse transcription was done with oligo dT primers to obtain complementary DNA (cDNA). Amplification by PCR was performed with cDNA as a template and specific primers of the gene of TES-120, yielding an amplicon size of 530 bp. The amplicon was cloned into pRSETa expression vector. The recombinant clones were transformed into BL21 (DE3) pLysS *E. coli* expression host and the clone was confirmed by colony PCR, restriction enzyme analysis and sequencing. The nucleotide sequencing of TES-120 gene of TN isolate showed 99.8 % homology with the previously published sequences of UK (U39815.1) and Venezuela isolate (KU951901.1) and 97.0 % homology with the published sequences of Iraq isolate (LC328969.1). Phylogenetic tree revealed close relationship of TN isolate with Venezuela isolate, which had a common ancestor with the UK isolate and it is distantly related to Malaysia isolate of *T. cati* (KP71707).

Keywords: Cloning, Excretory-Secretory antigen, Sequencing, TES-120, *Toxocara canis*

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INTRODUCTION

Toxocarosis is one of the neglected tropical diseases in human beings. It is caused by the larval stage of *Toxocara canis*, the most common canine round worm (McGuinness and Leder, 2014). Human infection occurs

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by accidental ingestion of embryonated eggs of *T. canis* present in the environment. After ingestion, the eggs hatch in the intestine, the larvae migrate to various visceral organs (Despommier, 2003). Based on the organs involved and clinical symptoms, human toxocarosis can be classified into visceral larva migrans (VLM), ocular larva migrans (OLM), covert or common toxocarosis (CT) and neurotoxocarosis (NT) (Magnaval *et al.*, 2001; Chen *et al.*, 2018). Currently, the diagnosis of human toxocarosis is based on serology using excretory secretory (TES) antigens from second-stage (L2) larvae (Smith *et al.*, 2009).

The harvesting of TES antigens is laborious, time-consuming, antigens vary from batch to batch, and their composition is heterogeneous, which may increase the risk of cross-reactivity with other parasitic nematodes; false positive results are common; and there is a lack of reproducibility (Iddawela *et al.*, 2007; Yamasaki *et al.*, 1998; 2000). The effectiveness of any serodiagnostic methods depends mainly on the type of antigens used and their purity. Therefore, alternative to TES antigens, recombinant antigens were used to improve the specificity of serodiagnostic assays.

Recombinant DNA technology helped standardization of the antigen source and hence, offered reproducibility of well-defined polypeptides in large quantity which was not possible with native antigen. Several researchers made attempts to clone and express the immunodominant proteins of *T. canis* ES antigen and used them as potential

candidates for developing immunodiagnostic assays (Yamasaki *et al.*, 2000; Fong *et al.*, 2003; Mohamad *et al.*, 2009; Yunus *et al.*, 2018). TES-120 glycoproteins of *T. canis* larvae are closely related to mucins which form the major constituents of the larval surface coat and ES antigens. Tc-muc-1 to 4 genes that encode these glycoproteins that have been cloned and described (Loukas *et al.*, 2000). Tc-muc-1 is one of the genes that have been shown to be widely expressed, and its encoded product is highly antigenic (Gem and Maizels, 1996; Tetteh *et al.*, 1999). In India, no research on the specific TES 120 kDa *T. canis* larval excretory-secretory protein has been done. Hence, the present study was attempted to clone the TES 120 gene of the excretory-secretory protein from infective larvae of *T. canis*.

MATERIALS AND METHODS

In vitro cultivation of *Toxocara canis* larvae

Adult female *T. canis* worms were collected from naturally infected non-descript puppies after deworming. The worms were washed in normal saline solution, the uteri of gravid female worms were dissected and the eggs were collected. *In vitro* culture of *T. canis* larvae was described previously by Kavitha *et al.* (2019). The hatched out larvae were counted and mixed with 10 times volume of an RNA stabilization reagent (RNAlater®, Qiagen) and stored at -80°C .

Isolation of total RNA and cDNA synthesis

Total RNA was isolated from *T. canis* larvae using RNeasy Mini Kit (Qiagen, Germany) following the manufacturer

protocol. Complementary DNA (cDNA) was synthesized from the total RNA using iScript™ cDNA synthesis kit (Bio-Rad, USA) as per the manufacturers instructions.

PCR amplification of TES 120 gene

Primers for gene encoding TES 120 of *T. canis* larvae were designed along with restriction sites for *Bam* HI in forward primer and *Hind* III in the reverse primer based on accession number U39815 (Mohamad *et al.*, 2009). The primers with the following sequences were used. TES - 120 - F:5'CGGCGGATCCATGCACGTCCTTACCGTCGCT3' TES-120-R:5'CGCAAGCTTACAGAAGCCGCACGTCAGTGG3'. The PCR was performed with the total reaction volume of 20 µL which comprised of 6 µL Nuclease-free water, 10 µL of Ampliqon Master mix, 1 µL of forward and reverse primers (20 pmol/ µL each), 2 µL of cDNA template. The amplification process was then performed with the following conditions: Initial denaturation at 95° C for 2 min; 40 cycles of denaturation at 95°C for 30 s, template-primer annealing at 60°C for 30 s and extension at 72°C for 1 min; and final extension at 72°C for 5 min. Analysis of the PCR-amplified product was conducted using 1.5% agarose gel electrophoresis.

Cloning of TES-120 gene

The purified product of TES-120 and the pRSETa (Invitrogen) circular vector were double digested with *Bam* HI and *Hind* III restriction enzymes. Ligation of the purified product of TES-120 and the pRSETa vector was performed using T₄ DNA Ligase.

Competent cells of *E. coli* BL21 (DE3) pLysS were prepared following the protocol of Sambrook and Russel (2001). A volume of 2 µL of the ligated product was transformed into 100 µl of *E. coli* BL21 (DE3) pLysS competent cells. Heat shock was applied for 45 sec at 42°C in a water bath. 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 chloramphenicol (34 µg/ml) at 37°C overnight.

Confirmation of insert was done by colony PCR, restriction digestion and sequencing of the recombinant clones. Five recombinant (white) clones were selected and tested by colony PCR using gene specific primers. The recombinant plasmid DNA was isolated from transformed BL21 *E. coli* cells using the QIAprep® spin Miniprep kit (Qiagen, Germany) according to the manufacturer's instructions. The plasmid DNA was subjected to restriction digestion using *Bam* HI and *Hind* III restriction enzymes. The restriction digestion was carried out at 37°C for 1 hour and then the reaction was stopped by adding 2 µl of 6X gel loading dye. The digested product was electrophoresed on 1.5% agarose gel to confirm the release of insert TES-120. Positive recombinant clones harboring the TES-120 fragment were verified via nucleotide sequencing (Eurofins Genomics India Pvt. Ltd) using vector-specific T7 promoter forward and T7 terminator reverse primer. The sequence information received was analyzed using BLAST and Gene Tool

software.

RESULTS AND DISCUSSION

In the present study, the total amount of RNA recovered from *T. canis* second stage larvae ranged from 0.9 to 1.2 g/mL, with an OD260/OD280 purity of 1.9. The purified 530 bp (Fig.1) TES-120 amplicon was cloned in pRSETa expression vector. A 530 bp product was amplified from the positive clones (Fig. 2). The identity of the recombinant plasmids were confirmed by the release of a 530 bp insert (Fig.3). The nucleotide sequence of TES-120 gene of *T. canis* TN isolate used in the present study showed 99.8 % homology with the published sequences of UK (U39815.1) and Venezuela isolate (KU951901.1) and 97.0 % homology with the previously published sequences of Iraq isolate (LC328969.1). The phylogenetic tree constructed with the sequences obtained from clones of TES-120 of Tamil Nadu using maximum likelihood method showed close relationship with the Venezuela isolate which had a common ancestor with the UK isolate. All the above mentioned sequences were distantly related to the Malaysia isolate of *T. cati* TES-120 (KP71707) (Fig. 4).

Many studies reported that native TES antigen is cross-reacting with serum samples from patients with other helminthic infections like ascariasis, trichinellosis, fasciolosis, filariosis, strongyloidosis, schistosomosis and gnathostomosis, when tested by either IgG-ELISAs or IgG-Western blots (Magnaval *et al.*, 2001; Watthanakulpanich *et al.*, 2008;

Smith *et al.*, 2009). In addition, the production of native TES antigen is laborious and time-consuming and the protein yield is limited because of the difficulty involved in obtaining adult worms for cultivation of *T. canis* larvae (Yamasaki *et al.*, 1998). The presence of shared carbohydrate (glycosyl) moieties in their peptides is one of the main reasons for cross-reactivity among helminth protein antigens. Few investigators have reported TES-120 recombinant antigen is potentially useful for serodiagnosis of human toxocarosis (Fong *et al.*, 2003; Mohamad *et al.*, 2009).

The present study was aimed to clone the excretory-secretory antigen of TES-120 gene from the larvae of *T. canis* as well as its phylogenetic relationship with other published sequences, since no study has been carried out in India on the particular TES protein. TES-120 is the major surface coat component of larvae and runs as a set of four closely migrating bands with apparent mobility of 120 kDa on SDS-PAGE. They protect the parasite from antibody and eosinophil attack, as the entire coat is shed upon binding of these molecules (Smith *et al.*, 1981; Badley *et al.*, 1987). In the future, research along these lines will aid in the development of more reliable diagnostic approaches for the detection of cryptic visceral larva migrans in humans, as well as the investigation of accurate diagnostic markers for toxocarosis. We conducted a preliminary study on cloning of TES-120 gene of infective *T. canis* larvae and further research is needed to produce the recombinant protein for use in the diagnosis of *T. canis* infection in humans and dogs.

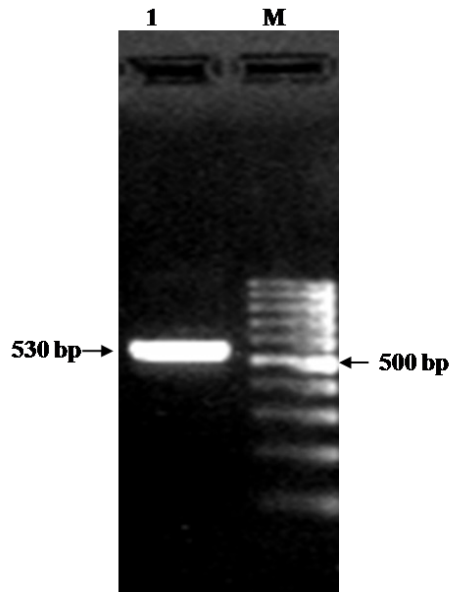


Fig. 1. PCR amplification of TES-120 gene. Lane M: 100 bp plus DNA ladder, Lane 1: PCR amplification of the 530 bp TES-120 gene

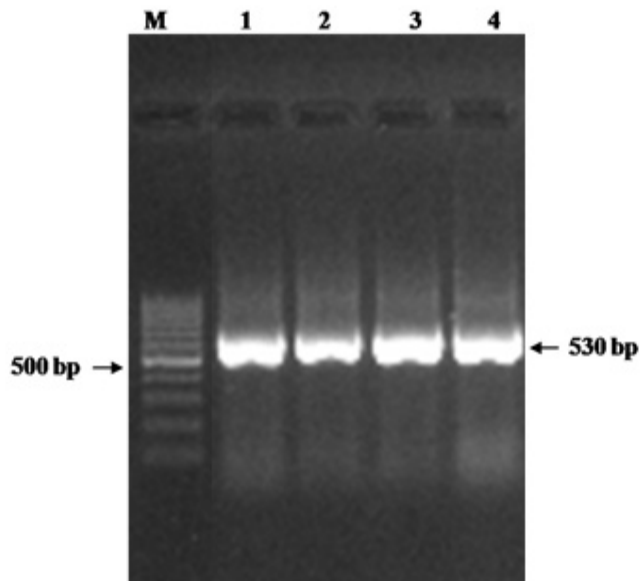


Fig. 2. Colony PCR of recombinant TES-120 clones in BL21 (DE3) pLysS *E. coli* cells. Lane M: 100 bp plus DNA ladder, Lanes 1 to 4: TES-120 (530 bp) amplicons from positive clones

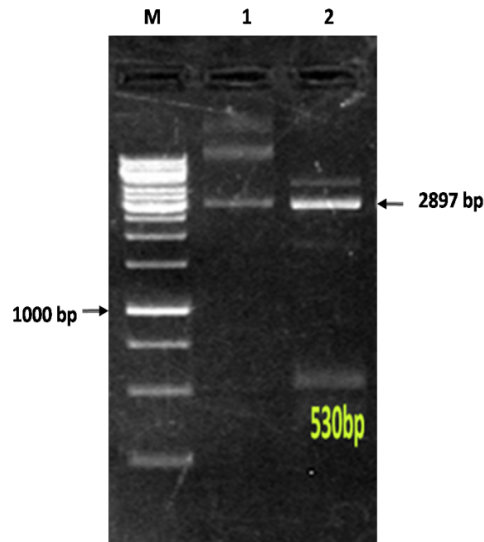


Fig. 3. Restriction enzyme analysis of TES-120 gene in pRSETa expression vector. Lane M: 1Kb plus DNA ladder, Lane 1: Undigested recombinant plasmid (BL21 (DE3) pLysS *E. coli* cells), Lane 2: TES-120 insert (530 bp) released by digestion with *Bam* HI and *Hind* III restriction enzymes

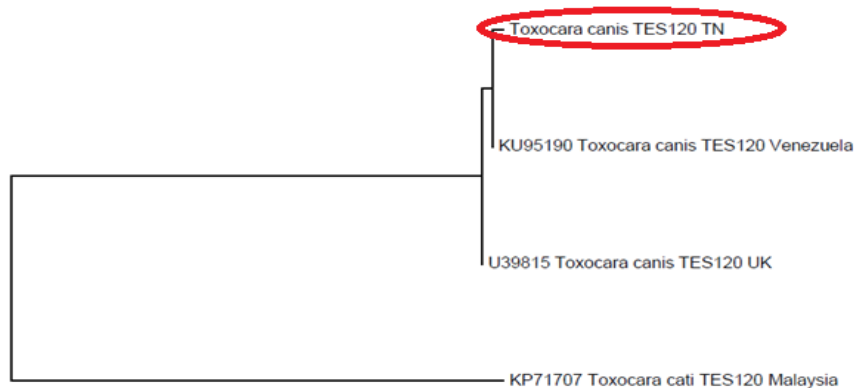


Fig. 4. Phylogenetic analysis of TES-120 gene by maximum likelihood method showing Tamil Nadu isolate is close relationship with Venezuela isolate and common ancestor with UK isolate.

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