Serodiagnosis of Human Toxocariasis Using Adult Somatic and Excretory-Secretory Antigens of *Toxocara canis*

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**Abstract:** Human toxocariasis is one of the most common zoonotic helminthiasis. Serological diagnosis remains the main tool for the diagnosis of toxocariasis. Serum samples collected from 57 confirmed cases of human toxocariasis and 26 healthy subjects (negative controls) were tested for anti-*Toxocara* antibodies by the enzyme-linked immunosorbent assay (ELISA) and enzyme linked immunotransfer blot (EITB) using adult *Toxocara canis* antigens; somatic antigen (TeSA) and its purified fractions (P-F1, P-F2 and P-F3) and excretory-secretory antigen (TeESA) to evaluate their sensitivity and specificity for the diagnosis of human toxocariasis. ELISA results showed that TeESA is the antigen of choice for diagnosis of human toxocariasis as it showed the highest specificity and sensitivity (100 and 92.30%, respectively). The immunoblot profile of TeESA, TeSA, P-F1, P-F2 and P-F3 reacted with rabbit hyperimmune serum prepared against TeSA showed common immunoreactive bands at 88.00, 85.00, 80.00, 47.462, 40.687, 35.706, 30.527, 21.983 and 19.285 kDa. The immunoblot patterns of them reacted with positive human sera displayed common bands at 47.462, 40.687, 21.983 and 19.285 kDa. The immunoblot profile of TeESA reacted with positive human (58.739, 47.462, 40.687, 30.527, 29.190, 21.983 and 19.285 kDa) and rabbit hyperimmune (94.00, 88.00, 85.00, 80.00, 47.462, 40.687, 35.706, 30.527, 21.983 and 19.285 kDa) sera showed common bands at 47.462, 40.687, 30.527, 21.983 and 19.285 kDa. So, we may suggest that 58.739 and 29.190 kDa polypeptides of the adult *T. canis* secretory-excretory antigen are specific for *Toxocara* infection in human and merit further evaluation as candidates for use in the diagnosis of human toxocariasis.

**Key words:** *Toxocara canis*, visceral larva migrans, ELISA, excretory-secretory protein

**INTRODUCTION**

Toxocariasis is a world-wide zoonotic infection caused by the ascarid nematodes *Toxocara canis* and *Toxocara cati* that routinely infect dogs and cats throughout the world. *T. canis* has a worldwide distribution and is regarded as the main cause of human toxocariasis (Barriga, 1988; Maepherson, 2005).

Infection in humans, particularly children, is frequently caused by accidental ingestion of embryonated *Toxocara* eggs present in soil, water, food, dirty hands and vegetables or by ingestion of larvae in under-cooked giblets. *Toxocara* cannot complete its life cycle in humans and the parasite development is arrested at the second larval (L2) stage. Larvae hatch in small intestine and migrate through somatic organs, preferably liver and eyes (Schatz, 1989).

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There are some clinical forms of toxocariasis: Visceral Larva Migrants (VLM), Ocular Larva Migrants (OLM) and Covert Toxocariasis (CT). The presentations of VLM include fever, abdominal pain, malaise, weight loss, skin rash, hepatomegaly, hypergammaglobulinemia and respiratory symptoms/signs with eosinophilia (Mgavnal et al., 2001). Toxocariasis has also been proposed as a potential etiology in neurologic disorders when the larvae migrate to the central nervous system (Nicoletti et al., 2002, 2007).

Diagnosis of human toxocariasis normally relies on a combination of the presence of clinical signs and symptoms backed by positive serology because of the difficulty in detecting larvae from tissues (Schantz, 1989). Savigny (1975) described a technique for in vitro maintenance of T. canis larvae with concomitant production of Excretory/Secretory (ES) or exo-antigen (TEX). TEX was used in ELISA for testing patients with visceral toxocariasis. This assay showed a high degree of sensitivity and specificity (Fan et al., 1999; Taranto et al., 2000; Iddawela et al., 2003; Noordina et al., 2005). Ragaa (2006) reported that TEX ELISA was able to discriminate positive and negative toxocariasis samples better than Toxocara canis embryonated egg antigen (TEE) ELISA in children and young adults with ocular toxocariasis.

Experiences during the past few years have shown several shortcomings in the use of adult somatic and adult excretory-secretory antigens of Toxocara canis for diagnosis of toxocariasis. Therefore, the aim of the present study was to evaluate the efficacy of purified adult somatic and adult excretory-secretory antigens for diagnosis of human toxocariasis using ELISA and enzyme linked immunotransfer blot (EITB).

MATERIALS AND METHODS

Human Sera
A total of 83 serum samples (collected from many Egyptian hospitals) were tested, comprising 57 from patients with clinical (visceral toxocariasis), hematological and serological evidence of toxocariasis (T. canis L2 larval ES antigen ELISA (TES-ELISA) and 26 non-Toxocara infected serum samples on TES-ELISA used as negative controls.

Parasitological Studies
T. canis adults were collected from the small intestine of naturally infected stray-dogs; they were identified as previously (York and Maplestone, 1962).

Preparation of Antigens
Adult Somatic Antigen
Whole worm extracts from adult T. canis flukes were prepared as described by Oldham (1983). The worms were washed several times in 0.01 M phosphate buffer saline, PBS pH 7.4 and homogenized with tissue homogenizer at 4°C until a uniform suspension was obtained. The homogenate was then subjected to ultrasonication at maximum amplitude (peak to peak) for 10 min, two times, with an interval of 1 min using an Ultrasonicator (Misonix, USA). After sonication, the preparation was centrifuged at 12,000 rpm for 45 min at 4°C. The supernatant was collected and designated as somatic antigen of T. canis (TeSA) and its protein concentration was measured as described by Lowry et al. (1951). TeSA was purified by gel filtration and produced three peaks P-F1, P-F2 and P-F3.

Adult Excretory-Secretory Antigen
E/S products were isolated from the live adult worms and processed as described by Diaz et al. (1998). Briefly, the adult worms were washed three times at room temperature with 0.9% NaCl.
worms were then individually incubated at 37°C in sterile RPMI-1640 medium without protein supplements. After incubation, the medium containing the E/S products was pooled and centrifuged at 1500 g for 30 min. The supernatant was immediately mixed with proteinase inhibitor (8 mM Phenyl Methyl Sulphonyl Fluoride (PMSF), concentrated, designated as TeESA and its protein concentration was measured as described by Lowry et al. (1951).

Preparation of Rabbit Anti-Sera Against T. canis Somatic Antigen

Two white New-Zealand rabbits were immunized subcutaneously (0.05 mg protein antigen/rabbit) with crude extract of TeSA emulsified in equal volume of Freund’s complete adjuvant and two rabbits were kept as control. Two weeks later, three booster injections in Freund's incomplete adjuvant were given with one week interval (Alkarni and Faulb, 1985). Serum samples were collected 4 days after the last booster injection. The serum was designated RaTeSA.

Analysis of Antigens and Antibodies

Enzyme Linked Immunosorbent Assay (ELISA)

ELISA was carried out according to Zimmerman et al. (1982) with some modifications. The optimal antigen (TeSA, P-F1, P-F2, P-F3 and TeESA) concentration, antibody and conjugate dilutions were chosen after preliminary checker board titration. In the present study, the optimum conditions were 20 μg mL⁻¹ coating buffer antigen concentration, 1:100 serum dilutions and 1:1000 alkaline phosphatase anti-human IgG (Sigma Co.) as conjugate and 1 mg p-nitrophenyl phosphatase dissolved in 1 mL substrate buffer as substrate. The absorbance of the colored reaction was read within 30 min at 405 nm using a flatbed multiskan ELISA reader. All incubation steps were carried out at 37°C in a moist chamber. The positive threshold value was determined to be two-fold the mean cut-off value of negative sera. Evaluation of ELISA results was carried out according to Timmreck (1994) as follows;

\[
\text{Sensitivity} \% = \frac{\text{True positive}}{\text{True positive} + \text{False negative}} \\
\text{Specificity} \% = \frac{\text{True negative}}{\text{True negative} + \text{False positive}}
\]

Sodium Dodecyle Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The gel cast comprised 12% resolving and 4% stacking gels with applied 10 μg well⁻¹ of the different antigens; TeSA, P-F1, P-F2, P-F3 and TeESA. Mini-protein II Dual slab cell (Bio-Rad Labs, Richmond, CA) was used to conduct electrophoresis using discontinuous system of Laemmli (1970). The fractionated antigens were visualized by Coomassie staining. Analysis of the separated bands was performed by soft ware analysis (Gel Proliner).

Enzyme Linked Immunotransfer Blot (EITB)

The fractionated T. canis antigens (TeSA, P-F1, P-F2, P-F3 and TeESA) were electrically transferred onto nitrocellulose (NC) membrane. NC sheets were cut into 0.5 cm strips (Towbin et al., 1979) followed by blocking in 5% BSA in PBS for 2 h on a rocker platform. Sera diluted at 1:100 in 5% BSA/PBS-T were reacted with fractionated NC strips for 2 h on a rocker platform. Following washing, alkaline phosphatase-labeled anti-rabbit IgG and alkaline phosphatase-labeled anti-human IgG (Sigma Co.) diluted at 1:1000 in PBS-T were added to T. canis antigens NC strips for 1 h on a rocker platform. The chromogen BCIP/NBT substrate was added to NC strips and allowed to develop for 30 min. The reaction was visualized by the naked eye.

RESULTS AND DISCUSSION

ELISA results showed that all the toxocariasis patients (n = 57) had antibodies against all the used (100% sensitivity) T. canis antigens (TeSA, P-F1, P-F2, P-F3 and TeESA). Also, control persons
possessed antibodies against TcESA (2/26, 7.69%), TcSA (6/26, 23.07%), P-F1 (6/26, 23.07%), P-F2 (6/26, 23.07%) and P-F3 (5/26, 19.22%). So, we can say that TcESA, TcSA, P-F1, P-F2 and P-F3 gave specificity level of 92.30, 76.92, 76.92, 76.92 and 80.76%, respectively. Cut off OD value was 0.3 for all the *T. canis* antigens.

Electrophoretic analysis of TcSA, P-F1, P-F2, P-F3 and TcESA revealed approximately 8 to 13 bands ranging from 250.00-14.054 kDa (Fig. 1).

The immunoblotting profile of TcSA, P-F1, P-F2, P-F3 and TcESA reacted with anti TcSA and positive human sera detected by ELISA showed approximately 10 to 12 and 7 to 10 bands ranging from 117.00-19.285 and 113.50-19.285 kDa, respectively (Fig. 2, 3). While, the immunoblotting profile of all the prepared *T. canis* antigens reacted with the control negative human sera detected by ELISA showed no bands (Fig. 4).
Toxocariasis is still an important and actual problem in human medicine. Although well recognized and studied in developed countries, canine parasitic zoonoses pose a lowly prioritized public health problem in developing countries. Definitive histopathological evidence of toxocariasis is rarely forthcoming, while clinical diagnosis is hampered by the lack of pathognomonic signs and symptoms. Both early detection of toxocariasis and the assessment of its public health significance rely heavily upon serology (Gillespie et al., 1993; Ajayi et al., 2000). The development of specific and reliable antigens to demonstrate the presence of toxocariasis is an important step towards improving diagnosis.

ELISA results recorded that all the used *T. canis* antigens demonstrated 100% sensitivity. TcESA demonstrated the highest degree of specificity (92.30%), while TcSA, P-F1, P-F2 and P-F3 gave lower specificity level of 76.92, 76.92, 76.92 and 80.76%, respectively. So, we can suggest that TcESA is the most sensitive and specific adult *T. canis* antigen for serodagnosis of human toxocariasis using ELISA. Abdel Aal et al. (1996) studied the role of crude adult worm antigen of *T. canis* and each of its purified fractions (P-F1, P-F2, P-F3, P-F4 and P-F5) in the serodiagnosis of human toxocariasis using...
ELISA. They reported that ELISA test using P-F1 is the test of choice for diagnosis of human toxocariasis.

Electrophoretic analysis of TeSA and TeESA revealed 13 and 8 polypeptide bands at 250, 125.00, 117.00, 90.435, 69.250, 56.70, 42.50, 40.687, 38.00, 35.70, 27.912, 21.983 and 19.285 kDa. Aida (1999) reported that the SDS profile of TeSA consists of 7 bands; 125.37, 117.73, 90.00, 69.25, 58.36, 47.13 and 46.53 kDa. Also, Mona et al. (2004) identified the electrophoretic profile of adult TeESA to consist of 8 protein bands at 127.66, 94.30, 64.00, 58.81, 50.01, 46.61, 40.09 and 19.263 kDa.

The anti serum raised in rabbits against TeSA (Ra TeSA) was utilized in EITB to identify the specific and the cross reacting antigens (TeSA, P-F1, P-F2, P-F3 and TeESA). The immunoblotting profile of TeSA with Ra TeSA showed 12 immunoreactive bands ranging from 117.00 to 19.285 kDa. Aida (1999) identified the immunoblot profile of TeSA reacted with rabbit hyperimmune serum raised against the parasite to consist of 4 prominent bands (125.37-69.25 kDa) with 2 common reactive bands with our result at 117.00 and 90.00 kDa. The immunoblotting profile of TeSA with Ra TeSA showed 10 immunoreactive bands ranging from 94.00 to 19.285 kDa. Mona et al. (2004) reported that blot analysis of the T. canis adult E/S antigen with experimentally immunized rabbit with the corresponding antigen showed 12 immunoreactive bands (234.32-36.71 kDa) with 3 common reactive bands with our result at 94.00, 88.00 and 36.00 kDa.

Morales et al. (2002) suggested that the 92 and 35 kDa polypeptides of T. canis second stage larvae E/S antigen are specific to Toxocara infection. Also, Iddawela et al. (2007) reported that the 57 kDa fraction of the larval T. canis E/S antigen (TeES-57) is specific to T. canis infection and does not cross react with sera of other related infections. In the present study, the immunoblotting profile of TeESA reacted with positive human (58.739, 47.462, 40.687, 30.527, 29.190, 21.983 and 19.285 kDa) and Ra TeSA (94.00, 88.00, 85.00, 80.00, 47.462, 40.687, 35.706, 30.527, 21.983 and 19.285 kDa) sera showed 5 common immunoreactive bands at 47.462, 40.687, 30.527, 21.983 and 19.285 kDa. So, we may suggest that 58.739 and 29.190 kDa polypeptides of the adult T. canis E/S are specific for Toxocara infection in human and these antigens merit further evaluation as candidates for use in the diagnosis of human toxocariasis.

REFERENCES


