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Characterization of Excretory and Secretory Larval Antigen of *Toxocara canis* by Western Blotting

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Toxocara canis is a dog helminth which causes visceral larva migrans (VLM) in humans. The identification of specific antigens of *T. canis* is important in order to develop better diagnostic techniques. The excretory-secretory larval antigens of *T. canis* (ESLA) were prepared by in vitro culturing of T. canis larvae in RPMI 1640 medium. These antigens were separated using sodium dodecyl sulphate-poly acrylamide gel electrophoresis (SDS-PAGE) which revealed 9 protein bands at a molecular weight of 17, 18, 22, 24, 26, 28, 30, 32 and 120 kDa. The immuno reactivity of excretory-secretory larval antigens of *T. canis* was checked by Western blotting using hyper immune serum raised in rabbits against ESLA antigen which showed six immuno reactive bands at a molecular weight of 17, 18, 22, 24, 30 and 32 kDa. These antigens merit further evaluation as candidate for use in diagnosis of toxocariasis in humans and adult dogs.

Introduction

Human toxocariasis is a major parasitic zoonosis, caused by infection with the larvae of *Toxocara canis*, the common roundworm of dogs and less frequently, of *Toxocara cati*, the roundworm of cats (Despommier, 2003). Humans, especially young childrens are more susceptible because of their habits of geophagy, onchophagy, poor hygienic conditions and their larger risk of exposure to soil contaminated with parasitic eggs (Smith *et al.*, 2009). *Toxocara* cannot complete its life cycle in humans and parasite development is arrested at the larval stage. The migrating larvae give rise to the clinical syndromes of visceral larva migrans (VLM), ocular toxocariasis (OT) and a non-symptomatic infection covert toxocariasis (CT) (Magnaval *et al.*, 2001).

The diagnosis of human toxocariasis currently depends on immunological examinations because it is extremely difficult to detect an

infective *Toxocara* larva in biopsy samples. In immunological tests, the excretory-secretory antigens of *T. canis* larvae (TES) are widely used for both the diagnosis and seroepidemiological studies (Smith *et al.*, 2009).

These antigens are obtained from *in vitro* maintenance of infective larvae and are a mixture of highly immunogenic glycoproteins (Maizels *et al.*, 1993). Since the first description of TES antigen production (De Savigny, 1975), few modifications in the method had been reported by many researches to increasing the parasite yield up to five fold, improving the larval purity and reducing the execution time of the protocol (Ponce-Macotela *et al.*, 2011; Thomas *et al.*, 2016). Recently number of diagnostic candidates has been investigated like *Toxocara* excretory-secretory antigen (TES-57) and recombinant *Toxocara* excretory-secretory antigens (rTES-120, rTES-26, TES-30USM) (Suharni *et al.*, 2009).

Therefore this paper includes an improved protocol for obtaining *T. canis* larvae, isolation of excretory-secretory (ES) antigen and characterization of antigenic components present in the larval excretory and secretory antigen of *T. canis*.

Materials and Methods

Parasite

Adult *Toxocara canis* worms were collected from naturally infected puppies kept at Blue cross of India, Tamil Nadu, after deworming with Piperazine hydrate (Virbac, India) at the dose rate of 100 mg/kg orally. The eggs were isolated from adult female worms following hysterectomy (Thomas *et al.*, 2016). The eggs were incubated in 2 per cent formal saline at room temperature (~26°C) for 28 days to induce embryonation.

Excretory and secretory larval antigen production

The embryonated eggs were repeatedly washed with sterile phosphate buffered saline (PBS), pH 7.2 to remove the formalin. Subsequently the eggs were treated with 6 per cent sodium hypochlorite for 5 min at room temperature to lyses the chitin-protein layer (Schonardie *et al.*, 2014). The eggs were washed again with PBS to remove the sodium hypochlorite. Hatching was induced by incubating decorticated eggs for overnight at 37°C in RPMI 1640 medium with glutamine and sodium bicarbonate. The hatched larvae were filtered through a polystyrene membrane with a 20 µm pore size.

The live larvae were transferred to RPMI 1640 medium supplemented with L-glutamine and sodium bicarbonate (Sigma-Aldrich Co, USA) containing antibiotics and antifungal solution (100 U/ml Penicillin-G, 100 µg/ml Streptomycin and 25 µg/ml Amphotericin B) in the T_{25} tissue culture flask at a concentration 10^3 larvae/ml and kept at 37°C in 5 per cent CO_2 incubator. The culture supernatant was removed 5 days interval, pooled and centrifuged to precipitate all debris.

The resulting supernatant was filtered through a 0.22 µm syringe filter (Millipore, USA) and stored at -20°C with 1 mM phenyl methyl sulfonyl fluoride (PMSF). The stored ES antigen was then mixed, dialyzed (molecular weight cutoff 10 kDa, Sigma-Aldrich Co, USA) against PBS, pH 7.2 for 12 h at 4°C and then concentrated to one tenth of initial volume using polyethylene glycol (PEG 6000-Himedia, India) at 4°C. The protein concentration of resultant ESLA antigen was determined by bicinchoninic acid (BCA) kit (Genei, Bangalore) method. The ESLA was stored in aliquots at -20°C.

Characterization of ESLA antigen

SDS-PAGE analysis

The protein fractions of the ESLA antigen of *T. canis* were separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) using discontinuous system in a Mini-PROTEAN II Electrophoresis unit (Bio-Rad, USA). The antigen (20 µg/lane) was diluted in 2X concentrate Laemmli sample buffer and boiled for 5 minutes to denature the protein then loaded in the 12 % polyacrylamide gel. A standard prestained molecular weight marker (MW 10 to 250 kDa, Bio-Rad) was used for calibrating the gel.

The electrophoresis was performed at a constant voltage of 100V till the tracking dye reaches 1 cm above the lower extremity. The gel was subjected to staining with 0.1% Coomassie Brilliant Blue R 250 (Sigma, B-0149) overnight followed by destaining. The gel was photographed with gel documentation system (Bio-Rad Gel Documentation system XR+ with Image Lab software version 3.0, USA).

Identification of immunogenic fractions

Raising of hyper immune serum

Two adult, New Zealand white rabbits of either sex aged about one year old were maintained as per CPCSEA guidelines (Approved Protocol No. 2345/16/DFBS dated. 26.10.2016). The rabbits were immunized with 0.5 mg of ESLA antigen with equal volume of Montanide (Seppic) adjuvant on 0 day intra muscularly. The booster doses were given on 14 and 28 days after primary immunization with same antigen. The rabbits were bled by ear vein 10 days after the last injection and serum was separated and preserved at -20°C.

Western blotting

The immunogenic fractions were identified by Western blot analysis according to the method described by Towbin *et al.,* (1979). Following electrophoresis, the proteins were transferred to nitrocellulose membrane (Sigma, USA) having a pore size of 0.45 µm by semidry blotting apparatus (Bio-Rad, USA) at 25V for one hour. The prestained protein marker carrying nitrocellulose membrane was cut separately. The rest of the nitrocellulose membrane was incubated in 5 per cent skim milk powder overnight at 4°C. The membrane was washed in washing buffer thrice, each for 5 minutes. The nitrocellulose membrane was incubated in hyper immune serum at a dilution of 1:100 in PBS for 1 hour at 37°C, washed in washing buffer thrice each for 5 minutes. The membrane was incubated with anti-Rabbit-IgG Horse Radish Peroxidase (HRP) conjugate (Sigma, USA) 1:1000 dilution for 1 hour at 37°C, washed in PBST three times and then substrate Diamino benzidine (DAB) solution was added. When brown colour bands appeared, the reaction was stopped by decanting the substrate solution and replacing it with distilled water. Thereafter, the membrane was allowed to dry.

Results and Discussion

To carryout studies aimed to improving the diagnosis of VLM, an important first step is to obtain sufficient quantities of ESLA. We modified few steps in the standard protocol for obtaining ESLA producing *T. canis* larvae to improve larval yield, purity and shorten the duration of procedures. On an average, about 90,000 eggs were isolated from each adult female worm of *T. canis* (Fig. 1). Embryonation of 62 % of the eggs were observed after 7 days of cultivation while after 28 days of incubation the frequency of embryonation reached up to 80 % (Fig. 2).

The embryonated eggs with second stage larvae were washed and decorticated using 6 per cent sodium hypochlorite solution. It was found that after 5 minutes of incubation with sodium hypochlorite solution the chitin-protein layer of the eggs got dissolved to a thin membrane around the larvae (Fig. 3). Decortication of larvated eggs using different concentrations of sodium hypochlorite was tried elsewhere (Roldan *et al.,* 2006, Ponce-Macotela *et al.,* 2011 and Thomas *et al.,* 2016). Hatching was induced by incubating the eggs with RPMI 1640 medium overnight at 37°C in an incubator. The hatched larvae were filtered through a polystyrene membrane with a 20 µm pore size. About 70 per cent of the viable larvae were recovered by this method (Fig. 4). Hatched larvae were cultured in RPMI-1640 medium containing antibiotics at a concentration of 10^3 larvae/ml with replacement of medium at 5 days interval and maintained up to 2 months (Fig. 5). The protein concentration of ESLA was obtained 2.5mg/ml of antigen.

In the present study, the excretory secretory larval antigens (ESLA) of *T. canis* were characterized by SDS-PAGE using 12% gel and stained with coomassie brilliant blue which revealed 9 protein bands with a molecular weight of 17, 18, 24, 26, 28, 30, 32 and 120 kDa (Fig. 6). Colli *et al.,* (2011) reported that the SDS-PAGE profile (10 per cent) of larval ES antigen of *T. canis* when stained with silver stain showed at molecular weight of 105-120, 70, 55, 44 and 31-34 kDa protein bands.

Fig.1 Unembryonated eggs teased from the uterus of *Toxocara canis* worms

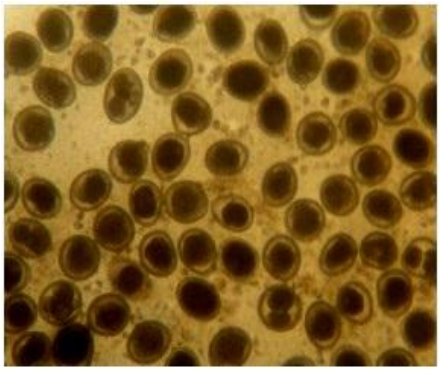


Fig.2 Embryonated eggs in 2% formal saline containing second stage larvae

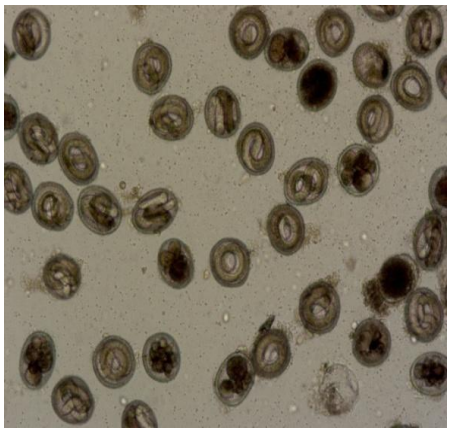


Fig.3 Decorticated eggs of *T. canis* using 6% sodium hypochlorite

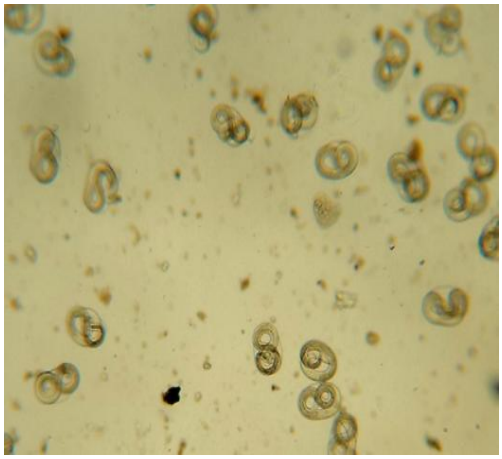


Fig.4 Hatched out second stage larvae of *T. canis*

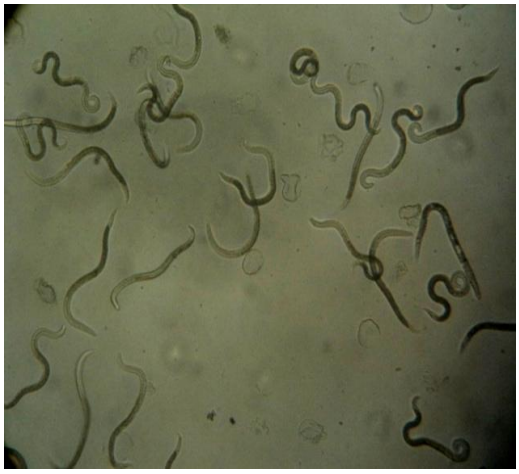


Fig.5 Larvae cultivated in RPMI 1640 medium

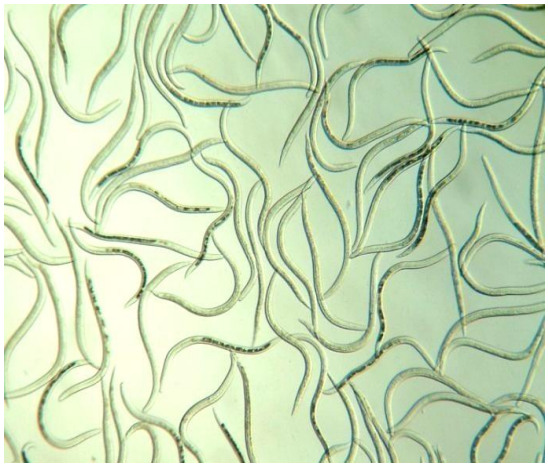


Fig.6 SDS – PAGE profile of ESLA antigen of *Toxocara canis* stained with Coomassie blue stain

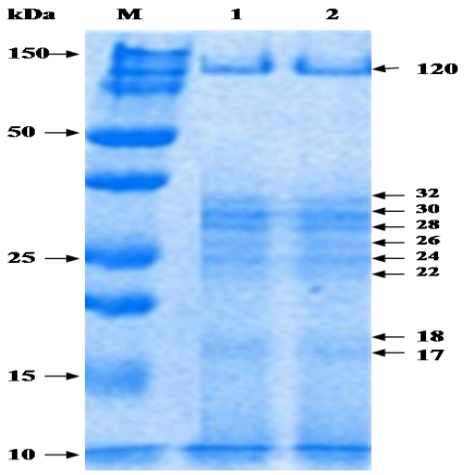
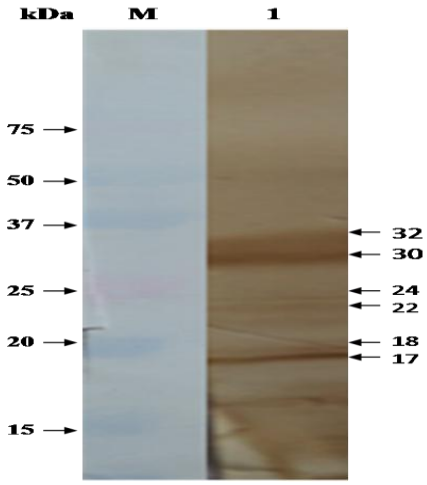


Fig.7 Western blot studies of ESLA antigen using hyperimmune sera raised in rabbits



Protein bands of larval ES antigen of *T. canis* recovered in this study appear similar in molecular weight to those associated with larval ES antigen of *T. canis* reported by Colli *et al* (2011) at mol. wt. of 120 (105-120), 32 (31-34) kDa. SDS-PAGE of *T. canis* ES has produced variable results between laboratories. Sugane and Oshima (1983) described a single band at 35 kDa, while Maizels *et al.*, (1984) demonstrated 5 major components (ES labelled with radio-iodination) at 32, 55, 70, 120 and 400 kDa. Meghji and Maizels (1986), carrying out extensive molecular and biochemical characterization of ES from long-term cultures, using labelled ES, concluded that there were a number of macromolecules secreted, of which the major components were glycoproteins that differed in essential characteristics, i.e., 32, 120 and 400 kDa.

The immuno reactivity of ESLA antigens of *T. canis* was checked by Western blotting using hyper immune serum raised in rabbits which revealed six immuno reactive bands at a molecular weight of 17, 18, 22, 24, 30 and

32 kDa (Fig. 7). Present study shows *T. canis* excretory–secretory proteins from larvae, which similar to those found in the crude antigen of *T. canis* larval protein bands at 28, 30 and 120 kDa (Jin *et al.,* 2013) and also relate to those found in the ES antigen of adult *T. canis* at molecular weight band of 30 kDa (Sudhakar *et al.,* (2014). The differences in the banding pattern can be attributed to differences in the preparation of antigen, age of larval culture (Iddawela *et al.,* 2007), contamination with somatic antigens in culture due to dead larvae, variation in the running condition of gel (Roldan and Espinoza, 2009) and variation due to larval strain differences (Badley *et al.,* 1987). These antigens merit further evaluation as candidate for use in diagnosis of toxocariasis in humans and adult dogs.

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