Comparison of fusion protein and excretory-secretory antigens of *Toxocara canis* in detection of antibodies in experimental toxocariasis

Choong MF[1], Mak JW[2], Ngah Zasmy[3], Navaratnam V[3] and Suresh Kumar[4] *[1]Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur; [2]Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia; [3]Centre for Drug Research, Universiti Sains Malaysia, 11800 Penang, Malaysia; [4]Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia*

Abstract

The immunodiagnosis of visceral larva migrans due to *Toxocara canis* infection is based on the detection of specific antibodies with the enzyme-linked immunoassay (EIA) using excretory-secretory (ES) antigens of second stage larvae of the parasite. As ES antigens are cumbersome to produce, a specific recombinant protein would be an attractive alternative. A recombinant protein produced previously (Yamasaki *et al.*, 1998) was compared with ES antigens in the EIA and immunoblot detection of antibodies in the sera of *Macaca fascicularis* experimentally infected with 5000 – 10000 *T. canis* embryonated eggs. The EIA using recombinant protein and ES antigens gave optical density readings that correlated significantly well with each other ($p<0.02$). Antibody response increased rapidly from week 1, and peaked at 6-8 weeks post-infection. In immunoblot assays, infected monkey sera at 4-8 weeks post-infection, recognized the recombinant protein and produced a single prominent band at 38 kDa relative molecular weight. In contrast, multiple bands between 38 – 98 kDa were obtained with ES antigens. Sera from uninfected control monkeys and pre-infection sera did not produce these bands. In conclusion, the recombinant protein is suitable for use in the EIA and immunoblot assay for detection of specific anti-*T. canis* antibodies.

Key words: visceral larva migrans; toxocariasis; recombinant protein; toxocarial antibodies

Introduction

The enzyme-linked immunosorbent assay (EIA) using excretory-secretory (ES) antigens produced from in vitro culture of the second stage larvae (L2) of *Toxocara canis*, is currently used for the diagnosis of visceral larva migrans (VLM) due to this parasitic infection (De Savigny, 1975; De Savigny *et al.*, 1979). However, ES antigen production involves the recovery of adult gravid female worms from puppies, 3-4 weeks to embryonate eggs recovered from the nematode uteri, egg hatching and in vitro cultivation of L2 in RPMI 1640 medium, followed by harvesting and purification of the antigen from the spent culture medium. Cross reactions with antibodies from infection with related nematodes like *Toxocara cati* (Maizels *et al.*, 1987), *Ascaris lumbricoides* and *Ascaris suum* (Glickman *et al.*, 1985; Kennedy *et al.*, 1989), have been reported. Although immunoblot analysis of antibodies reactive to SDS-separated parasite antigens (Normaznah *et al.*, 1988) can improve serological diagnosis of *VLM*, there is still a need for a constant supply of defined antigen with equal or better sensitivity and specificity.

Recently, Yamasaki *et al.* (1998) produced a recombinant antigen derived from a cDNA encoding an ES antigen from the L2 of *T. canis*. The antigen was found to specifically react with sera from toxocariasis patients. The objective of the present study is to compare the use of this recombinant antigen with ES antigens in EIA and immunoblot detection of antibodies in monkeys experimentally infected with embryonated eggs of *T. canis*.

Materials and Methods

Preparation of embryonated eggs

Toxocara canis adult gravid female worms were obtained from the intestine of stray puppies put down at the Kuala Lumpur City Veterinary Services. Eggs obtained from the uteri were washed twice with normal saline, suspended in 1% sulphuric acid and cultured at room temperature for four weeks. Embroynated eggs were washed several times with normal saline, and used for infection of experimental animals.

Preparation of ES antigens

Embyonated *T. canis* eggs were washed twice with sterile normal saline, resuspended in 1% sodium hypochlorite and incubated for an hour at 37°C. The eggs were then washed five times and then resuspended in sterile normal saline. Larvae were hatched from the embryonated eggs by bubbling CO_2 through the suspension. The hatched L2 were cultured in RPMI 1640 medium by the method of De Savigny (1975) for the production of ES antigen.

Production of recombinant Toxocara canis antigen

The construction of a recombinant plasmid pET-32b

(+) expression vector (Novagen, USA), its transformation into *E. coli* BL21 (DE3)·pLysS, and the subsequent expression of a thioredoxin-*T. canis* fusion protein by induction with IPTG has been described (Yamasaki *et al.*, 1998). The induced cells were sonicated in 20 mM Tris-HCl, pH 8.0, containing 100 mM NaCl and 1% Triton X-100. The fusion proteins expressed as inclusion bodies were solubilized completely with 8 M urea in 20 mM Tris-HCl, pH 8.0, and 100 mM NaCl, and then purified using a TALON™ Metal Affinity Resin Column (Clontech, USA). The selected fraction was dialysed with bicarbonate buffer (pH 9.6) for 24 hours and the protein concentration estimated using a Protein Assay Kit (Bio-Rad™, USA). This r*T. canis* was used in the EIA and immunoblot experiments.

EIA and immunoblot analysis

Checkerboard titration was carried out to determine the optimal concentrations of antigens (0.05 mg r*T. canis* or ES), serum, and conjugates for the indirect EIA. Peroxidase-conjugated goat anti-monkey IgG and TMB peroxidase substrate were used.

For the immunoblot analysis, 5 µg/well of either r*T. canis* or ES proteins were separated using SDS-PAGE, and transferred to nitrocellulose paper using the method of Towbin *et al.* (1979). Sera were pre-absorbed with *E. coli* lysate at 1: 5 dilution before use. The nitrocellulose paper with the transferred proteins was pre-incubated in PBS, pH 7.4 with 3% skimmed milk for 1 hour, washed, and then incubated with monkey sera diluted 1:40 (for r*T. canis*) or 1:100 (for ES), with 0.05% bovine albumin at 4°C overnight. After incubation with peroxidase-conjugated goat anti-monkey IgG, the antigen-antibody complexes formed were visualized with 4-chloro-1-naphthol.

Experimental infection of monkeys

Twenty *Macaca fascicularis* (Long-railed macaques),

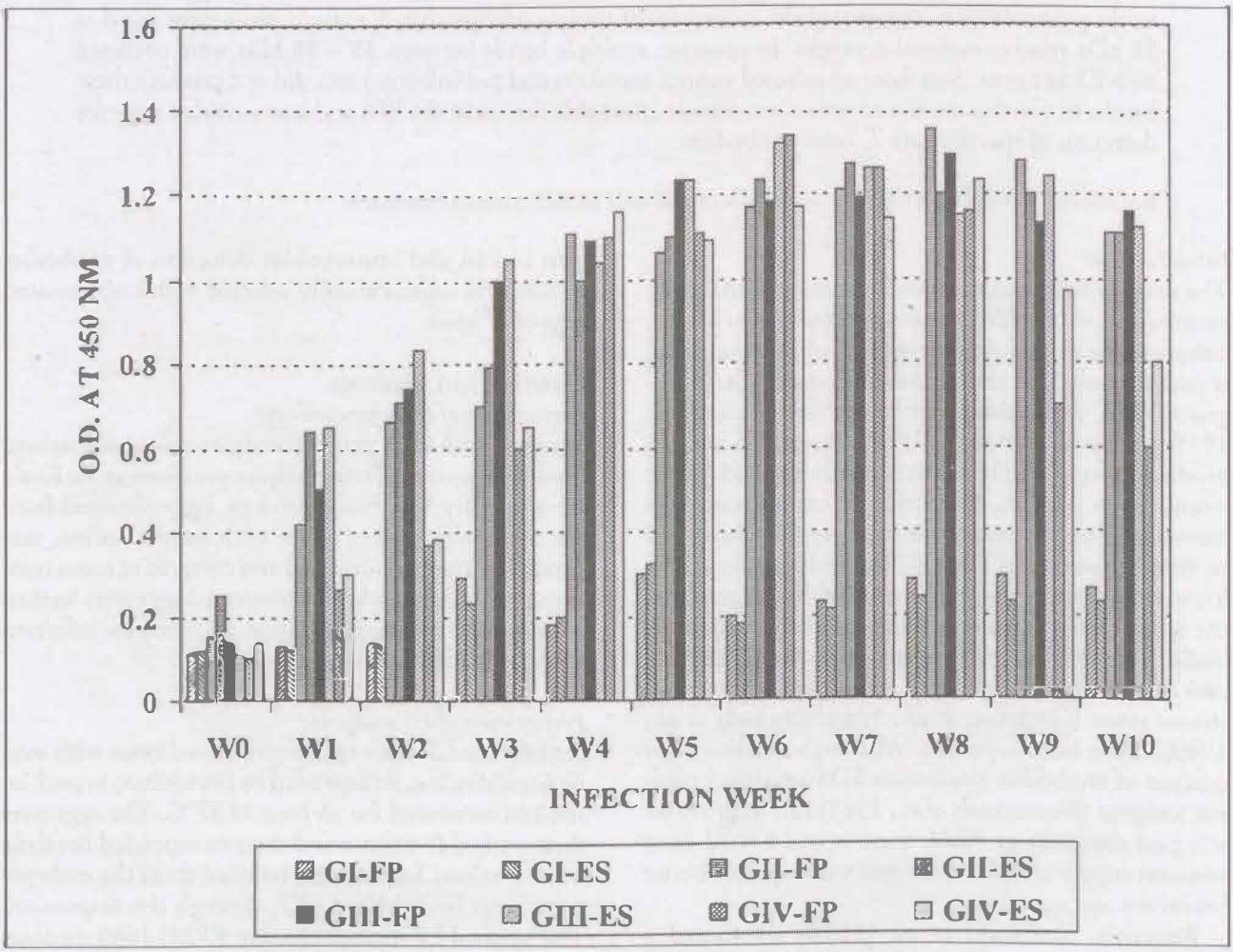

Fig. 1. Enzyme-linked immunosorbent assay optical density (O.D.) readings at 450 nm, using recombinant protein (FP-EIA) or ES antigen (ES-EIA), in *Macaca fascicularis* infected with different oral doses of embyonated *Toxocara canis* eggs*

*GI-FP, GII-FP, GIII-FP & GIV-FP = FP-EIA mean OD values for groups I (control), II (infected with 5000 eggs), III (infected with 10000 eggs) & IV (infected with 2000 eggs daily x 5 days) respectively; GI-ES, GII-ES, GIII-ES & GIV-ES = ES-EIA mean OD values for groups I, II, III & IV respectively.

weighing approximately 2.0 kg each were randomly assigned to a control (Group I) and three experimental groups (Groups II, III & IV) of five animals per group. Groups II and III animals were each infected orally via stomach tube, with 5000 and 10000 eggs at a single dose respectively. Group IV animals were each infected with 2000 eggs daily x 5 days. Sera were collected from the monkeys at pre-infection, and at weekly intervals until 10 weeks post-infection. The Animal Use Committee, Institute for Medical Research, Ministry of Health Malaysia, passed the experimental protocol and study.

Statistical analysis

Analysis of variance was performed to determine differences in EIA readings within and between control and experimental groups, using SPSS® for Windows Version 7.5.

Results

IgG-EIA

Antibodies were detected in the EIA with both r*T. canis* and ES antigens, the optical density (OD) readings correlating significantly well with each other within groups ($p < 0.02$; Fig. 1). There was no significant difference in the OD readings between groups during the pre-infection week. Both assays detected a rapid increase in antibody levels in infected animals, starting from week 1 post-infection, and reaching a peak between 6-8 weeks postinfection in all the experimental groups. By week 4, both assays showed mean antibody levels in experimental groups to be significantly higher than those in the control group. However, there was no significant difference in mean readings between experimental groups at the corresponding post-infection weeks.

Immunoblot analysis of Toxocara canis infected Macaca fascicularis sera

Similar results were obtained with sera of infected animals from all experimental groups (Groups II, III & IV). A single protein at 38 kDa molecular weight in r*T. canis* was recognized by sera of experimental animals collected at 4 – 10 weeks post-infection (Fig. 2). This was more prominently seen in animals infected with 10000 eggs each (Groups III & IV) than in Group II animals given 5000 eggs each. Only faint bands were

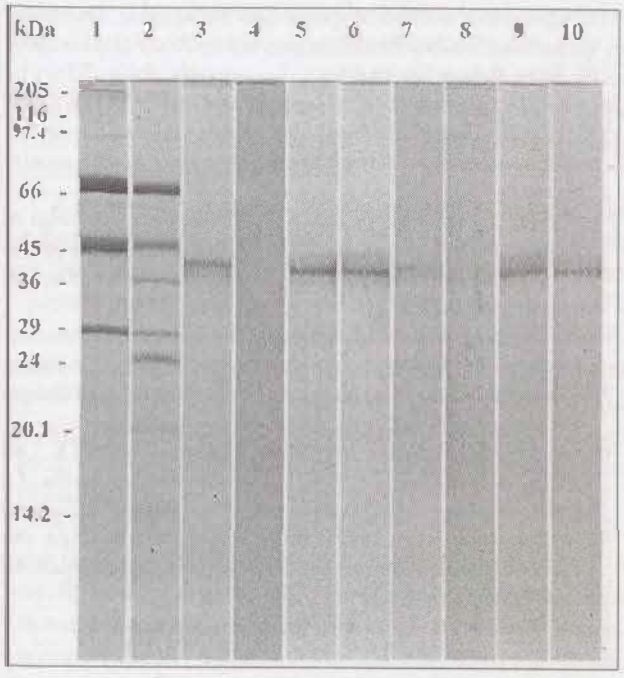

Fig. 2. Immunoblot analysis of sera from *Macaca fascicularis* experimantally infected with *Toxocara canis* embryonated eggs, with r*T. canis* protein. lane 1: high molecular weight marker; lane 2: low molecular weight marker; lane 3: r*T. canis* protein stained with Coomassie Blue; lane 4: pre-infection serum; lane 5: serum at week 4 post-infection, from group III animal (infected with 10000 *T. canis* embryonated eggs); lane 6: serum from group III animal at week 8 post-infection; lane 7: serum at week 4 post-infection, from group II animal (infected with 5000 embryonated eggs); lane 8: serum from group II animal at week 8 post-infection; lane 9: serum at week 4 post-infection, from group IV animal (infected with 2000 embryonated eggs daily x 5 days); lane 10: serum from group IV animal at week 8 post-infection.

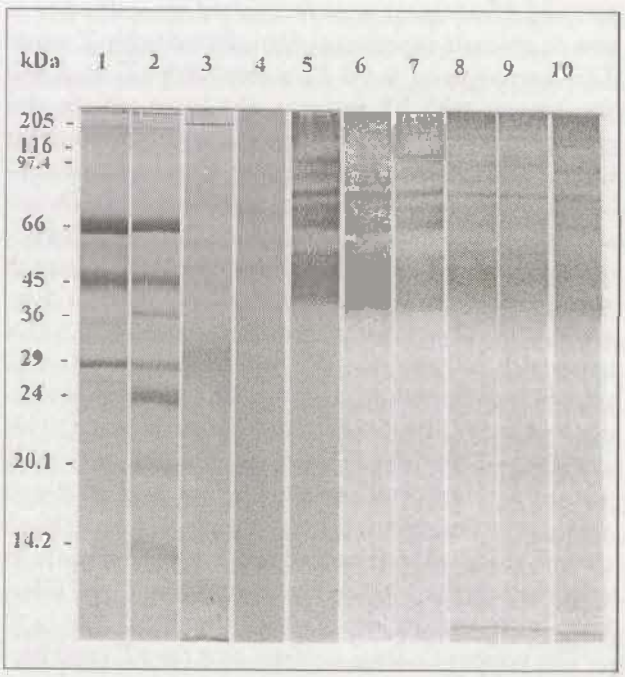

Fig. 3. Immunoblot analysis of sera from *Macaca fascicularis* experimantally infected with *Toxocara canis* embryonated eggs, with ES antigens. lane 1: high molecular weight marker; lane 2: low molecular weight marker; lane 3: ES antigens stained with Coomassie Blue; lane 4: pre-infection serum; lane 5: serum at week 4 post-infection, from group III animal (infected with 10000 *T. canis* embryonated eggs); lane 6: serum from group III animal at week 8 post-infection; lane 7: serum at week 4 post-infection, from group II animal (infected with 5000 embryonated eggs); lane 8: serum from group II animal at week 8 post-infection; lane 9: serum at week 4 post-infection, from group IV animal (infected with 2000 embryonared eggs daily x 5 days); lane 10: serum from group IV animal at week 8 post-infection.

seen in sera collected at weeks 2 and 3 post-infection (not shown in figures).

With SDS-PAGE separated ES proteins, sera from infected experimental animals produced multiple bands between 38-98 kDa, the prominent ones being at 38, 66, 80 and 98 kDa (Fig. 3).

Discussion

Monkeys that were infected with 5000 – 10000 *T. canis* embryonated eggs orally, all produced anti-toxocarial antibodies as early as week 1 post-infection. Both the FP-EIA and ES-EIA detected these antibodies, and the mean OD readings were similar and correlated very well with each other, at the corressponding time points. The mean antibody levels were significantly higher in experimental groups than in control groups at the corresponding weeks. Peak antibody levels were detected between 6-8 weeks post-infection, and these levels declined only slighrly in the subsequent weeks of observation. In general, there was no significant difference in mean antibody levels in animals infected with 5000 (group II) or 10000 eggs in a single dose (group III) or divided doses (group IV).

Immunoblot analysis showed that the r*T. canis* protein and ES antigens were recognized by antibodies in sera of animals experimenrally infected with *T. canis*. The reactive band at 38 kDa seen with r*T. canis* was also present with ES antigens, the latter antigen also giving other reactive bands. It is interesring to note that Lam *et al.* (1992) also reported that monoclonal antibodies developed from mice immunized with *T. canis* antigens also recognized a 38.5 kDa polypeptide in ES, embryonated eggand L2 *T. canis*antigens. Normaznah *et al.* (1988) also showed thar sera from patients diagnosed with visceral larva migrans recognized a 38.5 kDa band with L2 antigens. We believe that the r*T. canis* protein is probably similar to the 38.5 kDa protein recognized in these previous studies. Yamasaki *et al.* (1998) also showed that the r*T.canis* protein also specifically reacted with toxocariasis sera but not that of *Brugia malayi* infection, dirofilariasis, or ascariasis. Anasakiasis sera pre-absorbed with homologous antigen did not react with the protein. However, patients with *T. cati* infection also recognized the r*T. canis* protein.

The present findings confirm that the r*T. canis* protein can be used as a substitute for ES antigens for the detection of specific anti-toxocarial antibodies. This recombinant protein can therefore be used for the immunodiagnosis of visceral larva migrans due to *T. canis* infection.

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