

SEROPREVALENCE OF *TOXOCARA CANIS* ANTIBODIES AMONG ORANG ASLI (ABORIGINES) IN PENINSULAR MALAYSIA

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Abstract. An enzyme-linked immunosorbent assay using excretory-secretory antigens of the second stage larvae maintained *in vitro* was used to determine the seroprevalence of *Toxocara* antibodies in Orang Asli (aborigines) of Peninsular Malaysia. The mean + 3 SD optical density of 30 healthy subjects was used as the cut-off point. Overall prevalence was found to be 31.9%. No significant relationship was found between positive rates with sex and age groups, though children between 0 to 9 years recorded the highest positive rates. Eosinophil counts were found to be closely related to the proportion of positivity to toxocaral infection and mean optical densities. There was some degree of cross-reaction with *Trichuris trichuria* positive sera.

INTRODUCTION

Toxocariasis in man is caused by infection with the second stage larva of *Toxocara canis*, a nematode of dogs. The infection is acquired mainly through the ingestion of embryonated eggs from the soil (Schant and Glickman, 1978). Upon hatching, the larvae migrate through somatic organs giving rise to the condition known as the visceral larval migrans. Since the larvae migrate in tissue and do not develop into adult worms, definitive diagnosis through tissue biopsy and stool examination is very unlikely. The diagnosis of toxocariasis therefore depends heavily on immunological tests.

Since the adaptation of ELISA test for toxocariasis by de Savigny (1979) using excretory-secretory (ES) antigens of the second stage (L2) larvae of *T. canis*, a number of reports on the seroprevalence of toxocariasis in the European and Western Pacific countries have been produced. Rather less is known of the prevalence of *Toxocara* infection in the tropical region, where parasitic infections are common.

In Peninsular Malaysia, Aborigines (Orang Asli) who are seminomadic, have been observed to be closely associated with dogs. It was therefore of interest to determine the seroprevalence of *Toxocara* antibodies among Orang Asli by ELISA test using ES antigens of the L2 maintained *in vitro* (de Savigny, 1975).

MATERIALS AND METHODS

Subjects

Two ml of blood from patients and accompanying relatives admitted to the Gombak Hospital were collected, centrifuged at 5,000 rpm for 10 minutes and the serum stored at -70°C until used. The age, sex, registration number, ethnic group and address of the patients were recorded.

Physical examinations were carried out on the subjects. The total white blood cell and differential counts, and stool examination for parasitic ova were performed.

Sera from 30 healthy volunteers from the Malaria/Filaria Division of the Institute were also obtained for the determination of the 'normal' ELISA values.

Excretory-secretory antigens

Gravid female *T. canis* obtained from the intestines of 4 - 8 week old puppies were dissected for ova which were then washed twice with normal saline. The ova were then resuspended in 1% sulphuric acid and left at room temperature for 4 weeks to embryonate.

The embryonated eggs were washed twice with sterile normal saline. The eggs were then resuspended in a mixture of equal volumes of 2% NaOH and 2% sodium hypochlorite (clorox) solution for

3 minutes and then centrifuged at 1,000 rpm for 5 minutes. Subsequently the supernatant was discarded and the decoated eggs were washed 5 times with sterile normal saline.

The following procedures were then carried out under lamina flow with strict aseptic technique. The eggs were washed 3 times with the culture medium (RPMI 1640) containing 100 iu/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml fungizone. Each time after removing the supernatant, repeated aspiration up and down the pasteur pipet were performed to disrupt the eggs and releasing the larvae. The larvae were then resuspended in culture medium placed in the tissue culture flask. The larvae were then incubated at 37°C.

Each week, the spent medium containing the ES antigens was collected, pooled and filtered through cameo IFS filter (MSi microseparation inc). Aliquots of the ES antigens were stored at -70°C until used. The larvae were resuspended in fresh culture media and reincubated as described above.

ELISA test

The ELISA was carried out according to the method of Voller *et al* (1976) with modification. The optimum dilutions for antigen, serum and conjugate (peroxidase) determined by chequer-board titration were found to be 1 : 2, 1 : 1,000 and 1 : 10,000, respectively. The protein concentration of the antigen used was about 5 µg/ml as determined using the Bio-rad protein assay kit II (Biorad^l).

Two hundred microliters of the antigen diluted in coating buffer were added to each well of the microtiters plate. The plates were then left overnight at 4°C. The plates were then washed thrice with PBS-Tween and kept at -70°C until used. Two hundred microliters of each test serum diluted in PBS-Tween were added into two wells of the antigen coated plates and incubated at room temperature for two hours. Each plate contained PBS-Tween, positive and negative serum controls. The positive serum control was taken from a clinically diagnosed toxocariasis patient with a high antibody titer. After incubation, the plates were washed as above. Two hundred microliters of enzyme-labeled goat anti-human IgG horseradish peroxidase con-

jugate (Cappel Labs) diluted in PBS-Tween were added to each well and incubated for 3 hours at room temperature.

After washing, 200 µl of substrate solution were added to each well and incubated for 30 minutes in the dark. The enzyme reaction was stopped by adding 50 µl of 2.5 M sulphuric acid in each well. The absorbance value of the tests at 492 nm were read using an automatic micro ELISA reader (Dynatech, MR 600).

RESULTS

Prevalence by sex and age group

The mean ± SD ELISA OD values for the 30 sera of the healthy subjects was 0.244 ± 0.133 (range: 0.048 to 0.640) with a median value of 0.112. The mean OD + 3SD of the healthy subjects was taken as the cut-off point.

A total of 480 sera were collected from Gombak Hospital, 206 were from males (42.9%) and 274 from females. Of these, 153 had positive titers giving an overall prevalence of 31.9%. The prevalence among male was higher (33.2%) as compared to female (30.2%) but the difference was not statistically significant ($X^2=0.147$, $p>0.05$).

Table 1 shows the prevalence of *Toxocara* antibody according to age groups. All age groups showed evidence of exposure to the infection with the highest prevalence among children below 10 years ($X^2=18.09$, $p<0.01$). The prevalence rates appeared to decrease with increasing age but the correlation was weak and not significant ($r=-0.56$, $p>0.05$).

Intestinal parasites and *Toxocara* positive sera

Results of stool examination were available from 136 patients (Table 2). None of the 5 sera from individuals with *Ascaris lumbricoides* (single infection) were positive to *Toxocara* and similarly, none of those with *Giardia* and *E. coli* infections. The mean OD for *Toxocara* in patients with *Ascaris* was very much lower than those who were free from intestinal parasites ($t=3.77$, $p<0.05$). However, 4 of the 10 sera from patients with *Trichuris trichuria* infection had positive ODs for *Toxocara*. The mean OD between *Trichuris* infected and negative sera did not differ significantly ($t=0.29$, $p>0.05$).

Table 1
Positivity of *Toxocara* ELISA by age group.

Age group (years)	Mean age	n	No. positive	% Positive
0 - 9	4.8	104	47	45.2
10 - 19	14.3	69	26	37.7
20 - 29	24.3	94	18	19.1
30 - 39	33.5	84	25	29.8
40 - 49	43.9	54	15	27.8
50 - 59	53.0	42	14	33.3
≥ 60	70.0	33	8	24.2

Table 2
Positivity of ELISA *Toxocara* according to intestinal parasites.

Parasites	n	No. positive	% Positive	Mean OD ± SD
Negative	97	36	37.11	0.531 ± 0.392
<i>A. lumbricoides</i>	5	-	0	0.222 ± 0.088
<i>T. trichuria</i>	10	4	40.0	0.568 ± 0.290
<i>E. nana</i>	6	1	16.67	0.241 ± 0.229
<i>Giardia</i>	5	-	0	0.329 ± 0.208
<i>E. coli</i>	4	-	0	0.356 ± 0.080
Hookworm	3	-	0	0.247 ± 0.087
Mixed infection	6	2	37.11	0.531 ± 0.280

OD = optical density at 492 nm.

Eosinophil count (% of the total white cell count).

Table 3 shows the number of sera positive for *Toxocara* in relation to eosinophil count. The proportion of positive sera increased with increasing eosinophil counts ($r=0.96$, $p<0.05$). There was a marked difference in the proportion of positive sera between eosinophil count of $<10\%$ and those with $\geq 10\%$ ($X^2=14.1$, $p<0.05$). Strong positive correlation between mean optical densities and mean eosinophil counts ($r=0.92$, $p<0.05$) was also noted.

DISCUSSION

Serodiagnosis plays an important role in the detection of *Toxocara* infection since clinical symptoms are non-specific. However, uncertainty in the interpretation of results still occurs, particu-

larly in the definition of positivity. There are marked differences of positivity criteria between laboratories. In our study, we used the mean + 3SD optical density of the 30 healthy subjects as the cut-off value. Though it was arbitrarily determined, this is higher than the 3x median value used by de Savigny *et al* (1975) and Matsumara and Endo (1983).

Many authors have cautioned about the analysis of tropical sera in view of the potential cross-reactivity with wide range of other parasites since polyparasitism is common in the tropics. The ELISA technique utilising ES antigen has been shown to be sensitive and specific (de Savigny *et al*, 1975). Although 40% of those with *Trichuris* infection had positive sera, the mean OD between those with and without the infection was not significantly different. There has been a report that immune *T. trichuria* sera cross-react with *Toxocara*

Table 3

Positivity of *Toxocara* ELISA by % eosinophil count.

No. of E	n	Mean E	No. positive	% Positive	Mean OD \pm SD
0	55	0	15	27.27	0.491 \pm 0.347
0 - 4	77	2	21	27.27	0.455 \pm 0.340
5 - 9	49	7	13	26.53	0.472 \pm 0.391
10 - 14	16	12	7	43.75	0.544 \pm 0.368
≥ 15	19	21	10	52.63	0.695 \pm 0.458

E=% eosinophil count

ES antigen on immunoblots but the profile of antigens recognized was distinct from that obtained with *T. trichuria* antigen (Lillywhite *et al*, 1991). It is possible that the 40% of the *T. trichuria* infected subjects with positive OD values could also be exposed to toxocarasis.

It is interesting to note that the eosinophil count was found to be closely related to both the positivity rates and mean OD values. This finding further supports the differential diagnosis of toxocarasis in patient with hypereosinophilia (Beaver, 1962) and a positive ELISA OD value.

In the present study, no significant relationship was found between sex and a positive *Toxocara* ELISA OD reading in contrast to other findings (de Savigny *et al*, 1975; Worley *et al*, 1984). Although children between 0 to 9 years of age were found to have the highest prevalence, the decrease in prevalence rate with increasing age was found to be not significant.

We therefore conclude that exposure to toxocarasis is not uncommon among the Aborigines in Peninsular Malaysia, affecting both sexes and all ages. Further work should be carried out to determine the extent and distribution of the infection in other subpopulation in the country.

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