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Seroprevalence of toxocariasis among Orang Asli (Indigenous people) in Malaysia using two immunoassays

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Abstract. Toxocariasis is a zoonotic helminthic infection of humans caused by the dog roundworm (*Toxocara canis*) or cat roundworm (*Toxocara cati)*. There are two main human syndromes: visceral larva migrans (VLM), which are characterized by symptoms associated with major organs and ocular larva migrans (OLM), in which pathological effects on the host are restricted to the eye and the optic nerve. The present study evaluated the seroprevalence of toxocariasis among the Orang Asli with an IgG4-ELISA using recombinant antigens (rTES-26, rTES-30 and rTES-120) and an IgG-ELISA commercial kit (Cypress Diagnostic, Belgium). A total of 188 serum samples were analyzed using IgG4-ELISA recombinant antigens while 83 were tested using IgG-ELISA. Overall, 9 out of 188 (4.8%) samples were positive with the former assay: rTES-26 (2.7%) and rTES-30 (2.1%); and 63 out of 83 (75.9%) were positive with the IgG-ELISA. In general, the seroprevalence of toxocariasis among males (9.5%) was higher compared to females (1%). Children below 12 years (6.3%) have higher seroprevalence rate compared to adults (1.2%). Out of 59 IgG positive samples, 56 (94.9%) were also positive with soil-transmitted helminth (STH) infections which may indicate high false positivity. None of the IgG4-ELISA positive samples were positive with STH infections. Of 9 positive samples with IgG4-ELISA, 7 were also positive with IgG-ELISA giving the probability of true cases. The present finding indicated that exposure to *Toxocara* infection is not unusual among Malaysian aborigines, and it affects both sexes and all age groups. As a prevention strategy, more effective public health programmes to promote better understanding on the consequences of toxocariasis among the Orang Asli communities are deemed necessary.

INTRODUCTION

Toxocariasis is a zoonosis caused by the second stage larvae (L2) of ascarid of cats (*Toxocara cati*) and dogs (*Toxocara canis*). Of these two species, *T. canis* is regarded as the main cause of human toxocariasis. Cats usually bury their faeces and the infective eggs are not exposed to susceptible individuals. The morphology of the larvae in tissue section and in early serology failed to implicate *T. cati* in human cases. However, there were some studies which reported that *T. cati* has been implicated as the main cause for ocular toxocariasis in human (Smith & Rahmah, 2006). Hence, the role of *T. cati* in causing human toxocariasis should not be ignored and may require further investigation. Other species such as *Toxascaris leonine* (Fisher, 2003), *Toxocara malaysiensis* (Gibbons *et al*., 2001) and *Toxocara lyncus* (Macchioni, 1999) have also been reported in both cats and dogs. Nonetheless, it is still controversial whether these species could cause human infection resulting in clinical disease (Despommier, 2003).

The eggs of *Toxocara* are unembryonated when passed in the faeces of animals into the environment. Under

optimal temperatures and humidity, these eggs develop into embryonated eggs that are infectious to both final and paratenic hosts. Infective eggs are reported to survive under optimal circumstances for at least one year. Human may acquire the infection by oral ingestion of infective *Toxocara* eggs from contaminated soil (sapro-zoonosis) from unwashed hands or consumption of raw vegetables and ingestion of undercooked meat which contains the larvae (Smith & Rahmah, 2006).

In human, the infective larva cannot complete its lifecycle and does not develop into adult stage. Therefore, after the eggs hatch and larvae released, the larvae will wander around in the tissue and somatic organs and cause damage to the tissue they enter. The disease manifests itself as two distinct forms, namely visceral larva migrans (VLM) and ocular larva migrans (OLM). VLM is caused by the migrating larvae through the somatic organs. This condition is characterized by fever, hepatomegaly, eosinophilia, abdominal pain, weight loss and skin rash. VLM can also involve the central nervous system leading to seizures, neuropsychiatric symptoms or encephalopathy while migration to the lungs may cause asthma (Gillespie, 1993). An outbreak of spinal VLM due to *T. canis* infection has been reported in Japan (Umehara *et al*., 2006). As for OLM, it is caused by the larvae migrating in the eye, leading to uveitis and optic papillitis (de Andrade *et al*., 2005). The most serious consequence of OLM is the invasion of the retina by larvae leading to granuloma formation peripherally or in the posterior pole. Sometimes, OLM is misdiagnosed as retinoblastoma, which is a malignant tumor of the eye. Therefore, a correct diagnosis for OLM is required to avoid unnecessary eye surgery (Kenny *et al*., 1995).

There is no definitive method in diagnosing *Toxocara* infection. As the larvae of *Toxocara* are arrested in the paratenic host during migration, they do not mature into adults, hence faecal examination of the patient will not give any clue about the infection. There are also difficulties in locating the infective *Toxocara* larvae in biopsy samples due to the patchy distribution and very small size of the larvae (Wickramasinghe *et al*., 2008). Therefore, serodiagnosis using enzyme-link immunosorbent assay (ELISA) remains the best method to diagnose toxocariasis (de Savigny *et al*., 1979). Serodiagnostic test based on crude somatic antigens derived from *Toxocara* embryonated eggs, larvae and adults were used previously (de Savigny *et al*., 1979) before *Toxocara* excretory-secretory (TES) antigens were used widely (Jacquier, 1991; Akao *et al*., 1997; Yamasaki *et al*., 1998; Smith & Rahmah, 2006). TES antigens are used to detect antibody response and currently provide a greater specificity for toxocariasis serodiagnosis compared to crude water-soluble antigens. Lower molecular weights TES antigens, especially those of molecular weights 24-35 kDa increase the specificity (Magnaval *et al*., 1991). However, in general, the specificity of native TES is still not sufficient for use in tropical and developing countries were STH infections (i.e., *Ascaris lumbricoides*, *Trichuris trichiura*, hookworm and *Strongyloides stercoralis*) are prevalent (Lynch *et al*., 1988) and cross reactivity with false positive results have been reported with other parasitic nematodes (Noordin *et al*., 2005).

This method has been modified by using recombinant TES antigens (rTES) to increase the specificity of toxocariasis serodiagnosis (Yamasaki *et al*., 2000). Recombinant antigens must share important antigenic and immunogenic structures with the native antigens that were produced during the infection. The immunoglobulin G4 (IgG4)-ELISA involving three rTES antigens have been found to give good specificity compared to commercial IgG-ELISA for toxocariasis detection. Suharni *et al*. (2009) have demonstrated that rTES-26, rTES-30 and rTES-120 showed 96.2%, 93.9% and 92% specificities, respectively. Moreover, rTES ELISA using rTES-30 antigen was reported to have fewer false positive results

compared to the commercial IgG-ELISA (Norhaida *et al*., 2008). Previous study has reported that IgG4-ELISA has higher specificity (78.6%) compared to the IgG-ELISA (36%) with samples from patients with clinical signs and symptoms associated with toxocariasis (Noordin *et al*., 2005).

In Malaysia, there are limited reports of human toxocariasis. There were only sporadic studies on toxocariasis carried out among Malaysian population with prevalence ranging from 19.6% to 31.9% (Hakim *et al*., 1992; 1997). Toxocariasis is not a primary health priority in Malaysia due to several reasons: infrequent reports on *Toxocara* seroprevalence among Malaysians (Hakim *et al*., 1992); the test employed in the previous studies lacked specificity (Smith & Rahmah, 2006) and the clinical requests for *Toxocara* serology are infrequent due to lack of awareness and suspicion of the infection. Within this context, we conducted the present study to determine the seroprevalence of toxocariasis among Orang Asli subgroups with IgG4-ELISA using recombinant antigens (rTES-26, rTES-30 and rTES-120) and commercial IgG-ELISA kit (Cypress Diagnostic, Belgium).

MATERIALS AND METHODS

Study population

A total of 188 Orang Asli from two subgroups from two states randomly participated in the present study. One hundred and eleven samples were obtained from Semelai subgroup (Pahang state) and 77 samples were obtained from Temiar subgroup (Kelantan state).

Blood and faecal samples collection

An oral briefing explaining the objectives of the study was given to the participants before voluntary written informed consent was taken from each participant. Venous blood sample was taken by trained medical assistants and nurses from each participant using disposable syringes and placed into plain tube (without anticoagulant). The blood samples were kept in a standard ice packed storage box and transported back to the Department of Parasitology, Faculty of Medicine, University of Malaya for further analysis. Blood sample was separated at 1500 rpm for 10 minutes and stored in -20ºC until used. A wide mouth and screw capped faecal container with an attached scoop was labeled, coded and distributed to each participant. The collected faecal samples were processed and examined for the presence of soil-transmitted helminths (STH) by the formalin ether concentration technique. Formal ethics approval was granted by the Ethics Review Committee of the University Malaya Medical Centre (UMMC) before commencement of the study.

Enzyme-link immunosorbent assay (ELISA)

IgG4-ELISA (rTES-26, rTES-30 and rTES-120)

The serological diagnosis for toxocariasis was performed with Ig4-ELISA using *T. canis* recombinant antigens at Universiti Sains Malaysia. The 96 well microtiter plates (NUNC Immuno Maxisorp, Denmark) were coated with the recombinant antigens (rTES-26, rTES-30 and rTES-120), in separate wells (Suharni *et al*., 2009). The plates were incubated overnight at 4ºC. On the next day, after a washing step using 300µL/well of phosphate buffer saline in Tween-20 (PBS-T), 1x blocking solution (Roche Diagnostic, Germany) at 200µL/well was added and incubated for 1 hour at 37ºC. The microtiter plates were then washed and serum samples, positive and negative controls diluted 1:50 with 1x phosphate buffer saline (PBS) were loaded into each well (100µL/well), and incubated at 37ºC for 2 hours. The microtiter plates were again washed and 100µL/well of monoclonal anti-human IgG4 HRP was incubated at 37ºC for 30 minutes. After washing, 100µL ABTS substrate (Roche Diagnostics, Germany) was added to each well for colour development and incubated again at 37ºC for 30 minutes. Results for this assay were determined by an ELISA

reader (TECAN Sunrise, Sweden) at 405nm, reference at 490nm and expressed as optical density (OD). PBS well was used as the blank. As previously determined, the cut-off value (COV) for toxocariasis was OD of 0.2. Each serum sample was tested in duplicate.

IgG-ELISA (Cypress Diagnostic, Belgium)

The samples were tested at University of Malaya using the commercial IgG-ELISA *Toxocara* kit (Cypress Diagnostic, Belgium) according to the protocol provided by the manufacturer. The kit consisted of native TES antigens precoated microtiter ELISA plates, secondary reagent (protein A conjugated to HRP), urea peroxide (substrate) and TMG (chromogen). Serum samples were diluted to 1:50 prior to use. Optical density (OD) value was recorded in an automatic ELISA reader (Biotek/ELx 800, USA) at 450 nm. The COV was calculated according to the manufacturer's formula by dividing the test OD by the average OD of two negative controls plus 0.15 OD units. The value of sample ratio was evaluated as follows: negative (COV: <0.9); equivocal (COV: 0.9-1.1); positive (COV: >1.1). Similarly as above, the serum samples were each tested in duplicate.

RESULTS

The overall seroprevalence of toxocariasis among 188 Orang Asli was 4.8% (9 of 188) as determined by the IgG4-ELISA (Table 1). The seroprevalence rate was slightly higher among Semelai subgroup (5.4%) compared to Temiar subgroup (3.9%). Of the three recombinant antigens, 2.7% and 2.1% serum samples were positive with rTES-26 and rTES-30 respectively. None of the serum samples were positive with rTES-120. The seroprevalence of toxocariasis was further analyzed according to gender and age groups (Table 2). In general, the seroprevalence of toxocariasis among males (9.5%) was higher compared to females (1%). Children below 12 years (6.3%) have higher seroprevalence rate compared to adults (1.2%). The results were further analyzed by comparing IgG4/IgG-ELISA assays (Table 3). All 77 Temiar subgroup serum samples and the 6 Semelai subgroup serum samples which were positive with IgG4-ELISA were tested using IgG-ELISA. For the Temiar serum samples, 76.6% were positive with IgG-ELISA compared to 3.9% (3 of 77) obtained with IgG4-ELISA. Of the Semelai subgroup, of the six which were positive by IgG4-ELISA, 4 (66.7%) were positive with IgG-ELISA. There were 7 serum samples from both subgroups that were tested positive with both assays (i.e. 3 Temiar and 4 Semelai serum samples). The overall prevalence of STH infections among Orang Asli (i.e., Temiar subgroup) was 85.7% (66 of 77) (Table 4). By comparing IgG-ELISA positive samples with STH infections, 94.9% (56 of 59) were also positive for STH infections. None of the IgG4-ELISA positive serum samples were from persons with STH infection.

Table 1. Seroprevalence of toxocariasis based on IgG4-ELISA according to Orang Asli subgroups

Orang Asli subgroups	N	Recombinant antigens						Total	
		rTES-26		rTES-30		rTES-120			
		n	%	n	%	n	%	n	%
Semelai	111	3	2.7	3	2.7	0	0	6	5.4
Temiar	77	2	2.6	1	1.3	0	0	3	3.9
Total	188	5	2.7	4	2.1	0	0	9	4.8

N: Number examined; n: number positive; %: percent

Table 2. Seroprevalence of toxocariasis based on IgG4-ELISA according to gender and age

Variables	N	Toxocariasis (IgG4-ELISA)	
		n	%
Gender			
Male	84	8	9.5
Female	104	1	1
Age groups			
Children (< 12 years)	128	8	6.3
Adults (>13 years and above)	60	1	1.2

N: Number examined; n: number positive; %: percent

Table 3. Comparison of positive samples between IgG4-ELISA and IgG-ELISA

Orang Asli subgroups	IgG4-ELISA		IgG-ELISA	
	n	%	n	%
Semelai	6	100	*4(6)	66.7
Temiar	3	3.9	59	76.6

n: number positive; %: percent
* 6 samples which were tested positive by IgG4-ELISA, 4 were found to be positive by IgG-ELISA
7 samples were positive with both ELISA assays (3 Temiar's and 4 Semelai's)

Table 4. Comparison result of IgG-ELISA with STH infection among Temiar subgroup

IgG-ELISA	N	STH infections			
		Positive		Negative	
		n	%	n	%
Positive	59	56	94.5	3	5.1
Negative	18	10	55.6	8	44.4
Total	77	66	85.7	11	14.3

N: Number examined; n: number positive; %: percent

DISCUSSION

Orang Asli, the indigenous group represents approximately 0.6% of the total population of Malaysia. They are a diverse group comprising of 18 ethnic subgroups. Economically, the Orang Asli is still regarded as one of the most impoverished group in the country. Although the Orang Asli are the indigenous inhabitants of Malaysia, unfortunately they have not benefited much from the country's rapid pace of development. Parasitic diseases such as malaria, filariasis, intestinal parasitic infection as well as zoonotic infections which are closely associated with poverty, environmental and personal hygiene practices are still causing major health problems among them, which is indicative of their prevailing low socioeconomic status.

The present study found that the overall seroprevalence of toxocariasis among Orang Asli based on IgG4-ELISA with three different recombinant antigens (rTES-26, rTES-30 and rTES-120) was 4.8%. This is in contrast with a previous local study, which reported a high seroprevalence of toxocariasis (31.9%) among Orang Asli communities in Malaysia (Hakim *et al*., 1992). Among the general Malaysian populations, however, it is difficult to estimate with certainty the current overall seroprevalence of toxocariasis as the last large-scale survey of toxocariasis on the three major races (i.e. Malay, Chinese and Indian) was undertaken in 1992. The study reported that the Indian community has the highest seroprevalence rate (35.5%) followed by Malays (14.8%) and Chinese (10.9%), with significantly more infections among children below 12 years (Hakim *et al*., 1997). A similar study conducted among the Taiwanese aboriginal populations reported high seroprevalence of toxocariasis ranging from 46% to 76.6% (Fan *et al*., 2004a,b).

More recently, Gracia *et al*. (2008) reported high seroprevalence of toxocariasis (57.5%) among children in a Mexico-United States border region. The finding of the present study is in contrast with both studies conducted among Taiwanese aboriginal communities. The variation in the results may be due to differences in geography, ethnicity, and cut off titer used. Thus, the prevalence of toxocariasis among aboriginals in different countries may not be directly comparable (Fan *et al*., 2004a). Seroepidemiological studies in rural areas from other countries also reported high seroprevalence of toxocariasis. Dar *et al*. (2008) reported 32.9% serum samples were seropositive for *Toxocara* in India. Dogan *et al*. (2007) conducted a seroepidemiological study in Turkey and found that the seroprevalence of toxocariasis to be 13%. High seroprevalences of toxocariasis has also been reported in Brazil, Sri Lanka and Czech Republic with the seroprevalence rates of 26.9%, 43% and 36%, respectively (Uhlikova & Hubner, 1998; Iddawela *et al*., 2003; Muradian *et al*., 2005).

The lower seroprevalence of toxocariasis (4.8%) in the present study compared to other previous studies may be due to the use of recombinant antigen-based IgG4-ELISA which has been shown to be highly specific (92% to 96.2%) (Suharni *et al*., 2009). Serum samples that were positive for toxocariasis were positive with only one type of rTES. Of the 9 positive serum samples, 5 were positive with rTES-26. The previous study has shown that rTES-26 is the most specific of the three recombinant antigens used in this study (Suharni *et al*., 2009). All serum samples tested negative with rTES-120 and this may be due to its lower antigenicity. Similarly, another study has also reported that rTES-26 has the highest antigenicity, followed by rTES-30 and rTES-120 had the least (Suharni *et al*., 2009).

In the present study, with regard to age groups and gender, children below 12 years (6.3%) and males (9.5%) tend to be more commonly infected compared to adults (1.2%) and females (1%), respectively. However, as there were only a small number of positive toxocariasis cases, statistical analyses may not be accurate. The higher seroprevalence of toxocariasis among children in the present study is

consistent with previous local studies in Malaysia (Hakim *et al*., 1992; 1997), as well as in Taiwan (Fan *et al*., 2004a;b), India (Dar *et al*., 2008), Turkey (Dogan *et al*., 2007), and Brazil (Muradian *et al*., 2005). The present study also showed that males tend to be infected more compared to females. Comparable result was also reported among aboriginal population in Taiwan (Fan *et al*., 2004a).

The higher seroprevalence of toxocariasis among the children is probably due to several factors such as lack of awareness of personal hygiene and good cleanliness practices or not realizing the significance of exposing themselves to pathogenic organisms due to their young age. Unhygienic habit such as placing contaminated hands into their mouths is very common among children and this could act as source of *Toxocara* infection via ingestion of embyronated eggs in contaminated soil. This condition is further aggravated by some degree of negligence and lack of parental supervision in personal hygiene and cleanliness in cases where both parents are working and children are without sufficient parental guidance.

During our visit to the Orang Asli villages, cats and dogs ownership were found to be very common. Children had very close contact and were noted playing with their pets (i.e., cats and dogs) especially puppies. Moreover, it has been reported previously that the presence of cats and dogs has a significant role in the high seroprevalence of toxocariasis among children (Hakim *et al*., 1997; Fan *et al*., 2004a,b; Muradian *et al*., 2005). Hunting with the help of dogs is a common activity among Orang Asli communities. Hunting activities are only practiced by males, therefore, they have close contact with dogs and is at higher risk of acquiring *Toxocara* infections. A previous study also indicated that dogs infected with *Toxocara* may infect human by direct contact because of the high density of embryonating and embryonated eggs found on dog's fur (Wolfe & Wright, 2003).

Based on the commercial IgG-ELISA (Cypress diagnostic, Belgium), the present study reported a high seroprevalence of toxocariasis (76.6%) among the Orang Asli. This is in agreement with many other studies which were also based on IgG-ELISA using native TES antigens. Thus the high seroprevalence of toxocariasis in the present study and other studies may be caused by the low specificity of native TES antigens used (de Andrade *et al*., 2005). The present study also indicated that of the 59 IgG-ELISA positive individuals, 94.9% were also infected with STH, thus indicating that the IgG-ELISA may have high false positive results. Previous studies have also reported that the specificity of native TES antigen for serodiagnosis of toxocariasis is often low, especially in developing countries, where helminth infections particularly STH infections that cause cross-reaction are prevalent (Lynch *et al*., 1988; Noordin *et al*., 2005). Native TES consists of multiple components with a wide range of molecular masses, compared to the single or homogenous molecule of rTES. In addition rTES is produced in bacteria, so the molecule is not glycosylated compared to the glycosylated nature of the native TES. The latter exhibits more cross reactivities with antibodies that recognized sugar moieties of native TES (Suharni *et al*., 2009). Furthermore, the use of native TES antigens is laborious, time consuming with limited production capacities (Smith & Rahmah, 2006). On the other hand, recombinant antigens have limitless production capacity.

Although the sensitivity and specificity of both IgG4-ELISA and IgG-ELISA assays were not calculated in the present study, previous study which compared the sensitivity and specificity of both assays showed that the sensitivity of the IgG-ELISA was 97.1%, while that of the IgG4-ELISA was 45.7%; while the specificities were 36% and 78.6%, respectively (Noordin *et al*., 2005). The investigators also demonstrated that the results of IgG-ELISA were twice

more sensitive than IgG4-ELISA and the results of IgG4-ELISA were twice more specific than IgG-ELISA (Noordin *et al*., 2005). Indeed, a more specific assay is needed in areas where STH infections are high due to high possibility of cross-reaction with other nematodes.

Exposure to *Toxocara* infection is not uncommon among Malaysian aborigines, and it affects both sexes and all age groups. More effective public health programmes to promote better understanding on the consequences of toxocariasis for prevention strategies against *Toxocara* infection and other parasitic health problems among Orang Asli communities are deemed necessary. The way forward is through an integrated approach with community participation in order to provide better understanding of the importance of environmental sanitation and personal hygiene practice and awareness of potential zoonotic infections in the prevention of this disease. There is also an urgent need to address whether *Toxocara* infection really is a hazard to the health of Orang Asli community. In fact, VLM and OLM are practically unknown to local physicians in Malaysia. Therefore aspects such as whether these asymptomatic aboriginal communities with positive serology are susceptible to development of vision impairments or other *Toxocara*-associated clinical features, such as partial epilepsy, should be further evaluated.

In conclusion, the present study highlighted the shortcomings of the commercial native TES antigens, IgG-ELISA, in which cross-reactions with STH infection were found to be high. The use of IgG4-ELISA based on recombinant antigens will enable more accurate serodiagnostic result of human toxocariasis due to high specificity especially in areas where cross reactions with other nematode infection may occur. The combination of three rTES antigen instead of single rTES antigens will provide a more sensitive and robust assay for serodiagnosis of *Toxocara* infection. Further investigation to improve sero-diagnostic method such as developing an inexpensive rapid rTES IgG4 test without performing ELISA will help to enhance the existing body of knowledge on *Toxocara* infection.

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