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Seroepidemiology of *Toxocara canis* infection among mountain aboriginal schoolchildren living in contaminated districts in eastern Taiwan

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Summary We conducted a seroepidemiological study of *Toxocara canis* infection among mountain aboriginal schoolchildren aged 7–12 years living in contaminated districts in eastern Taiwan. To detect sera IgG (≥1:64) we used a *T. canis* larval excretory-secretory antigen-based enzyme-linked immunosorbent assay. A short questionnaire elicited information on the practices of raising dogs, playing with soil, eating raw vegetables, or whether the child normally washed his/her hands before eating. The overall seroprevalence was quite high, reaching 76.6% (252/329). Neither age nor gender seemed to be important factors related to a positive serology. Aboriginal schoolchildren who raised dogs (OR = 1.83, 95% CI: 1.04–3.19, $P = 0.03$), or played with soil (OR = 2.52, 95% CI: 1.49–4.25, $P < 0.001$) seemed to be more susceptible to *T. canis* infection than those who did not. Children who habitually washed their hands before eating (OR = 0.57, 95% CI: 0.33–0.97, $P = 0.04$) had a lower chance of acquiring *T. canis* infection than those who did not.

keywords aboriginal schoolchildren, enzyme-linked immunosorbent assay, eastern Taiwan, *Toxocara canis*

Introduction

Toxocariasis is an infection predominantly caused by migration of the roundworm *Toxocara canis* larvae to organs and tissues. The major clinical consequences of prolonged migration of *T. canis* larvae in humans are visceral larva migrans (VLM) and ocular toxocariasis (OT) (Glickman 1993). Humans can be infected either by ingesting parasite eggs, by contact with larvae contaminating the teats of bitches that have recently given birth or the muzzle of puppies, or by means of the paratenic hosts of the parasite. Young children up to the age of 12 years are the main population supposedly susceptible to *T. canis* infection due to dirt pica, poor hygiene, or frequent contact with dogs (Glickman 1993). In the last 10 years, child toxocariasis cases associated with endomyocarditis, generalized lymphadenopathy, endophthalmitis, asthma, hepatosplenomegaly, and meningoencephalitis have been widely reported (Szczepanski *et al.* 1996; De Cock *et al.* 1998; Kincekova *et al.* 1999; Chan *et al.* 2001; Vidal *et al.* 2003). It is noteworthy that there is now considerable interest in the role of *T. canis* in epilepsy, particularly partial epilepsy (Nicoletti *et al.* 2002).

As the larvae migrate in tissues and do not develop into adult worms, making a definitive diagnosis through tissue biopsy and a stool examination is unlikely; diagnosis of toxocariasis mainly relies on a *T. canis* larval excretory-secretory (TcES) antigen-based enzyme-linked immunosorbent assay (ELISA), which reportedly shows 78% sensitivity and 92% specificity (Glickman *et al.* 1978). The seroprevalence of toxocariasis among children in different countries has been reported to be within a range of 4.0–86% using the TcES-ELISA (Holland *et al.* 1995; Jimenez *et al.* 1997; Luo *et al.* 1999; Ajayi *et al.* 2000; Alonso *et al.* 2000; Sadjjadi *et al.* 2000; Alderete *et al.* 2003). In general, reports concerning the seroprevalence of *T. canis* infection in aboriginal children living in mountainous areas are rather rare (Hakim *et al.* 1992).

Taiwanese aboriginal adults hunt wild animals with dogs (Fan *et al.* 1998a). It is unknown whether *T. canis* eggs shed from infected dogs contaminate the environment, thus causing *T. canis* infection in aboriginal populations. Our previous study revealed that the prevalence of *T. canis* embryonated eggs in stools deposited in the soil was fairly high (18%, 3/17) from a survey in Bunun aboriginal districts in eastern Taiwan (Fan *et al.* 2000). In this study,

we investigated the seroprevalence of and risk factors for *T. canis* infection among schoolchildren through screening for sera anti-*Toxocara* IgG antibodies by means of a TcES-based ELISA and a questionnaire interview in those aboriginal districts of Bunun that are contaminated.

Materials and methods

Study population and subject selection

In 1993, there were 10 aboriginal tribes, with the Bunun (approximately 41 000 people) being the fourth largest aboriginal population in Taiwan. Most Bunun populations live at elevations of 100–600 m in eastern Taiwan (Xiun 1996). Bunun aboriginal schoolchildren living in mountainous areas at elevations of 300–400 m in Hei-dong and Yen-ping districts in Taitung County of eastern Taiwan, respectively, were chosen as the study population (Figure 1). All 12 primary schools located in five villages in these two districts were included in the present study and the total number of schoolchildren in these primary schools was approximately 618.

In total, 329 serum samples were obtained by venipuncture, 164 serum samples from boys and 165 from girls, all randomly collected from apparently healthy schoolchildren. The mean ages were similar in both genders and ranged between 7 and 12 years for all schoolchildren. All serum specimens were kept at −30 °C until laboratory examination. Most aboriginal adults in both districts were labourers, or farmers (Ministry of the Interior, ROC 2000).

Questionnaire interview to assess risk factors

Informed consent was obtained from the parents or guardians of schoolchildren, giving the participation rate of 53.2% (329/618). Each participating child completed a short self-administered questionnaire, the results of which were reviewed by three trained public health nurses. The questionnaire requested various personal details including age, sex, and residential district, and whether the children had been raising dogs, playing with soil, eating raw vegetables, and whether they washed their hands before eating. This study was approved by the Taipei Medical University ethical committee.

Egg culture

Adult *T. canis* were collected from the intestines of necropsied stray dogs (Fan *et al.* 1998b). Eggs harvested from the anterior third of the uterus were cultured according to the method of Bowman *et al.* (1987) with slight modifications. Briefly, eggs were stirred in 1% sodium hypochlorite solution and incubated for 5 min at room temperature; thereafter, the mixture was centrifuged for 5 min at 2000 ***g***. The pellet was washed twice with distilled water and once with 2% formalin. The eggs were resuspended in 2% formalin and placed in a 250-ml Erlenmeyer flask, to which 2% formalin was added to bring the liquid level to approximately 1 cm deep. The flask was covered with parafilm, and incubated at room temperature for 8–9 weeks with gentle weekly agitation. They were then stored at 4 °C until use.

Preparation of larval excretory-secretory antigens

Toxocara canis larvae were hatched according to De Savigny (1975). All solutions were sterile and experimental procedures were carried out under aseptic conditions. Briefly, *T. canis* embryonated eggs were washed with PBS, resuspended in 1% sodium hypochlorite and incubated in an atmosphere containing 5% CO_2 at 37 °C for 30 min. After several washings with PBS containing antibiotics (100 IU/ml penicillin, 250 μg/ml streptomycin, and 25 μg/ml nystatin; Biochrom KG, Berlin, Germany), the pellet was resuspended in RPMI-1640 medium (JRH Biosciences, Lenexa, KS, USA) containing the same concentration of antibiotics. Motile larvae were collected from the bottom of the jar containing a modified Baermann apparatus (Yen *et al.* 1987) made up of two layers of cotton cloth in a steel sieve that had been kept in an atmosphere containing 5% CO_2 at 37 °C for 12 h. They were transferred to new RPMI-1640 medium containing antibiotics in 50-ml tissue culture flasks (BD Biosciences, Franklin Lakes, NJ, USA) with a larval concentration of 1×10^4/ml. Supernatants containing ES antigens derived from the *T. canis* larvae (TcES) were collected weekly, pooled, and centrifuged to precipitate all debris. The resulting supernatant was sterilized by membrane filtration (pore size 0.2 μm) and dialysed (molecular weight cutoff, 6000–8000 kDa) against PBS for 12 h at 4 °C, or until the phenol red disappeared. Protein content of the dialysate was estimated by the method of Bradford (1976), it was then concentrated by lyophilization (Labconco, Kansas City, MO, USA) and stored at −70 °C until use.

Enzyme-linked immunosorbent assay

Determination of the serum IgG specific for TcES was performed according to the method of Jimenez *et al.* (1997) and our previous study with modifications (Fan *et al.* 2003). Optimal dilutions of the antigens and antibodies (Abs) were pre-determined by check board titration. Briefly, wells of microtitre plates (Nunc, Roskilde, Denmark) were coated

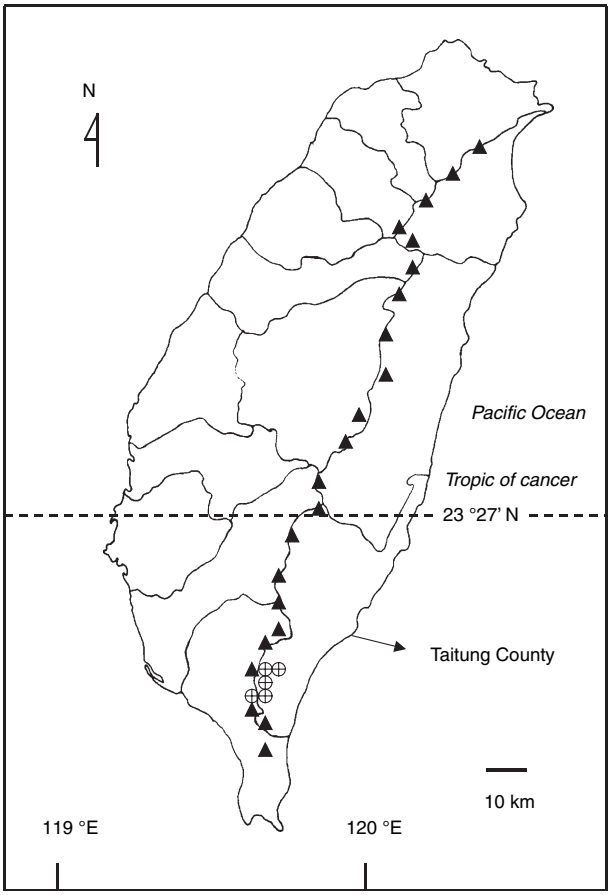

Figure 1 Map of Taiwan showing selected study areas: (⊕) Bunun villages; (▲) mountain areas.

with TcES antigen (protein concentration: 10 μg/ml) diluted in carbonate buffer at pH 9.6 and 2% skimmed milk in PBS was used as the blocking solution. Optimally diluted test sera (1:64), which were further verified by immunoblotting analysis, were used as the primary antibodies. Positive control sera were from patients with toxocariasis with proven clinical and laboratory diagnosis (kindly provided by Dr B. Gottstein, Institute of Parasitology, Berne University, Switzerland). Horseradish peroxidase-conjugated goat anti-human IgG (heavy and light chains) (Amersham, Piscataway, NJ, USA), diluted 1:1000 in PBS, were used as the secondary antibodies, and 2,2-azinobis[3-ethylbenzo-thiazoline-6-sulfonic acid] diammonium salt (ABTS) solution (Calbiochem, Darmstadt, Germany) was used as the substrate and the reaction was stopped by 1% sodium dodecyl sulphate (SDS). Duplicate tests were run on each test serum. The absorbance at 405 nm was determined in individual wells with an automated spectrophotometer (EIA reader model EL312e Bio-Tec, Virginia Beach, VA, USA). Positive and negative control sera were included in each plate. Tested serum whose mean OD value was equal to or higher than the mean OD value minus 2 standard deviations of the positive control serum was considered to be positive.

Immunoblotting verification

The serum titre set at 1:64 selected by an ELISA titration study was further verified by immunoblotting (IB) analysis using negative and positive control sera. Briefly, TcES Ag preparations (9 μg per slab) were separated by 12.5% SDS-PAGE and transferred to a nitrocellulose membrane (Amersham) in a semi-blotter (Hoffer, Fremont, CA, USA). Strips were then incubated with sera diluted at 1:64. A Western Lightning® kit (PerkinElmer Life Sciences, Boston, MA, USA) was employed to detect the immunoreactions, and positive reactions were ascertained by the presence of low-molecular-weight bands of either 24, 28, 30, or 35 kDa which specifically correlate to *T. canis* infection (Magnaval *et al.* 1991). Some ELISA-positive and -negative tested sera were also subjected to IB confirmation.

Statistical analysis

In the present study, subjects were categorized into six age groups (7-, 8-, 9-, 10-, 11- and 12-year-old groups). Statistical analysis was performed using the SAS software system (SAS Institute, Cary, NC, USA). The increasing trend of age-specific seropositive rates was tested for statistical significance using the chi-square test for trends. Multivariate adjusted odds ratios (ORs) with their 95% CIs were estimated by means of multiple logistic regression analysis. The statistical significance of differences in age-, gender- and risk factor-adjusted seropositive rates among comparison groups was examined by testing the statistical significance of the regression coefficients.

Results

The optimal titre for detection of *T. canis* infection as determined by a titration study using TcES-ELISA was 1:64. Further verification by IB revealed that only positive but not negative control serum at titre of 1:64 could recognize both of the specific low-molecular-weight bands at 30 and 35 kDa which are specifically related to toxocariasis (Figure 2). A subsequent ELISA study revealed that mean OD values ± SD calculated from positive control sera from each plate were 0.440 ± 0.047, thus the OD cutoff was 0.346 (mean −2SDs of positive control sera OD values). Randomly selected ELISA-positive tested sera

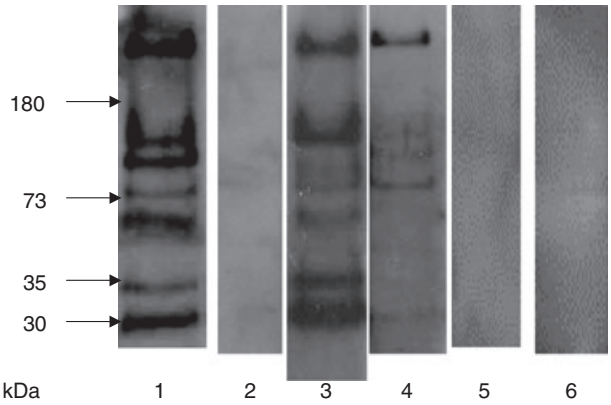

Figure 2 Immunoblotting analysis of clinically proven positive control sera and randomly selected ELISA-positive sera showing reactive low-molecular-weight bands at 30 and 35 KDa specifically related to toxocariasis. Lane 1: positive control serum; lane 2: negative control serum; lane 3: ELISA-positive tested serum 1 (mean ± SD: 1.369 ± 0.053 OD); lane 4: ELISA-positive tested serum 2 (0.656 ± 0.062 OD); lane 5: ELISA-negative tested serum 1 (0.313 ± 0.088 OD); lane 6: ELISA-negative tested serum 2 (0.269 ± 0.022 OD).

could recognize both specific low-molecular-weight bands at 30 and 35 kDa, whereas ELISA-negative tested serum did not react with either of these bands (Figure 2).

Of 329 serum samples studied, 252 (76.6%; 252/329) were positive for *Toxocara* IgG antibody for aboriginal schoolchildren as determined by TcES-ELISA. The overall seroprevalence was 78.0% (128/164) in boys and 75.2% (124/165) in girls (Table 1). Seroprevalence was highest (83.9%, 47/56) in 8-year-olds, followed in sequence by 81.8% (45/55) in 7-year-olds, 77.6% (45/58) in 10-year-olds, 73.7% (42/57) in 12-year-olds, 73.3% (33/45) in 9-year-olds and 69.0% (40/58) in the 11-year-old age group (Table 1). Seropositive values for the IgG antibody against TcES in aboriginal schoolchildren were 79.4% (197/248) in those who had histories of raising dogs, 82.5% (179/217) in children who played with soil, 73.0% (100/137) in those who ate raw vegetables, and 72.3% (133/184) for those who washed their hands before eating (Table 1). In the multiple logistic regression analysis, gender, age and risk factors were included in the regression model. As shown in Table 2, gender did not seem to have a significant association with seropositivity for *T. canis* antibody (OR = 0.85, 95% CI = 0.71–1.96, $P = 0.53$). Age was also not an important factor related to *T. canis* infection due to there being no significant differences in seroprevalence among the 7-, 8-, 9-, 10-, 11- and 12-year-old age groups ($P > 0.05$). Moreover, the multivariate-adjusted ORs were 1.83 and 2.52 for those who had histories of raising dogs or playing with soil against those without (OR = 1.83, 95% CI = 1.04–3.19, $P = 0.03$; OR = 2.52, 95% CI = 1.49–4.25, $P < 0.001$).

Table 1 Demographic characteristics of the seroprevalence of *Toxocara canis* IgG antibody among mountain aboriginal schoolchildren in eastern Taiwan

Variable	Group	No. tested	No. positive	Percentage
Gender	Boys	164	128	78.0
	Girls	165	124	75.2
Age (years)	7	55	45	81.8
	8	56	47	83.9
	9	45	33	73.3
	10	58	45	77.6
	11	58	40	69.0
	12	57	42	73.7
Risk factors				
Raising dogs	No	81	55	67.9
	Yes	248	197	79.4
Playing in the soil	No	112	73	65.2
	Yes	217	179	82.5
Eating raw vegetables	No	192	152	79.2
	Yes	137	100	73.0
Washing hands before eating	No	145	119	82.1
	Yes	184	133	72.3
Total		329	252	76.6

Table 2 Multivariate-adjusted ORs for various risk factors associated with seropositivity of *Toxocara canis* antibodies among aboriginal children in Taiwan

Variable	Group	Multivariate-adjusted ORs (95% CI)	*P*-value
Gender	Female	1.00* (referent)	
	Male	0.85 (0.71–1.96)	0.53
Age (years)	7	1.00† (referent)	
	8	1.16 (0.43–3.12)	0.77
	9	0.61 (0.24–1.58)	0.31
	10	0.77 (0.31–1.93)	0.58
	11	0.49 (0.20–1.19)	0.11
	12	0.62 (0.25–1.54)	0.30
Risk factors			
Raising dogs	No	1.00‡ (referent)	
	Yes	1.83 (1.04–3.19)	**0.03**
Playing in the soil	No	1.00‡ (referent)	
	Yes	2.52 (1.49–4.25)	**<0.001**
Eating raw vegetables	No	1.00‡ (referent)	
	Yes	0.71 (0.43–1.19)	0.19
Washing hands before eating	No	1.00‡ (referent)	
	Yes	0.57 (0.33–0.97)	**0.01**

* Adjusted variables included age and risk factors.
† Adjusted variables included gender and risk factors.
‡ Adjusted variables included gender and age.

Schoolchildren who had the habit of washing their hands before eating were less likely to acquire *T. canis* infection than those who did not (OR = 0.57, 95% CI = 0.33–0.97, $P < 0.001$). We found no significant difference in seroprevalence between schoolchildren with and without a history of eating raw vegetables (OR = 0.71, 95% CI = 0.43–1.19, $P = 0.19$).

Discussion

Serological tests are of considerable importance in the detection of infection by *T. canis*, as the clinical symptoms of toxocariasis are of limited value in the differential diagnosis (Schantz 1989). The use of TcES-ELISA to detect antibodies against *T. canis* does not require the pre-absorption of sera with embryonated *Ascaris suum* egg antigen because TcES-ELISA is now widely recognized as having good specificity in the diagnosis of toxocariasis (Glickman & Schantz 1981; Hakim *et al.* 1992; Gueglio *et al.* 1994; Luo *et al.* 1999). In the present study, a fixed ELISA cutoff titre of 1:64 in the screening of sera for the anti-*Toxocara* IgG antibody in Taiwanese aboriginal schoolchildren by TcES-ELISA appeared to be valid as further verified by immunoblotting analysis that not only positive control sera (1:64) could react with the specific low-molecular-weight bands at 30 and 35 kDa which were correlated with toxocariasis (Magnaval *et al.* 1991), but also ELISA-positive tested sera exhibited similar results, while negative control sera as well as ELISA-negative tested sera showed no reactive bands.

Our study revealed that the overall seroprevalence among healthy aboriginal schoolchildren living in egg-contaminated districts was quite high (76.6%, 252/329). Comparison of seroprevalence data between the present result and other studies is difficult due to the use of different cutoff titres and the difficulty in evaluating relationships between titre level, infection, and clinical findings (Alderete *et al.* 2003). However, lower seroprevalences in healthy schoolchildren ranging from 4.0% to 49.1% have been observed in Argentina, Brazil, China, Iran, Ireland, Nigeria, Spain and the US (Holland *et al.* 1995; Jimenez *et al.* 1997; Luo *et al.* 1999; Ajayi *et al.* 2000; Alonso *et al.* 2000; Sadjjadi *et al.* 2000; Alderete *et al.* 2003). A higher seroprevalence (86%) was observed in St Lucia (Thompson *et al.* 1986).

Gender did not seem to be an important factor related to *T. canis* infection among healthy Taiwanese aboriginal children due to a lack of a significant association between gender and frequency of *Toxocara* seropositivity in the present study. Similar findings have been reported in China, Iran, Nigeria and Spain (Jimenez *et al.* 1997; Luo *et al.* 1999; Ajayi *et al.* 2000; Sadjjadi *et al.* 2000); while some reports indicated that boys had a greater opportunity to acquire *T. canis* infection, as they had more contact with dogs (Holland *et al.* 1995; Alonso *et al.* 2000). Seroprevalence of *T. canis* infection in aboriginal schoolchildren did not increase with age, perhaps due to the age range being too small to find a discrepancy of age *vs.* seroprevalence. Nevertheless, the children older than 7 years might have acquired *T. canis* infection earlier in their childhood, as antibody titres might persist for a long period of time (Schantz 1989). Similar findings of a lack of an age-related increase in seroprevalence were also reported in children in Argentina, Iran, Nigeria and Spain (Jimenez *et al.* 1997; Ajayi *et al.* 2000; Alonso *et al.* 2000; Sadjjadi *et al.* 2000).

On the other hand, a significant association was observed between ownership of dogs and *Toxocara* infection in aboriginal schoolchildren. They probably acquired the infection through inadvertently ingesting eggs contaminating a dog's body. A recent study indicated that dogs infected with *T. canis* might infect people by direct contact because of the high density of embryonating and embryonated eggs in their fur (Wolfe & Wright 2003). Nevertheless, high seropositive rates of 79.4% in dog owners and 67.9% in non-owners of dogs in the present study suggest that these two groups are equally at risk of being infected. The results are in line with those of Woodruff *et al.* (1978) who observed that 50% of patients with clinical toxocariasis had never owned a dog or had close contact with pets. However, controversy exists regarding the importance of contact with dogs as a risk factor for human toxocariasis. Some authors reported a higher frequency of infection for individuals who maintained contact with dogs (Matsumura & Endo 1983); while others found no association of ownership and professional contact with dogs with the frequency of *Toxocara* infection (Woodruff *et al.* 1978). Nevertheless, the presence of dogs seems to be important for the determination of human infection, as the eradication of dogs in Iceland during the 1940s led to the disappearance of human *Toxocara* infection in that country at the beginning of the 1980s (Woodruff *et al.* 1982).

The present results further suggest that toxocaral infection in Taiwanese aboriginal schoolchildren in the study area was also acquired by the ingestion of soil containing infective eggs, confirming our earlier report that contamination of soils with *T. canis* eggs was relatively high (18%) in the same Bunun districts (Fan *et al.* 2000). This confirms that children who do not wash their hands before eating are more likely to get infected. This high incidence of *T. canis* infection among Bunun schoolchildren may well exist in other aboriginal districts in Taiwan due to their similar lifestyles (Xiun 1996).

It was noteworthy that OT was reportedly more common in children over 6 years (Schantz 1989); in addition, decreased cognitive development or increased behavioural disorders were also observed in asymptomatic children (Marmor *et al.* 1987; Nelson *et al.* 1996). Recently, it was proposed that the cause of partial epilepsy was highly related to *T. canis* infection (Nicoletti *et al.* 2002). However, our laboratory has diagnosed (using patients' vitreal fluid and sera as determined by TcES-ELISA and Western blotting) several cases in Taiwanese children referred from different hospitals in Taipei City, who were suspected of being infected with OT (Fan & Su KE, unpublished data). There is thus an urgent need to address whether *Toxocara* infection really is a hazard to the health of Taiwanese schoolchildren. In fact, VLM and OT are practically unknown to local physicians in aboriginal districts; whether these asymptomatic aboriginal schoolchildren with positive serology are susceptible to development of vision impairments or other *Toxocara*-associated clinical features, such as partial epilepsy, should be further evaluated.

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