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Validation of a *Toxocara* ES antigen Enzyme Linked Immunosorbent Assay (TES-ELISA) for use in childhood toxocariasis in Sri Lanka

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Abstract

An enzyme-immunoassay detecting serum antibodies specific for excretory secretory antigens of *Toxocara* L2 larvae, a test recommended for seroepidemiological studies, was validated for use in a local population of children under 12 years. The cut off level, at antigen dilution of 10µg/ml and serum dilution of 1:100, determined by 1 tail confidence limit of 1.96 was 0.148 with a geometric mean of 0.16087. This conforms to the standard reference value for this test (OD=0.2) adopted in reference laboratories in other countries. The upper OD value of 0.7 accepted in these reference laboratories to indicate recent exposure or active infection corresponded to the 84th percentile in our population. Cross reactions were not seen against *Ascaris lumbricoides* L2 larval excretory secretory antigen and L3 larval antigen of *Necator americanus*. However, 25% of the positive sera cross reacted with *T. vitulorum*, the common buffalo parasite in Sri Lanka. This indicates that development and validation of species specific tests is essential for determining the role of each species of *Toxocara* in the etiology of human toxocariasis in Sri Lanka.

Key Words: Toxocara ELISA, Validation

Introduction

Human toxocariasis is caused by several species of the nematode round worm *Toxocara*. *Toxocara* spp. are common intestinal round worms parasitizing a wide range of domestic, agricultural and wild animals. In Sri Lanka the species include *T. canis* of dogs and wild canids, *T. cati* of cats, and *T. vitulorum* of ruminants. *T. canis* is very common in dogs throughout the world and thus

is considered as the primary cause of human toxocariasis (1).

Other than in the natural definitive hosts such as the dogs, cats or ruminants, *Toxocara* spp. cannot develop to the adult worm in humans and other paratenic hosts and in these hosts the development remains restricted to the larval form. The migrating larvae cause extensive damage in the organs involved and the condition, characterized by various clinical manifestations, is known as larva migrans. Worldwide, the highest prevalence rates of larva migrans is reported in children (2).

T. canis is unable to complete its life cycle in the human host because larval development is arrested at the L2 stage. Laboratory diagnosis, therefore, depends largely on immunodiagnostic techniques. The enzyme linked immunosorbent assay (ELISA) based on excretory-secretory antigen (ES) has proved to be the most sensitive and specific immunodiagnostic tool for toxocariasis and is the assay used extensively to date (3). In tropical countries, a major problem in the diagnosis of helminthiasis is the widespread sharing of helminth antigens that results in immunological cross reactions. Although De Savigny *et al*, (4), testing sera against several common helminthiasis, concluded that this was not a problem with the TES-ELISA, it needs to be rechecked in our population.

Objective

This study was carried out to determine

1. the cut off level for *Toxocara* ES antigen ELISA (TES-ELISA) for the Sri Lankan population.
- 2 to determine any cross reactions with other helminthiasis that are common in our childhood population.

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Material and Methods

The study population, for validating the cut-off level, consisted of randomly selected 1020 children, between 1-12 years of age resident in the Hindagala Community Health Project area who were apparently well. This comprised 18.7% of the total population of 5440 children aged 1-14 years (5).

Collection of blood

Ethical clearance was obtained from the Committee on Research and Ethical Clearance, Faculty of Medicine, University of Peradeniya. Approximately 2 ml of venous blood was collected into a plain bottle without anticoagulant by venepuncture, using disposable syringes and needles under sterile techniques.

Preparation of *T. canis*, *T. vitulorum* and *Ascaris lumbricoides* excretory-secretory antigens

Excretory-secretory antigens from *T. canis* (TcES), *T. vitulorum* (TvES), *A. lumbricoides* (AIES) were prepared by the method described by De Savigny (1975) and Rajapakse *et al.*, (6). Briefly, fertile eggs were collected from the faeces of *T. canis* infected puppies, *T. vitulorum* from naturally infected buffalo calves and *Ascaris lumbricoides* from infected human faeces. The eggs were left to embryonate, hatched by decortication using saturated calcium hypochlorite and larvae collected in a Baermann apparatus. The larvae were maintained at a concentration of 10^3 /ml at 37°C in Minimal Essential Medium. During this period culture medium was harvested weekly and concentrated against polyethyleneglycol (Aquacide-111, Biochem, USA). Concentrated culture medium was then dialyzed against phosphate buffered saline and protein content was estimated by the bicinchoninic acid protein assay kit (Sigma chemicals, UK).

Necator americanus larval antigen (NaL)

The hookworm egg- positive faecal samples collected from patients were cultured using the Harada – Mori technique (7). Approximately 0.5 g of the pooled faecal specimen was smeared along the length of the filter paper and placed in test tubes containing small amounts of normal saline. Strips were removed after 10 days of

incubation at room temperature. The test tubes having larvae were placed in a water bath at 50 °C for 15 minutes to kill the larvae. The tubes were then centrifuged at 1500 g for 15 min and the supernatant was discarded. The sediments were pooled, sonicated using ultrasonic sonicator (Labson 2000) at a frequency of 2500 Hz for 15 minutes. The NaL antigen was stored in aliquots of 0.5 ml at -20 °C until use.

Enzyme linked immunosorbent assay using *Toxocara canis*, excretory - secretory antigen

The indirect ELISA was carried out on the children's sera. Optimum antigen dilution and serum dilutions were determined by checkerboard titration. The microtitration plates were coated with 10 µg/ml TES antigens and incubated at 4°C overnight. Plates were then washed 3 times in washing buffer, post coated with 120 µl of PBS containing 1% bovine serum albumin and 2.5% sucrose and incubated at room temperature for one hour. Subsequently 100 µl of diluted (1:100) serum samples were added to the test wells in duplicate and incubated for one hour at room temperature (RT). Known negative and positive sera, along with a conjugate control were used as controls on each plate. Following the incubation, plates were washed three times in washing buffer to remove unbound serum and 100 µl of peroxidase-conjugated antihuman IgG diluted 1:250 added to each well. Plates were incubated for 1 hour at RT, washed and 100µl of the enzyme substrate o-phenylenediamine dihydrochloride, 2 mg tablets in 3% hydrogen peroxide solution (SIGMA- Aldrich corporation, India) was added. Hydrolysis was stopped with 100 µl of 3M H₂SO₄ and the optical density (OD) values determined photometrically at 490 nm in an ELISA reader (MINIREADER 11, Dynatech Laboratories Inc.). Each of the test sera was tested in duplicate and the mean absorbance was taken.

Enzyme linked immunosorbent assay using *T. vitulorum* and *A. lumbricoides* excretory - secretory antigen and *Necator americanus* larval antigen

The microtitration plates were coated with 10 µg/ml of *T. vitulorum*, *A. lumbricoides* ES

antigens and *N. americanus* larval antigens and ELISA test was carried out as stated previously. Serum samples used to detect specificity of TES-ELISA were as follows:

TES-ELISA high positive - 50 serum samples

TES-ELISA low positive - 30 serum samples

TES-ELISA negative - 20 serum samples

Results

Determination of TES-ELISA cut off point for the study population

Since the distribution of ELISA values was skewed (Figure 1) log-transformation of data was carried out to obtain an approximate normal distribution (Figure 2). Cut off level was determined by 1 tail confidence limit of 1.96. Geometrical mean was 0.160847 and the cut off level was 0.148. The upper OD level of 0.7, to indicate recent exposure or active infection, corresponded to the 84th percentile in our population.

Cross reactivity of the TES-ELISA

All 80 TES-ELISA positive serum samples were negative when tested against *A. lumbricoides* ES antigen and *N. americanus* larval antigen. However, against *T. vitulorum* ES antigen, 20 (25%) serum samples gave positive results. The 20 TES-ELISA negative sera remained negative with *A. lumbricoides*, *T. vitulorum* ES antigen and *N. americanus* larval antigen.

Discussion

This study attempted to determine the cut off ELISA value for a local reference population and to assess the specificity of TES-ELISA, a serodiagnostic test recommended for the diagnosis and seroepidemiological surveys of toxocariasis (4).

The TES-ELISA cut off level for this population was found to be 0.148. This conforms to the TES-ELISA cut off level of international reference, O.D. value= 0.2 (8, 9). De Savigny *et al* (4), testing a limited number of sera of patients diagnosed as clinical toxocariasis showed a 100% sensitivity

with this test. However, the predictive value, an important parameter of immunodiagnostic tests, cannot be determined in toxocariasis with accuracy because criteria, which constitute clinically diagnosed toxocariasis, have not been standardized and definitive diagnosis on histology is generally not possible. Hence for an upper cut off level, to indicate recent exposure or active infection, it was decided to adopt the value of 0.7 (84th percentile in our population), which is the standard accepted in the studies by Lynch *et al* (9) and Taylor *et al* (10).

Evaluation of the TES-ELISA in groups of patients with presumptive diagnosis of visceral larva migrans (VLM) indicated sensitivity and specificity of 78% and 92% respectively at a titer of > 1:32 (11). At a given cut-off titer, sensitivity of TES-ELISA for the diagnosis of ocular larva migrans (OLM) is less than that of VLM (12, 13, 14). Using the TES-ELISA, Speiser and Gottstein (15) reported sensitivity and specificity of 80% and 93% respectively and Jacquier *et al.*, (16) indicated sensitivity and specificity of 91% and 86% respectively.

Overall, the TES-ELISA test is accepted as the 'gold standard' for toxocariasis with a high sensitivity and high specificity using appropriate antigen dilutions and cut-off levels that exclude cross-reactions. In this study population, at serum dilution of 1:100 and antigen dilution of 10µg/ml, TES-ELISA positive sera (based on the cut off level of OD=0.2), did not cross react with *A. lumbricoides* and *N. americanus* antigens. Therefore we conclude that, in agreement with De Savigny *et al.*, (4), that the TES-ELISA assay can be used without adsorption of test sera with helminth antigens. The lack of cross reactivity with the common helminth antigens further suggests that this assay can even be used in tropics where multiple helminth infection is common.

Within the genus *Toxocara*, Kennedy *et al.* (17) demonstrated extensive cross reactions between *T. canis* and *T. cati* using SDS-PAGE with radio

immunoprecipitation assay and produced a monoclonal antibody (Ten 2) that was specific for *T. canis*. This study showed a 25% cross reactivity between *T. canis* and *T. vitulorum* antigens confirming that TES -ELISA is not species- specific. Therefore assays using TcES cannot distinguish between infections within the genus *Toxocara* such as that of *T. canis*, *T. cati*, and *T. vitulorum*. Although species specificity is not relevant in clinical diagnosis, it is of great importance in prevention and control of toxocariasis as each species deals with different host sources and human risk behavior. Moreover *T. vitulorum* being a buffalo parasite would be a risk to all age groups, more so to the adults handling the buffalo dung. This indicates that the development and validation of species specific diagnostic tests for use in seroepidemiology is essential for determining the role of each species in human toxocariasis in different populations in Sri Lanka.

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Figure 1 Distribution of TES-ELISA values of children in the Hindagala Community Health Project area

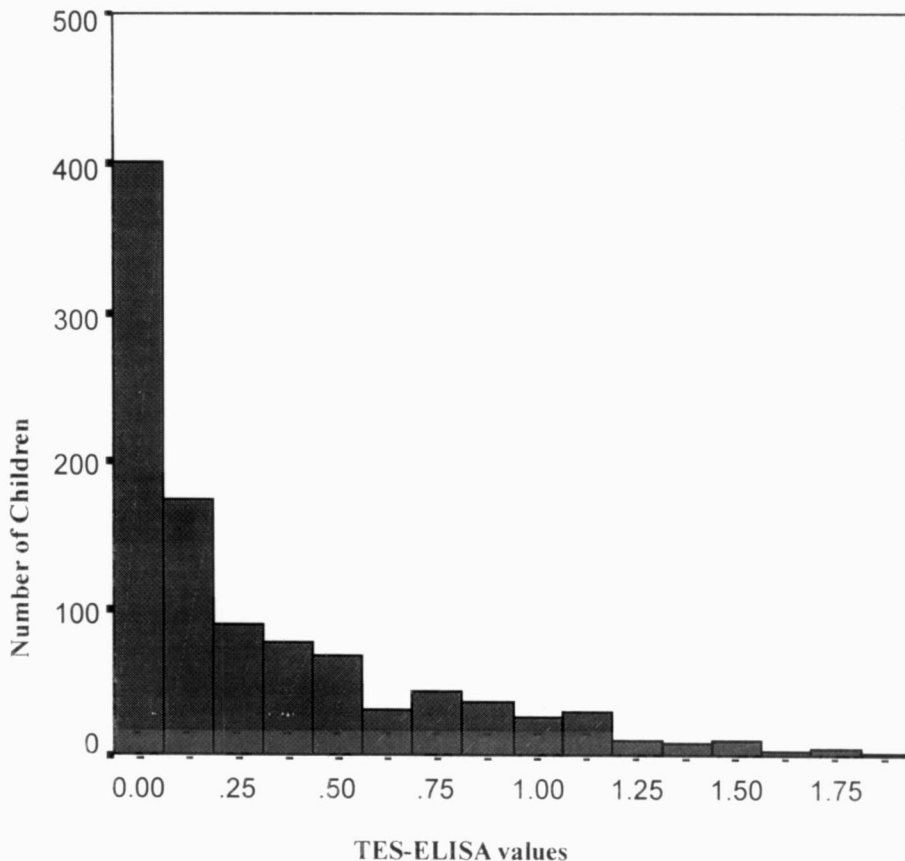


Figure 2 Log-transformed distribution of ELISA values

