

Serodiagnosis of ocular toxocariasis: a comparison of two antigens

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SUMMARY This study was designed to compare the sensitivity and specificity of enzyme linked immunosorbent assay (ELISA) for the serodiagnosis of ocular toxocariasis using *Toxocara canis* embryonated egg antigen (TEE) and toxocara excretory-secretory or exoantigen (TEX) produced in vitro. TEE and TEX ELISA were comparably sensitive, but TEX ELISA was better able to discriminate between serum samples from patients with ocular toxocariasis and those from patients with retinoblastoma. In addition, preabsorption of sera with *Ascaris suum* embryonated egg antigen seemed to be essential to prevent false positive results with TEE ELISA but was not so critical for TEX-ELISA. Further studies are still required to standardise TEX for serodiagnosis.

In 1950 Wilder described the occurrence of nematode larvae or larval remnants in 24 of 46 pseudogliomas from eyes that had been enucleated because of endophthalmitis and presumed retinoblastoma.¹ When five of these larvae were later re-examined they were identified as *Toxocara canis*.² Since 1950, more than 400 documented cases of ocular toxocariasis have been reported.³ Despite advances in medical technology such as computed tomography and ultrasonography, immunological methods are often relied on to confirm a clinical diagnosis of ocular toxocariasis or to rule out a suspected malignancy. Definitive histopathological diagnosis is possible only after enucleation.

An enzyme linked immunosorbent assay (ELISA) using *T canis* embryonated egg antigen (TEE) was found to be more sensitive for diagnosis of visceral toxocariasis than either bentonite flocculation or indirect haemagglutination using antigen prepared from adult *T canis* worms.⁴ When evaluated in 41 patients with clinically diagnosed ocular toxocariasis, the sensitivity and specificity of TEE-ELISA were 90% and 91% respectively, at a diagnostic titre of 1/8.⁵ Based on these findings the ELISA was adopted by the Centers for Disease Control for routine serodiagnosis of human visceral and ocular toxocariasis.

Experiences during the past four years have shown several shortcomings in the use of ELISA for

diagnosis of ocular toxocariasis. In general, patients with ocular toxocariasis have serum anti-*T canis* antibody titres that are significantly lower than those with visceral toxocariasis.⁶ As a result, at least one patient with a suspected retinoblastoma and a negative serum ELISA titre for *T canis* has had an eye enucleated, with a pathologist subsequently finding a *T canis* larva on microscopical examination.⁷ A possible solution to this problem was suggested when higher anti-*T canis* antibody titres were found in the vitreous than in the serum of patients with clinically diagnosed toxocaral ophthalmitis.⁸ It was noted, however, that vitreous aspiration poses some risks and, in the clinical setting, may limit testing to serum only.

In 1975 de Savigny⁹ described a technique for in vitro maintenance of *T canis* larvae with concomitant production of excretory-secretory or exoantigen (TEX). TEX was used with ELISA for seroepidemiological studies and for testing patients with visceral toxocariasis.⁹ This assay showed a high degree of sensitivity and specificity, but was not directly compared with a TEE ELISA.

The present study was designed to compare ELISA using TEE and TEX for the serodiagnosis of ocular toxocariasis. In addition, since adequate specificity of TEE-ELISA requires preabsorption of serum with *Ascaris suum* embryonated egg antigen (AEE),¹⁰ we determined whether preabsorption is also required with TEX ELISA.

Material and methods

SPECIMENS

TEE and TEX were evaluated with ELISA using sera previously submitted to our laboratory for immunological diagnosis of ocular toxocariasis. These samples had been tested by TEE ELISA with visual reading of the titre end points.⁴ This test requires that each 50 μ l of specimen be preabsorbed with 20 μ l of AEE to remove non-specific reactivity to ascaris. In addition, the samples had also been tested after sham absorption with 20 μ l of phosphate buffered saline (PBS). All samples had been stored at -70°C for up to two years. A diagnostic titre for ocular toxocariasis using this method is $\geq 1/8$.⁵

Sera were available from 11 patients with clinically diagnosed unilateral ocular toxocariasis. All of the specimens were positive, with TEE ELISA titres ranging from 1/8 to 1/256. Histological confirmation of ocular toxocariasis was not feasible. The referring ophthalmologist was contacted one to two years after submission of the serum specimen, however, and in every instance the original diagnosis of ocular toxocariasis was unchanged. Negative toxocara specimens were obtained from 12 patients with clinically diagnosed unilateral retinoblastoma whose sera were assayed to rule out a diagnosis of ocular toxocariasis. All of these samples had anti-*T canis* antibody titres of $< 1/8$. In addition, nine patients with suspected ocular toxocariasis were identified whose sera were positive (titre of $\geq 1/8$) by TEE ELISA after sham absorption with PBS and were negative after absorption with AEE. These sera were therefore designated as serologically cross reactive with TEE.

TEE AND TEX

TEE was prepared according to the method of Cypess *et al.*¹⁰ For the preparation of TEX, hatched *T canis* larvae were obtained as described elsewhere¹¹ and maintained in culture following the method of de Savigny.⁹ Hatched larvae were added to sterile 50 ml flasks in 10 ml of RPMI 1640 (KC Biological, Lenexa, KA), pH 7.2, supplemented with 1% glutamine at a concentration of 1×10^3 larvae per millilitre. Cultures were incubated in 5% CO_2 at 37°C and 95% relative humidity. At weekly intervals cultures were examined microscopically for contamination and larval viability. Cultures with contamination or those with greater than 5% larval mortality were discarded. At weekly intervals larvae were allowed to settle for 15 min and the culture medium aspirated aseptically and transferred to sterile centrifuge tubes. Fresh culture medium was added to the flasks and cultures were incubated as

before. Conditioned culture medium was centrifuged at 1000 rpm for 5 min and the supernatant fluid was collected aseptically. Weekly samples were collected for up to 16 weeks, pooled, and stored at -70°C . Medium was exhaustively dialysed against 0.05 M Tris buffer, pH 8.0, containing 0.02% sodium azide, and concentrated using an ultrafiltration system with a 10 000 molecular weight exclusion limit (Amicon Corp, Lexington, MA). Relative protein concentrations of TEE and TEX antigen were determined by spectrophotometric absorbance at 280 nm with comparison to a bovine albumin standard curve.

ELISA PROCEDURE

Ninety six round bottom well Linbro polystyrene microtitre plates (Flow Laboratories Inc, Hamden, CT) were coated by passive adsorption with either TEE or TEX. Fifty microlitres of antigen, 12 $\mu\text{g}/\text{ml}$ protein concentration in 0.1 M sodium carbonate buffer, pH 9.6, was added to each well, and the plates were dried overnight at 37°C . Before use, TEE and TEX adsorbed plates were washed three times with PBS containing 0.05% Tween 20 (PBS-Tween), pH 7.4, to remove unadsorbed antigen. Two aliquots of samples to be tested were preabsorbed, one with AEE (50 μ l serum plus 20 μ l AEE) and the other with PBS as a dilution control (sham preabsorbed). Absorbed sera were diluted 1/8 with PBS-Tween and 50 μ l aliquots were added to each of four wells on a TEE and a TEX coated microtitre plate. The plates were incubated at room temperature for 16 h, washed with PBS-Tween to remove unbound serum components, and incubated for 1 h at 37°C with 50 μ l of a 1/850 dilution of horseradish peroxidase conjugated goat antihuman IgG Fc fragment (Cappel Laboratories, Conchranville, PA) in 0.05 M phosphate buffer, pH 7.4.

After a wash, 50 μ l of 0.1 M phosphate buffer, pH 7.4, was added to each well followed by 50 μ l of enzyme substrate solution. Substrate solution consisted of 45 ml of 5-aminosalicylic acid (Sigma Chemical Co, St. Louis, MO) (1 mg/ml in distilled water) and 5 ml of 0.06% hydrogen peroxide (0.06 M). The colour reaction was allowed to proceed at room temperature and was monitored with an automated spectrophotometric reader (Artek model 210, Artek Systems Corp, Farmingdale, NY) at the optimum wavelength (405 nm). A mean absorbance value was calculated for four replicates.

STATISTICAL ANALYSIS

Statistical differences in ELISA values between groups—for example, AEE preabsorbed *v* sham preabsorbed; TEE ELISA *v* TEX ELISA—were determined by application of the paired t test at a

probability level of 0.05. The power of TEE and TEE ELISA to discriminate between positive and negative *T canis* sera was measured using discriminant function analysis.¹² The values derived from this analysis are not measures of statistical significance but rather are probabilities of misclassification using TEE ELISA or TEX ELISA and were used for comparative purposes.

Results

To evaluate the need for preabsorption with AEE, aliquots of all serum samples were preabsorbed with AEE or sham preabsorbed with PBS; the reciprocal log 2 TEX ELISA and TEE ELISA titres were determined by visual inspection at 60 min. The results are presented in the Table. Preabsorption of sera with AEE had no significant effect in any group when tested by TEX ELISA ($p > 0.80$; paired t test). In contrast, preabsorption with AEE resulted in significantly lower titres ($p < 0.05$) for the negative *T canis* and *T canis* cross reactive sera when tested by TEE ELISA; the reduction in titre was greatest for the *T canis* cross reacting sera.

To evaluate further the need for preabsorption with AEE antigen, positive, negative, and cross reacting serums were randomly selected, diluted 1/8, and monitored spectrophotometrically. The results are shown in Figs. 1 and 2. Absorption with AEE reduced the TEE ELISA values and the effect was strongest for the cross reacting *T canis* serum (Fig. 2). The reduction was less apparent with TEX than it was with TEE. These findings confirm that preabsorption of serum with AEE before TEE ELISA is necessary to remove TEE cross reactivity that could lead to false positive results. In contrast, preabsorption with AEE did not appear to be critical to the TEX ELISA.

To compare the sensitivity and specificity of TEX and TEE for diagnosis of ocular toxocariasis, all positive and negative sera were compared using two methods. Firstly, 50 µl aliquots of these sera were preabsorbed with AEE and tested by TEE ELISA.

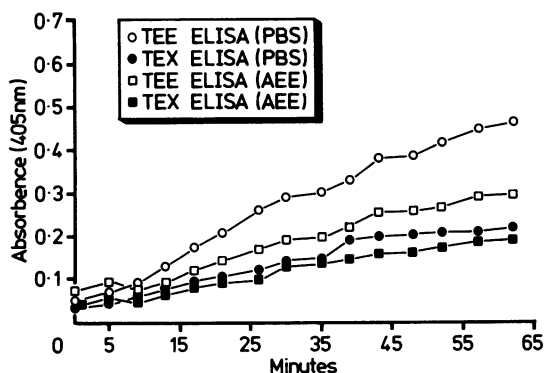


Fig. 1 *Toxocara canis* embryonated egg antigen (TEE) and *Toxocara exoantigen* (TEX) ELISA absorbance values for a patient with clinically diagnosed retinoblastoma whose serum was tested to rule out a diagnosis of ocular toxocariasis. The serum was either preabsorbed with *Ascaris* serum embryonated egg antigen (AEE) or sham preabsorbed with phosphate buffered saline (PBS) and assayed spectrophotometrically.

Secondly, aliquots were sham preabsorbed with PBS and tested by TEX ELISA. The results are shown in Fig. 3.

Mean (1 SD) absorbance values for the 11 positive sera using TEE ELISA and TEX ELISA were 0.073 (0.276) and 0.758 (0.271), respectively. This difference was not significant (paired t test; $p > 0.2$). Mean (1 SD) absorbance values for the 12 negative sera using TEE ELISA and TEX ELISA were 0.250 (0.096) and 0.170 (0.060), respectively. This difference was also not significant ($p > 0.05$). A greater distinction was evident, however, between the positive and negative sera using TEX ELISA compared with TEE ELISA. Linear discriminant analysis showed a probability of misclassification for TEX ELISA of 6% compared with a probability of misclassification for TEX ELISA of 13%. The probability of misclassification (6%) if both tests were applied concurrently to each sample was no less than for TEX ELISA alone (6%). Thus TEX ELISA was

Mean (SD) reciprocal log ELISA titre of human sera based on visual readings at 60 min.

Sera	No tested	ELISA antigen/absorption antigen			
		TEE/PBS	TEE/AEE	TEX/PBS	TEX/AEE
Negative toxocariasis	12	0.38 (0.50)	0	0.07 (0.26)	0.07 (0.26)
Ocular toxocariasis	11	5.66 (1.32)	5.11 (1.69)	5.44 (1.66)	5.33 (1.73)
TEE crossreactive	9	2.44 (1.66)	0.33 (0.70)	0.88 (1.36)	0.77 (1.09)

*Paired t test.
A positive ELISA titre for ocular toxocariasis is $> \log_2 3.0$.

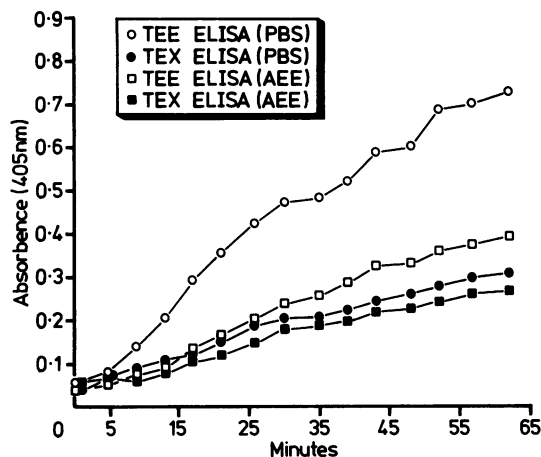


Fig. 2 *Toxocara canis* embryonated egg antigen (TEE) and *Toxocara* excretory-secretory or exoantigen (TEX) ELISA absorbance values for a patient with suspected ocular toxocariasis whose serum had tested positive (titre $\geq 1/8$) previously by TEE ELISA after sham preabsorption with phosphate buffered saline (PBS) and tested negative following preabsorption with *Ascaris* serum embryonated egg antigen (AEE) and visual reading. The serum was either preabsorbed with AEE or sham preabsorbed with PBS and assayed spectrophotometrically.

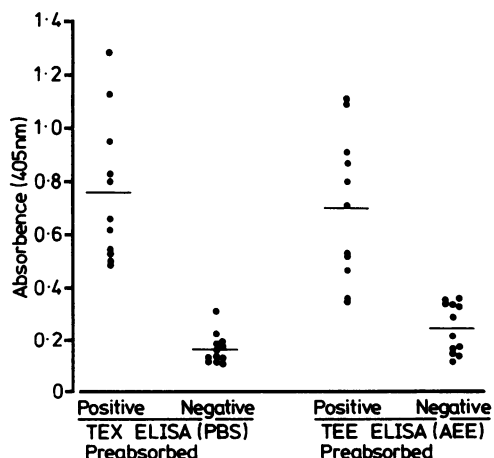


Fig. 3 *Toxocara canis* embryonated egg antigen (TEE) and *Toxocara* excretory-secretory or exoantigen (TEX) ELISA absorbance values for patients with clinically diagnosed ocular toxocariasis (positive) or retinoblastoma (negative). The sera were either preabsorbed with *Ascaris* serum embryonated egg antigen or sham preabsorbed with phosphate buffered saline (PBS) and assayed spectrophotometrically. Horizontal bar indicates the mean absorbance value.

better able to discriminate between positive and negative samples than TEE ELISA. In addition, testing samples by both TEX ELISA and TEE ELISA provided no additional diagnostic information to that provided by TEX ELISA alone.

Discussion

Sensitive immunodiagnostic tests are especially critical for parasitic infections characterised by a small infectious dose, lack of multiplication or egg production in the host, and low concentrations of circulating parasite specific antibody. Specificity is important when other parasites or pathological processes are capable of producing similar clinical manifestations but where medical management may differ significantly. Such is the case with ocular toxocariasis. Whereas children with visceral toxocariasis typically have pronounced eosinophilia and anti-*T. canis* specific antibody titres in their serum patients with ocular toxocariasis are usually asymptomatic and often have lower concentrations of eosinophils and circulating anti-*T. canis* specific antibody.⁶ These differences have been attributed to the level of infection; children with visceral toxocariasis are often geophagic or coprophagic and consume many eggs, while children with ocular toxocariasis lack this habit and have probably been inadvertently infected with few eggs.⁶

TEE ELISA for *T. canis* has proved extremely useful for the immunological diagnosis of ocular toxocariasis but has several inherent limitations. Each serum to be tested must be preabsorbed with AEE to remove cross reacting antibodies to reduce the number of false positive results. Another obstacle has been the production of adequate amounts of *T. canis* antigen from the stage concerned with the pathogenesis of the infection. The method of de Savigny⁹ for culture of second stage *T. canis* larvae in a defined medium afforded us the opportunity to produce adequate amounts to TEX for comparison with TEE in ELISA for ocular toxocariasis.

Results of our studies have shown that TEE and TEX ELISA are comparably sensitive. There was no significant difference in absorbance value for the 11 *T. canis* positive sera using TEE ELISA with sera preabsorbed by conventional methods and TEX ELISA with sham preabsorbed sera. Similarly, there was no significant difference in absorbance values when different antigens were used for the 12 negative *T. canis* sera. TEX ELISA, however, resulted in better discrimination between the positive and negative sera than TEE ELISA. If the lowest absorbance value for the positive sera had been selected as the cut off point using TEE ELISA, two of the 12 negative sera would have been falsely classified as posi-

tive (Fig. 3). It is encouraging that these results using TEX ELISA were obtained without preabsorption of the sera with AEE. Further work is still required to standardise TEX for the serodiagnosis of ocular toxocariasis.

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