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rTES-30USM: cloning via assembly PCR, expression, and evaluation of usefulness in the detection of toxocariasis

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Currently, the laboratory diagnosis of toxocariasis, caused by *Toxocara canis* or *T. cati*, mainly relies on serological tests. Unfortunately, however, the specificities of most of the commercial tests that are available for the serodiagnosis of this disease are not very high and this may cause problems, especially in tropical countries where co-infections with other helminths are common. In an effort to develop a serological assay with improved specificity for the detection of *Toxocara* infection, an IgG_4-ELISA based on a recombinant version (rTES-30USM) of the 30-kDa *Toxocara* excretory–secretory antigen (TES-30) has recently been developed. To produce the antigen, the TES-30 gene was cloned via assembly PCR, subcloned into a His-tagged prokaryotic expression vector, and purified by affinity chromatography using Ni^{2+}–nitrilotriacetic-acid (Ni–NTA) resin. The performance of the ELISA based on the recombinant antigen was then compared with that of commercial kit, based on an IgG-ELISA, for the serodiagnosis of toxocariasis (*Toxocara* IgG-ELISA; Cypress Diagnostics, Langdorp, Belgium). Both assays were used to test 338 serum samples, including 26 samples from probable cases of toxocariasis. Assuming that all the probable cases were true cases, the assay based on rTES-30USM demonstrated a sensitivity of 92.3% (24/26) and a specificity of 89.6% (103/115) whereas the commercial kit exhibited a sensitivity of 100% (26/26) but a specificity of only 55.7% (64/115). The high sensitivity and specificity exhibited by the new IgG_4-ELISA should make the assay a good choice for use in tropical countries and any other area where potentially cross-reactive helminthic infections are common.

Human toxocariasis is a zoonotic disease caused by the helminths *Toxocara canis* from dogs and *Toxocara cati* from cats (Glickman and Schantz, 1978). It occurs world-wide although seroprevalences tend to be lower in the developed countries than in the developing. In France, 2%–5% of apparently healthy adults from urban areas and 14.2%–37% of such adults from rural areas were found seropositive for *Toxocara* by Magnaval *et al.* (1994). A much higher seroprevalence (63.2%) was observed in Bali (Chomel *et al.*, 1993), whereas 20% of the subjects investigated by Hakim *et al.* (1993), in Malaysia, were found to be seropositive.

Most cases of human toxocariasis are caused by *Toxocara canis* and most acquire their infections from puppies that are themselves infected transplacentally or via milk. The ova voided in faeces mature in the soil to the human-infective second-stage larvae (L_2; Lescano *et al.*, 1998). Humans are infected when they accidentally ingest the embryonated ova in soil or on other

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contaminated surfaces. In humans the eggs hatch but the parasite is unable to develop past the L_2 stage, the larvae wandering throughout the body, sometimes for many years (Glickman and Schantz, 1978; Magnaval *et al.*, 2001). The wandering larvae in humans give rise to various clinical manifestations, known as visceral larva migrans (VLM), ocular larva migrans (OLM) and covert toxocariasis.

The presentations of VLM include fever, hyper-eosinophilia, abdominal pain, bronchial asthma, malaise, skin rash and hypergammaglobulinaemia (Glickman and Schantz, 1978; Patrick *et al.*, 2001). OLM occurs when L_2 migrate into the eye, causing glaucoma, leucocoria, strabismus or even sudden loss of vision (Sabrosa and de Souza, 2001; Mirdha and Khokar, 2002). Covert toxocariasis presents as a wide range of non-specific signs, including abdominal pain, cough, headache, and sleep and behavioural disturbances (Taylor *et al.*, 1988).

The early detection of human toxocariasis and the assessment of the disease's importance in any population rely heavily upon serological tests because it is extremely difficult to detect the larvae in biopsy samples (Gillespie *et al.*, 1993; Ajayi *et al.*, 2000). Although crude extracts of *Toxocara* excretory–secretory (TES) antigens, from L_2 cultured *in vitro*, have been used extensively in IgG-ELISA for the serodiagnosis of human toxocariasis (De Savigny *et al.*, 1979; Akao *et al.*, 1997), such assays generally have insufficient specificity for use in tropical countries where other helminthic infections are common (Rahmah *et al.*, 2005). The specificity can be improved by using just the smaller TES antigens (i.e. those of 24–35 kDa; Magnaval *et al.*, 1991), and a recombinant protein corresponding to the 30-kDa TES antigen (TES-30) has also been found to give good sensitivity and specificity in an IgG-ELISA for the detection of human toxocariasis (Yamasaki *et al.*, 1998, 2000). As an ELISA for the detection of IgG_4 reacting with TES was recently found to give fewer false-positive results than an IgG-ELISA based on the same antigens (Rahmah *et al.*, 2005), it seemed possible that an IgG_4-ELISA based on the recombinant TES-30 antigen would give the best specificity yet seen. The aim of the present study was to explore this possibility.

MATERIALS AND METHODS

Oligonucleotide Design

The sequence of the TES-30 gene was obtained from GenBank (as accession AB009305) and used, with version 3.2 of the MacDNAsis software package (Hitachi Software Engineering, San Francisco, CA), to design 34 overlapping oligonucleotides together covering about 660 bp of the gene sequence (Table 1). All complementary oligonucleotides were overlapped by about 20 nucleotides, so that adjacent oligonucleotides would prime each other.

Gene Assembly

The TES-30-gene assembly PCR was carried out as described by Stemmer *et al.* (1995), with some modifications. Stock solutions of the upper and lower oligonucleotides were prepared, each at 250 μM, and then either left undiluted or diluted 1:2, 1:5, 1:7, 1:9, 1:15, 1:20, 1:25 or 1:30 (v/v) with sterile water, for use as templates in the PCR. The Master Gradient thermocycler (Eppendorf North America, New York, NY) was set to give 5 min at 95°C, then 55 cycles, each of 30 s at 95°C, 30 s at 55°C and 2 min at 72°C, before a final 5 min at 72°C. The specific primers NHF (5′-TAG AAT TCA TGA TCG CCG CAAT-3′) and NHR (5′-GCA TAA GCT TCT AGA GAG GTC TCTT-3′) were used to amplify the correct construct from the mixture of products produced in the gene assembly.

Cloning of TES-30 Gene

The construct amplified using NHF and NHR was ligated into the pCR2.1-TOPO

cloning vector from the TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA). The resultant mixture was transformed into the TOP10 *Escherichia coli* host (Invitrogen) and the transformed colonies were then screened in PCR, using external (M13F and M13R) and internal primers (NHF and NHR). Plasmids produced from the two best clones were isolated and purified using a commercial kit (Wizard® *Plus* SV Minipreps DNA purification kit; Promega, Madison, WI) and then sequenced commercially, using universal M13 forward and reverse primers and gene-specific primers. The sequences of the engineered gene were then compared with the published sequence of the TES-30 gene, using version 6.0 of the Vector NTI Advance® software package (InforMax, Frederick, MD).

Repair of Base Mutations

Two nucleotide deletions (of 281 and 639 bp) were observed and repaired using a PCR-based site-directed mutagenesis kit (QuikChange®; Stratagene, La Jolla, CA), with some modifications. The specific

TABLE 1. *The 34 overlapping oligonucleotides of the TES-30 gene that were employed in the PCR assembly*

Name	Sequence
UPPER-STRAND OLIGONUCLEOTIDES	
NHF1	5′-ATGATCGCCATCGTCGTTTTGTTATGTCTCCACCTGT-3′
NHF2	5′-CCGCCACAAATGCTTGCGCCACCAACAATGACTGTGGTAT-3′
NHF3	5′-TTTCCAAGTGTGCGTTAACAACGTCTGCGTTGCCAATAAC-3′
NHF4	5′-CAAGGATGCAATCCGCCTTGTGTGCCTCCGCAGGTTTGCG-3′
NHF5	5′-TTGCACCAATGTGTGTCGCCCCTCCACCGGCAGCCACTAC-3′
NHF6	5′-AACTGCAGCTCCAGGCGTTACAACTACAAGACCACGTGCC-3′
NHF7	5′-TGCCCACCCAATTGGACGCCGTTCAACAACAATTGCTATA-3′
NHF8	5′-TCGCCTCTCTACCTGGACGCTTCCTTTTCAACCAAGCTAG-3′
NHF9	5′-GACCAGTCGACTGTAGGAAACTTCGGCAGCGAACTGAACT-3′
NHF10	5′-GACCAGTCGACTGTAGGAAACTTCGGCAGCGAACTGAACT-3′
NHF11	5′-TCGTCAACAGTTTCGCTCTCGGTCGTGGAGTAACAAGATA-3′
NHF12	5′-CTGGATAGGAGTGAACAGACAGTTCGGCCAGTGGGTGTTT-3′
NHF13	5′-ACGAATGGAAGTCCAGTGATATTTTCGAACTGGAGGCCTA-3′
NHF14	5′-GTCAACCGGACGGATGCTGTGGTTCCAACGTCACGTGTGC-3′
NHF15	5′-GTTCGTTAATTACGCGAACTTCCTCGGCCAATGGGATGAT-3′
NHF16	5′-GCCCCATGCGGAAGTTTGTTTACGACTCCACAAGGCTTCG-3′
NHF17	5′-TATGCAAGAGACCTCTCTAG-3′
LOWER-STRAND OLIGONUCLEOTIDES	
NHR1	5′-CTAGAGAGGTCTCTTGCATACGAAGCCTTGTGGAGTCGTA-3′
NHR2	5′-AACAAACTTCCGCATGGGGCATCATCCCATTGGCCGAGGA-3′
NHR3	5′-AGTTCGCGTAATTAACGAACGCACACGTGACGTTGGAACC-3′
NHR4	5′-ACAGCATCCGTCCGGTTGACTAGGCCTCCAGTTCGAAAAT-3′
NHR5	5′-ATCACTGGACTTCCATTCGTAAACACCCACTGGCCGAACT-3′
NHR6	5′-GTCTGTTCACTCCTATCCAGTATCTGGTTACTCCACGACC-3′
NHR7	5′-AACAGCGAAACTGTTGACGAAGTTCAGTTCGCTGCCGAAG-3′
NHR8	5′-TTTCCTACAGTCGACTGGTCGAACCACACAACTCTTGAGC-3′
NHR9	5′-CAGTCTGTGTACACCAGTCGCTAGCTTGGTTGAAAAGGAA-3′
NHR10	5′-GCGTCCAGGTAGAGAGGCGATATAGCAATTGTTGTTGAAC-3′
NHR11	5′-GGCGTCCAATTGGGTGGGCAGGCACGTGGTCTTGTAGTTG-3′
NHR12	5′-TAACGCCTGGAGCTGCAGTTGTAGTGGCTGCCGGTGGAGG-3′
NHR13	5′-GGCGACACACATTGGTGCAACGCAAACCTGCGGAGCCACA-3′
NHR14	5′-CAAGGCGGATTGCATCCTTGGTTATTGGCAACGCAGACGT-3′
NHR15	5′-TGTTAACGCACACTTGGAAAATACCACAGTCATTGTTGGT-3′
NHR16	5′-GGCGCAAGCATTTGTGGTGGACAGGTGGAGACATAACAAA-3′
NHR17	5′-ACGACGATTGCGGCGATCAT-3′

forward primers 30MF1 (5′-GCT CTC GGT CGT GGA GTA ACC AGGT-3′) and 30MF2 (5′-CCG CAA TCG TCG TTT TGT TAT GTCT-3′) and reverse primers 30MR1 (5′-ACC TGG TTA CTC CAC GAC CGA GAGC-3′) and 30MR2 (5′-AGA CAT AAC AAA ACG ACG ATT GCGG-3′) were designed according to the kit manufacturer's instructions. Each 50-μl PCR reaction mixture contained 4 μl plasmid template DNA, 5 μl 10 × mutagenesis buffer, 2 μl of a 20 μM solution of each mutant forward and reverse primer, 1 μl of a 10 mM solution of each deoxynucleotide triphosphate, and 2.5 U *Pfu* DNA polymerase, in sterile water. The thermocycler was set to give 5 min at 95°C, then 12 cycles, each of 30 s at 95°C, 1 min at 55°C and 5 min at 72°C, and then a final 5 min at 4°C. The PCR product was digested with 10 U *Dpn*I for 1 h at 37°C before being transformed into the TOP10 *E. coli* host. After the transformed colonies were screened by PCR, the plasmids produced from the best clones were isolated, purified and commercially sequenced.

Subcloning into a Bacterial Expression Vector

Both the expression vector employed in the subcloning step (pPROEX-HT; Life Technologies, Gaithersburg, MD) and the corrected recombinant plasmid were digested with *Bam*HI and *Xho*I. The recombinant plasmid fragment was subjected to electrophoresis and then extracted from the agarose gel, using a commercial kit (Wizard® SV Gel and PCR Clean-Up System; Promega), before being subcloned into the digested version c of the expression vector, ligated using the T4 DNA ligation kit (Roche Diagnostics, Mannheim, Germany) and finally transformed into competent TOP10 *E. coli*.

Expression and Purification of the rTES-30USM Antigenic Protein

The TES-30USM recombinant bacteria were cultured overnight in Terrific Broth containing 100 μg ampicillin/ml and then subcultured into the same medium. Expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM and incubating at 30°C in a shaker-incubator. The induced cells were harvested after 6 h and disrupted by sonication, in lysis buffer containing 50 mM NaH_2PO_4, 300 mM NaCl and 20 mM imidazole, before the sonicate was purified by affinity chromatography on Ni^{2+}–nitrilotriacetic-acid (Ni–NTA) resin (QIAGEN, Valencia, CA). The protein-containing fractions were pooled and analysed by SDS–PAGE and western blotting.

Human Serum Samples

Overall, 338 human sera were investigated. Since the parasitological confirmation of human toxocariasis is so difficult, there is no good 'gold standard' for the diagnosis of toxocariasis. In evaluating the performance of the ELISA, sera from 26 probable cases of human toxocariasis — who not only showed clinical signs and symptoms consistent with toxocariasis but were also found to have high titres of anti-*Toxocara* antibodies when tested using commercial serodiagnostic kits — were tested and assumed to be from true cases.

The other 312 sera investigated came from other patients — with soil-transmitted helminths (35 samples), a serological diagnosis of amoebiasis (31), or toxoplasmosis (20), *Brugia malayi* microfilaraemias (28), or *Gnathostoma spinegerum* in the eye (one) — and apparently healthy blood donors (197). The 338 samples came from the serum banks of two universities — the *Universiti Sains Malaysia* (Kelantan, Malaysia) and the *Fakultas Kedokteran Universiti Sam Ratulangi* (Manado, Indonesia) — and were obtained and tested with the approval of the ethical committee of each of these universities.

Western Blotting

For the western blotting (WB), the rTES-30USM antigen (at 20 μg/well) was

subjected to electrophoresis (10% SDS–PAGE) before the protein bands were transferred onto nitrocellulose paper (GE Osmonics Labstore, Minnetonka, MN), using a semi-dry Trans-Blot® electroblotting system (Bio-Rad, Hercules, CA) for 30 min at 12 V. The paper was then cut into strips, blocked with 1% casein solution (Roche Diagnostics) for 1 h and then probed with the serum samples diluted 1:100 in Tris-buffered saline at pH 7.5 (TBS). The secondary antibody employed, at a dilution of 1:2000, was a monoclonal anti-human-IgG_4 conjugated to horseradish peroxidase (CLB, Amsterdam), and the washing solution used was TBS containing 0.05% (v/v) Tween 20. A BM chemiluminescence blotting reagent (Roche Diagnostics) and X-ray films (Eastman Kodak, Rochester, NY) were then employed to investigate the blots.

IgG_4-based ELISA

An ELISA based on the detection of anti-rTES-30USM IgG_4 was developed. For this, 96-well microtitre plates (Maxisorp™; Nunc, Roskilde, Denmark) were coated with 0.02 M bicarbonate buffer (pH 9.6) containing 20 μg rTES-30USM/ml, at 100 μl/well. Each plate was incubated in a humid box overnight at 4°C and then for 2 h at 37°C before being washed. Each washing step was performed using a Columbus Pro™ microtitre-plate washer (Tecan Austria, Grödig, Austria) and five 5-min rinses in phosphate-buffered saline (pH 7.2) containing 0.05% (v/v) Tween 20. After being washed, the coated plates were blocked for 1 h with 2% casein solution (Roche Diagnostics), and then washed again before the test sera, each diluted 1:100 in 1% casein solution, were added, at 100 μl/well. The plates were incubated for 2 h at 37°C. After another washing step, 100 μl of a 1:500 dilution of the horseradish-peroxidase conjugate of the monoclonal anti-human-IgG_4 were added to each well and incubated for 30 min at 37°C. Following a final wash, 2′,2-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS; Roche Diagnostics) was added as the substrate and incubated for 30 min at 37°C. The optical density (OD) of each well was then read at 405 nm, in an ELISA spectrophotometer (Tecan Nordic, Mölndal, Sweden). Any serum giving an OD above 0.200 — the mean OD plus three S.D. for the sera from 30 healthy blood donors from an area endemic for soil-transmitted helminths — was considered positive for anti-*Toxocara* IgG_4.

Commercial *Toxocara* IgG Kit

Using all 338 test sera, the performance of the new IgG_4-ELISA was compared with that of a commercial IgG-ELISA for the serodiagnosis of toxocariasis. The commercial ELISA (*Toxocara* IgG-ELISA; Cypress Diagnostics, Langdorp, Belgium) was performed according to the manufacturer's instructions, using the conjugate, control sera, washing buffer, substrate, chromogen and stop solution provided. Briefly, 50 μl of 1:50 dilution of a test serum (or the control serum provided by the manufacturer) were pipetted into a coated well and incubated at room temperature (RT) for 15 min. The plates were then washed using a Columbus Pro microtitre-plate washer and five 5-min rinses in washing buffer. A drop (about 50 μl) of the solution of peroxidase-conjugated Protein A was added to each well. After 15 min at RT and a final wash, a drop of substrate and one of chromogen were added to each well. After another 15 min at RT, a drop of stop solution was added to each well and OD at 450 nm were then read on the ELISA spectrophotometer. For each test serum, a sample ratio was then calculated by dividing the sample OD with the cut-off value (the mean OD for the negative-control sample plus 0.150). A sample ratio of >1.1 indicated a positive result.

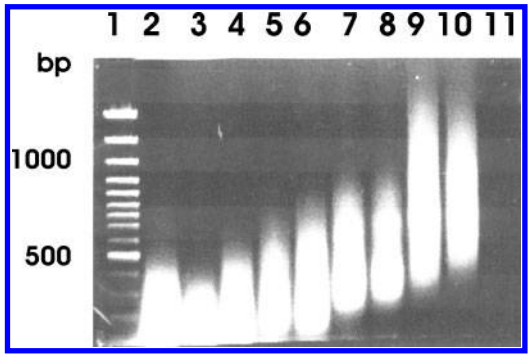

FIG. 1. The results of the agarose-gel electrophoresis of the PCR products from the TES-30 gene assembly, with the stock solutions of the 34 oligonucleotides undiluted (lane 2) or diluted 1:2 (lane 3), 1:5 (lane 4), 1:7 (lane 5), 1:9 (lane 6), 1:15 (lane 7), 1:20 (lane 8), 1:25 (lane 9) or 1:30 (lane 10). Also run on the gel were a 100-bp DNA marker ladder (lane 1) and a negative control (lane 11).

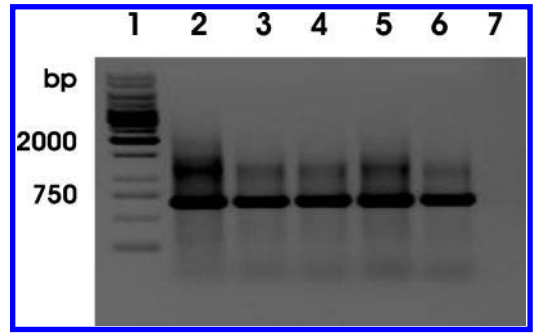

FIG. 2. The results of the agarose-gel electrophoresis of the products from the second PCR, with the internal specific primers (NHF and NHR) undiluted (lane 2) or diluted 1:2 (lane 3), 1:5 (lane 4), 1:7 (lane 5) or 1:9 (lane 6). Also run on the gel were a 1-kb DNA marker ladder (lane 1) and a negative control (lane 7).

Toxocariasis Western-blot Kit

As western blotting employing the TES antigen has been reported to produce specific and sensitive results in the serodiagnosis of toxocariasis (Magnaval *et al.*, 1991, 2002), the performances of the new IgG_4-ELISA and the commercial IgG-ELISA were also compared with that of a commercial western-blot kit (BLOT *Toxocara* IgG; Test-Line Clinical Diagnostics, Brno, Czech Republic), in which antigenic bands of 120, 32 and 26 kDa are considered highly specific indicators of toxocariasis. In the three-way comparison of the immunoassays, only 32 of the serum samples (12 from the probable cases of toxocariasis, 10 from patients with other infections and 10 from healthy blood donors) were employed.

Statistical Analysis

McNemar's χ^2 test (two-sided) was employed to determine whether the observed difference in specificities between the new IgG_4-ELISA and the commercial IgG-ELISA (with the results from the healthy blood donors excluded) was statistically significant. A *P*-value of <0.05 was considered indicative of a statistically significant difference.

RESULTS

Electrophoresis in agarose gel of the PCR products of the assembled TES-30 gene produced different banding patterns according to which dilution of the 34 oligonucleotides was used (Fig. 1). A 1:15 dilution (lane 7 in Figure 1), which gave the best result — in that the centre of the smear covered the expected amplicon size of 660 bp — was chosen for the second amplification step. Electrophoresis in agarose gel of the products of this second amplification gave banding patterns that varied little with the dilutions of the NHF and NHR primers that were used (Fig. 2); although all dilutions gave a thick band of about 660 bp, a 1:5 primer dilution (lane 4 in Figure 2) was chosen for the subsequent cloning step.

SDS–PAGE of the purified rTES-30USM gave a distinctive and thick band of approximately 28 kDa (Fig. 3). In the western-blot analysis of the recombinant antigen, all of the serum samples from the probable cases of toxocariasis (but none of the tested samples from patients with other helminth infections or those from the apparently healthy blood donors) gave a positive reaction at the expected band size of approximately 28 kDa (Fig. 4).

In the IgG_4-ELISA based on rTES-30USM, 24 (92.3%) of the 26 serum

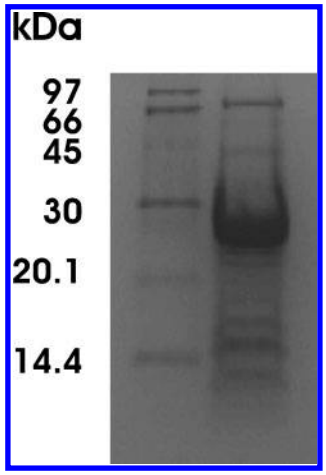

FIG. 3. The results of the SDS–PAGE of the rTES-30USM antigen, showing the low-molecular-weight markers in the lefthand lane and the antigen (an intense band of about 28 kDa) in the righthand lane.

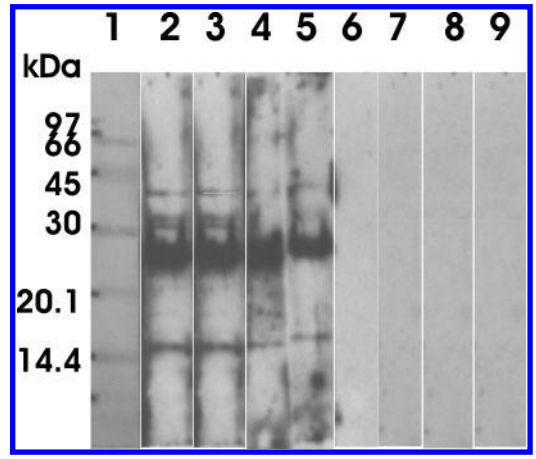

FIG. 4. Western blots in which the rTES-30USM antigen was probed with sera from probable cases of toxocariasis (lanes 2–5), from patients with *Trichuris trichiura* (lane 6) or *Toxoplasma gondii* infection (lane 7), and from apparently healthy blood donors (lanes 8 and 9). Lane 1 contained low-molecular-weight markers.

samples from the (probable) toxocariasis cases were positive. In the commercial IgG-ELISA, however, all 26 of these serum samples were found positive, apparently giving a slightly better sensitivity (100%). The newer IgG_4-ELISA did, however, appear much more specific than the assay from Cypress Diagnostics (89.6% *v.* 55.7%, when the results for the blood donors were excluded; $P<0.001$), with most cross-reactions in the commercial IgG-ELISA caused by soil-transmitted helminths (Table 2). Of the 197 sera from apparently healthy blood donors that were tested, six were found positive (presumably false-positive) in both the IgG_4-ELISA and IgG-ELISA.

When, using a subsample of the available sera, the performances of the two ELISA were compared with that of a commercial diagnostic kit based on western blotting, all three tests were found to have similar sensitivities (Table 3). In terms of specificity, however, the IgG_4-ELISA and western-blot kit were similar, both being superior to the commercial IgG-ELISA (Table 3).

DISCUSSION

The serodiagnosis of toxocariasis has been generally based on non-defined antigens,

TABLE 2. *The results of evaluating the specificities of the new rTES-30USM IgG_4-ELISA and of a commercial IgG-ELISA (Cypress Diagnostics) based on excretory–secretory antigens from cultures of* Toxocara *larvae*

	No. of sera		
		Found positive in the:	
Infections detected in serum donors	Tested	IgG_4-ELISA	Commercial IgG-ELISA
Ascaris lumbricoides, *Trichuris trichiura* and hookworm	30	2	22
Strongyloides stercoralis	5	0	4
Gnathostoma spinigerum	1	0	1
Entamoeba histolytica	31	5	8
Brugia malayi	28	3	16
Toxoplasma gondii	20	2	0
Any of the above	115	12	51

such as somatic L_2 antigens, soluble embryonated-egg extracts, soluble adult antigens or TES (Smith and Rahmah, 2006). Such antigens have been used in various types of assay, including radio-iodination, western blotting, ELISA, latex agglutination, radio-allergosorbent tests (RAST), indirect immunofluorescent antibody tests, counter immuno-electrophoresis (CIEP) and the rapid *Toxocara*CHEK test (Smith and Rahmah, 2006). Although the tests have been used to check sera for specific IgG, IgM, IgE or IgG_4, the most common test currently employed for the serological diagnosis of toxocariasis, in both laboratory-based assays and in commercial kits, is the indirect IgG-ELISA using TES antigens (Smith and Rahmah, 2006).

Although assays based on TES antigens (which can be produced from cultures of *Toxocara canis* L_2) usually have good sensitivity, their specificity is often too low for them to be used effectively in developing countries where the helminth infections that cause cross-reactions, particularly soil-transmitted parasites, are endemic (Lynch *et al.*, 1988; Jacquier *et al.*, 1991; Rahmah *et al.*, 2005; present study). In such countries, therefore, all the commercial ELISA that are based on TES antigens only have very limited value (Lynch *et al.*, 1988; Magnaval *et al.*, 2001). Although a negative result in such an ELISA can still be useful in these areas, helping to eliminate toxocariasis in a differential diagnosis, a positive result is only an uncertain indication that toxocariasis is present (Rahmah *et al.*, 2005).

The production of TES antigens is also limited to the capacity of the parasite culture and is time-consuming and subject to batch-to-batch variability in quality and purity. The heterogeneous composition of the TES antigens, with their wide variability in size, is also likely to increase the possibility of at least one of the antigens cross-reacting with antibodies produced to other helminth infections. Dead larvae in cultures may release non-TES proteins into the culture medium, and such proteins may very well 'contaminate' TES antigens recovered from cultures and contribute to the cross-reactions reported with these antigens (Smith and Rahmah, 2006).

To avoid most of the limitations of the crude extracts of TES antigens produced by cultured L_2, recombinant proteins based on the TES antigens, that can be produced with no variability and in unlimited amounts, have been developed. Assays based on one of these recombinant antigens generally have improved specificities because the recombinant antigen is a single molecule and, unlike a natural TES antigen, is not glycosylated. The absence of glycosylation avoids, or at least decreases, the cross-reactivity with antibodies that recognise the sugar moieties of the TES antigens produced by *Toxocara canis* larvae (Maizel *et al.*, 1987).

TABLE 3. *Comparison of the reactivities of the two types of ELISA with those of a commercial kit based on western blotting (WB)*

	No. of sera			
		Found positive in the:		
Infections detected in serum donors	Tested	IgG_4-ELISA	Commercial IgG-ELISA	Commercial WB kit
Toxocara	12	10	10	10
Ascaris lumbricoides, *Trichuris trichiura* and hookworm	10	2	7	2*
None (sera from apparently healthy blood donors)	10	3	3	3†

*The two samples found positive by western blotting were also found positive in the IgG_4-ELISA.
†The three samples found positive by western blotting were also found positive in both ELISA.

In IgG assays, the recombinant TES-30 antigen has been shown to demonstrate very good sensitivity and specificity in the detection of toxocariasis, although some cross-reactivities have been reported, especially in western blots (Yamasaki *et al.*, 1998, 2000). Another recombinant antigen, TES-120, expressed from the *Tc-muc 1* gene, has also been shown to be potentially specific and sensitive in the serodiagnosis of toxocariasis and merits further validation studies (Fong *et al.*, 2003; Fong and Lau, 2004).

It also seems to be possible to increase the specificity of a test for the serodiagnosis of toxocariasis by limiting the antibodies detected to anti-*Toxocara* IgG_4 (Wiechinger, 1998; Rahmah *et al.*, 2005). In the detection of lymphatic and non-lymphatic filariasis, limiting the assay to IgG_4 antibodies also greatly increases specificity, anti-filarial IgG_4 being a marker of active infection (Kwan Lim *et al.*, 1990; Weil *et al.*, 2000; Klion *et al.*, 2003).

The concept behind the present study was that a serodiagnostic ELISA based on the detection of IgG_4 reacting with a recombinant antigen should have very high specificity and so be useful even in those tropical countries where potentially cross-reacting helminth infections are co-endemic. The TES-30 gene was successfully cloned, via assembly PCR, subsequently allowing the production of the rTES-30USM antigen in a prokaryotic expression vector. An IgG_4-ELISA using the rTES-30USM was then developed and evaluated for its usefulness in the detection of toxocariasis. The test was found to exhibit a sensitivity of 92.3% (24/26) and a specificity of 89.6% (103/115). Although a commercial IgG-ELISA kit appeared to give slightly better sensitivity (100%), this was offset by the kit's rather poor specificity (55.7%). Western blotting tends to be highly specific but a commercial kit based on western blotting showed no better specificity than the new IgG_4-ELISA. It therefore seems possible that the 12 patients with geohelminth, *Entamoeba*, *Brugia* or *Toxoplasma* infections who were found positive in the IgG_4-ELISA were all co-infected with *Toxocara*.

In areas where potentially cross-reactive helminths (especially soil-transmitted helminths) are endemic, more specific IgG_4-ELISA based on rTES-30USM would have greater usefulness than at least one commercial IgG-ELISA based on TES antigens from cultures. Further studies are underway to develop a rapid TES-30-IgG_4 test that could be useful in areas without the laboratory facilities for performing ELISA.

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