APPLICATION OF RECOMBINANT SMR-DOMAIN CONTAINING PROTEIN OF *ANGIOSTRONGYLUS CANTONENSIS* IN IMMUNOBLOT DIAGNOSIS OF HUMAN ANGIOSTRONGYLIASIS

Apichat Vitta[1], Timothy P Yoshino[2], Thareerat Kalambaheti[3], Chalit Komalamisra[1], Jitra Waikagul[1], Jiraporn Ruangsittichai[4] and Paron Dekumyoy[1]

[1]Department of Helminthology, [3]Department of Microbiology and Immunology, [4]Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; [2]Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, WI, USA

Abstract. The aim of this study was to find novel proteins expressed from an *Angiostrongylus cantonensis* adult female worm cDNA library for serodiagnosis of angiostrongyliasis. An immuno-dominant clone, fAC22, was identified by immunoscreening with pooled positive sera from proven angiostrongyliasis patients. The clone contained an open reading frame of 2,136 bp encoding a 80.5 kDa protein with a predicted isoelectric point of 5.8. The deduced amino acid sequence (712 amino acids) contained the conserved domain of Small mutS related (Smr) superfamily protein, with similarity with the Smr domain protein of *Brugia malayi*. The fusion His-tagged 81 kDa recombinant protein expressed as inclusion body in *Escherichia coli* was solubilized and purified by Ni-affinity chromatography for use in immunoblot analysis. Its sensitivity, specificity, positive and negative predictive values in immunodiagnostic test was 93.5, 91.5, 79.0 and 97.5%, respectively. Although some cross-reactivity of the antigen was observed among gnathostomiasis, bancroftian filariasis, ascariasis, echinococcosis, paragonimiasis and opisthorchiasis, sera from 14 other infections were all negative. These data indicate its possible application in immunodiagnosis of clinically suspected angiostrongyliasis.

Key words: *Angiostrongylus cantonensis*, eosinophilic meningitis, recombinant fusion protein, immunodiagnosis

INTRODUCTION

Human angiostrongyliasis is a food-borne parasitic zoonosis caused by infection with the larval stage of the rat lungworm *Angiostrongylus cantonensis*. Among human helminthic infections, *A. cantonensis* is known to be the primary cause of eosinophilic meningitis or meningoencephalitis. In the early 2000s, 3 outbreaks of the disease were reported, from Taiwan (Tsai *et al*, 2001), the Caribbean (Slom *et al*, 2002) and China (Chen *et al*, 2005). Recently, at least 2,827 cases of the disease have been documented worldwide and over half of

Correspondence: Paron Dekumyoy, Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, 420/6 Ratchawithi Road, Bangkok 10400, Thailand.
Tel: 66 (0) 2354 9100 to 4 ext 9180-1; Fax: 66 (0) 2643 5600
E-mail: tmpdk@mahidol.ac.th

these cases (1,337) were reported from Thailand (Wang *et al*, 2008). Moreover, this parasite can occasionally be the cause of ocular angiostrongyliasis (Sawanyawisuth *et al*, 2007; Sinawat *et al*, 2008).

A definitive diagnosis of angiostrongyliasis is made by finding the immature worms in the eyes or cerebrospinal fluid (CSF) of patients. However, recovery of worms from human patients rarely occurs. Diagnosis of the disease is commonly made by the combination of clinical symptoms, laboratory findings and history of eating intermediate or paratenic hosts. To assist in the diagnosis of this disease, several serological tests have been reported, such as enzyme-linked immunosorbent assay (ELISA) and immunoblot (Chen, 1986; Yen and Chen, 1991; Akao *et al*, 1992; Eamsobhana *et al*, 1995, 1997; Nuamtanong, 1996; Maleewong *et al*, 2001). In the early studies, detection of antibody against *A. cantonensis* was done by using different kinds of antigens, such as crude or partially purified adult worm antigens (Welch *et al*, 1980; Chen, 1986), young adult worm antigens (Cross and Chi, 1982; Yen and Chen, 1991) or female adult antigens (Yen and Chen, 1991; Nuamtanong, 1996). Results obtained using these antigen preparations, however, have been unsatisfactory because of cross-reactivity with antibodies from other parasitic infections.

A group of the native antigens, including purified proteins of 29 kDa, 31 kDa and 32 kDa, from *A. cantonensis* have been widely used in IgG- and IgG_{1-4}–immunoblotting tests for diagnosis of human angiostrongyliasis (Nuamtanong, 1996; Maleewong *et al*, 2001; Intapan *et al*, 2003; Eamsobhana *et al*, 2004). All native antigens are derived by extraction of *Angiostrongylus* worms, all stages of which are maintained in the laboratory by cycling through snail hosts and experimental rats. Maintaining the parasite life cycle is laborious and time-consuming, and requires the slaughtering of experimental animals to obtain antigen materials. In addition, such antigens exhibit considerable variability in sensitivity and specificity in diagnostic tests. Therefore, novel antigens are still needed for more specific serodiagnosis of angiostrongyliasis.

In the present study, a molecular cloning approach was used to express recombinant antigenic fusion protein derived from cDNA of adult female worms of *A. cantonensis*. By immunoscreening, a clone encoding an 80.5 kDa protein was analyzed for its DNA sequence and antigenic property for detection of angiostrongyliasis.

MATERIALS AND METHODS

Experimental production of *A. cantonensis*

A. cantonensis worms were experimentally maintained in the rat definitive host, *Rattus norvegicus*, in the Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, Thailand. An intermediate host, the freshwater snail, *Biomphalaria glabrata*, was infected with fleshly isolated first-stage larvae from the rat feces. After 6 weeks of infection, the third stage (L3) larvae were harvested by HCl-pepsin digestion of chopped snails and isolated by the Baerman technique (Nuamtanong, 1996). Sixteen female rats, 6 weeks old, were used as the definitive host. They were individually infected with 30 L3 larvae by stomach intubation under light anesthesia. After 2-4 months of infection, fecal samples were examined for first stage larvae. Infected rats were then killed by an overdose of ether. Adult worms were harvested from the pulmonary arteries and right side of the heart. The worms

were washed with sterile 0.85% NaCl and used for extraction of total RNA. This study was approved by the animal care and use committee, Faculty of Tropical Medicine, Mahidol University (Project No. FTM-ACUC 009/2007).

Extraction of total RNA and purification of mRNA

Thirty freshly harvested female *A. cantonensis* (approximately, 100 mg wet weight) were homogenized in a glass tissue grinder. Total RNA was extracted with TRIzol® reagent according to the manufacturer's instructions (Invitrogen-Life Technologies, Carlsbad, CA). Messenger RNA (mRNA) was purified from 3 mg of total RNA using Oligotex mRNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To concentrate the mRNA by precipitation, 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol were added to mRNA solution. The tube was kept at -20ºC overnight and then centrifuged at 12,000*g* for 30 minutes at 4ºC. The pellet was washed with 75% (v/v) ethanol in RNase-free water, air-dried at room temperature for 10-15 minutes, and finally dissolved in 40 µl of RNase-free water and used for constructing cDNA library.

Construction and immunoscreening of cDNA library

A. cantonensis cDNA library was constructed using ZAP Express® cDNA Synthesis kit according to the manufacturer's instructions (Stratagene, La Jolla, CA). The phage titer was determined in order to plate the correct number of plaque forming units (PFUs) per plate before immunoscreening. The bacterial host strain, *E. coli* XL-1 Blue MRF/, was mixed with phage stock and incubated at 37ºC for 15 minutes. Then 3 ml of NZY top agarose, 15 µl of 0.5 M IPTG (in water) and 50 µl of X-Gal (250 mg/ml in DMF) were added to the mixture. After mixing well, the solution was immediately poured onto pre-warmed NZY agar plates. The plates were incubated in an inverted position at 37ºC overnight to develop the plaque color. Plaques were counted to determine the integrity of the library (PFU/ml).

In order to isolate and identify the antigenic clones, approximately 200,000 plaques from the amplified libraries were screened using pooled sera from 5 angiostrongyliasis patients. The pooled sera were pre-treated with *E. coli* phage lysate (Stratagene, La Jolla, CA) to eliminate any background cross-reactivity and false positive reactivity from antibodies against *E. coli*. Plaques were grown on NZY agar for 4 hours at 42ºC before they were overlaid with nitrocellulose membranes (Schleicher and Schuell) impregnated with 10 mM IPTG. After incubation at 37ºC for 4 hours, membranes were incubated with 1% bovine serum albumin (BSA) at 4ºC overnight. They were incubated with the pre-adsorbed pooled positive serum (diluted 1:2,000 with BSA solution) at 4ºC overnight, followed by incubation in alkaline phosphatase (AP)-conjugated goat anti-human IgG (Sigma, St Louis, MO) at a dilution of 1:20,000 for 2 hours at room temperature before the color reaction was developed with nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Positive purple plaques were removed from the master plate and subjected to secondary screening at a lower density as described above. Tertiary screening was performed to ensure clonality.

Preparation of plasmid DNA and estimation of cDNA insert size

Single-clone excision protocol was performed according to the manufacture's

instructions (Stratagene). Plasmids were purified using QIAprep Spin Miniprep kit according to the manufacturer's instructions (Qiagen, Valencia, CA). The size of cDNA insert was determined by double digestion with *Kpn*I and *Sac*I (Promega, Madison, WI), followed by visualization of the digested plasmids in 1% agarose gel and comparison with DNA molecular weight standard markers.

Sequencing and sequence analysis

Plasmid DNA sequencing of the selected clones was performed by the Biotechnology Center at the University of Wisconsin-Madison, USA. The nucleotide sequences were converted to amino acid sequences using BioEdit version 5.0.9 (www.mbio.ncsu.edu/BioEdit/BioEdit.html). The deduced amino acid sequences were subjected to a homology search against a non-redundant protein database using BLASTP program of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nim.nih.gov). ClustalW program was used to align between deduced amino acid sequences.

Expression and purification of recombinant protein

The cDNA insert of the clone, fAC22, was subcloned into pET-46 Ek/LIC expression vector according to the Ek/LIC cloning kit (Novagen, Madison, WI). Open reading frame (ORF) of fAC22 was amplified by PCR with forward primer 5'-GACGACGACAAG**ATG**CAACAGTATGCTTTTCATGC-3' and reward primer 5'-GAGGAGAAGCCCGG**TTA**TTTGCATTGAACCACAACTTC-3' [The start codon (ATG) and antisense stop codon (TTA) are indicated in bold]. PCR parameters were as follows: initial heating at 95ºC for 3 minutes, then 35 cycles of 30 seconds at 95ºC, 45 seconds at 55ºC, and 2 minutes at 72ºC; and a final step at 72ºC for 10 minutes. The PCR product was electrophoresed and gel-purified according to the Qiagen gel extraction kit (Qiagen, Valencia, CA), and then it was transfected into *E. coli* strain BL21(DE3). For induction of the His6-tagged protein, transformants were grown in LB-broth containing 100 μg/ml of ampicillin at 37ºC until the OD_{600} reached 0.4-0.6, followed by induction of expression with 1 mM IPTG for 4 hours. Bacterial cells were sedimented by centrifugation at 8,000*g* for 10 minutes and then resuspended in 5 ml of LEW lysis buffer (50 mM NaH_2PO_4 and 300 mM NaCl) and 2 ml of BugBuster® Master Mix (Novagen, Madison, WI). Cells were lysed by sonication on ice 6 times for 10 seconds with 10 seconds pauses (Sonicator® ultrasonical processor XL 2020) followed by centrifugation at 8,000*g* for 20 minutes at 4ºC. Recombinant fusion protein was resuspended in 5 ml of denaturant buffer (50 mM NaH_2PO_4, 300 mM NaCl, 8 M urea, pH 8) followed by sonication as described above. After centrifugation at 10,000*g* for 30 minutes at 4ºC, soluble recombinant His6 fusion protein was purified using Ni-NTA chromatography according to the manufacturer's instructions (Ni-NTA ProBond, Invitrogen, CA).

SDS-PAGE and immuno-characterization of recombinant fusion protein

The purified His6 fusion protein was electrophoresed in a 13% SDS-PAGE and then transferred to a nitrocellulose membrane. Membrane was incubated with 3% skim milk BSA in PBS-Tween20 (PBS-T) followed by incubation in a 1:50 dilution of pooled positive or pooled negative sera at 4ºC overnight with rocking platform. Membrane was washed, incubated with anti-human IgG (Southern Biotech, Birmingham, AL) conjugated with horseradish peroxidase (HRP) at a dilution of 1:1000

(in PBS-T) for 2 hours at room temperature. Membrane was washed 3 times with PBS-T before color development with 2, 6-dichlorophenol indophenol/H_2O_2 substrate. The reaction was terminated by placing in distilled water.

Application of purified fusion recombinant protein for serodiagnosis

All serum samples were supplied by Immunodiagnostic Unit for Helminthic Infections, Department of Helminthology, Faculty of Tropical Medicine, Mahidol University. Serum samples were divided into 3 groups: (i) normal group, which included samples from people ($n = 31$) proven to be parasite-free using fecal examinations comprising of a simple smear method, formalin-detergent floatation technique and the Kato technique (Garcia, 2007); (ii) Second group, which included samples from either confirmed angiostrongyliasis positive patients (worm-positive) ($n = 4$) or from patients showing clinical criteria ($n = 57$) and had consumed snail intermediate hosts or paratenic hosts and were immunoblot positive for 31 kDa band; (iii) Third group, which consisted of samples from patients with other parasitic infections ($n = 144$), including gnathostomiasis, strongyloidiasis, hookworm infection, trichinellosis, toxocariasis, ascariasis, trichuriasis, bancroftian filariasis, brugian filariasis, dirofilariasis, neurocysticercosis, taeniasis, echinococcosis, sparganosis, hymenolepiasis, haplorchiasis, paragonimiasis, opisthorchiasis, schistosomiasis and fascioliasis.

Purified recombinant fusion protein was electrophoresed in a single-well 13% SDS-PAGE slab gel and electrotransferred to a nitrocellulose membrane. After blocking nonspecific binding sites with 3% skim milk for 1 hour, the membrane was cut into ~3 mm wide strips. Each strip was incubated with individual human serum samples (diluted 1:50 in PBS-T containing 0.02% NaN_3) overnight at room temperature. After 3 washes with PBS-T, the strips were incubated for 2 $hour_3$ in 1:1,000 diluted goat anti-human IgG conjugated with HRP (Southern Biotech, Birmingham, AL). After 3 washes with PBS-T, positive reactions were developed with 2,6-dichlorophenol indophenol/H_2O_2 substrate. Reaction was terminated by placing in distilled water.

Data analysis

In order to evaluate the 81-kDa recombinant fusion protein for serodiagnosis based on immunoblotting, parameters of sensitivity, specificity, and positive and negative predictive values were calculated as previously described (Galen, 1980).

RESULTS

Construction and immunoscreening of *A. cantonensis* cDNA library

Total RNA was observed to be intact by electrophoresis in 1.2% agarose/ethidium bromide gel as evidenced by a single stained smear ranging from 200 to 6,000 bp in size. From this RNA preparation a primary *A. cantonensis* cDNA library was successfully constructed with a titer of 4.03 x 10^5 PFU/ml and 97.3% of the library producing white plaques. The titer of the amplified library was 3.63 x 10^8 PFU/ml. In a primary immunoscreening, approximately 200,000 plaques were screened with pre-absorbed pooled positive human serum. This yielded 67 positive clones. After secondary and tertiary screenings, 44 positive clones were identified as indicated by strong positive antibody staining (purple coloration). One clone, named fAC22, was subjected to *in vivo* excision phagemid for further sequence analysis.

Analysis of the cDNA insert and amino acid sequence

The cDNA insert was removed by an *in vivo* excision protocol. The pBK-CMV phagemid was isolated from *E. coli* XLOLR strain. The plasmid was double digested with *Kpn*I and *Sac*I restriction enzymes and following electrophoresis, the insert cDNA from clone fAC22 was estimated to be ~3.0 kbp in size (Fig 1).

The entire cDNA sequence (2,736 bp) and deduced amino acid sequences of clone fAC22 were determined (Fig 2). It consisted of a 57 bp 5′ untranslated region (5′ UTR), ORF of 2,136 bp encoding 712 amino acids, 516 bp of 3′ UTR and 24 bp of poly(A). A putative polyadenylation signal sequence (ATTAAA) was located 18 bp upstream of the poly(A) sequence. The predicted molecular weight and isoelectric point was 80.5 kDa and 5.8, respectively. After a BLASTP search, a putative Small MutS related (Smr) domain-containing peptide segment was found at the N-terminus of the protein. Its amino acid sequence exhibited 24% similarity/homology to the Smr-domain containing protein of the nematode parasite, *Brugia malayi* (accession no. XP_001893874) (Fig 3). In addition, the deduced amino acid sequence showed similarity (24-36% homology) to several other proteins containing Smr domains, including *Aspergillus flavus* (accession no. EED56380), *Talaromyces stipitatus* (accession no. EED23317), *Penicillium marneffei* (accession no. XP_0021 44766) and *Neosartorya fischeri* (accession no. XP_001257903).

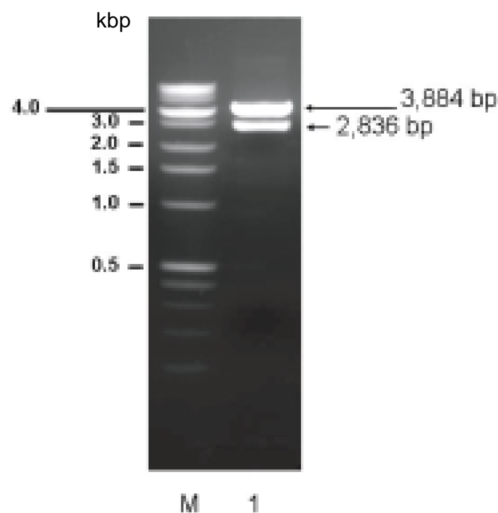

Fig 1–Double digestion of recombinant pBK-CMV phagemid (clone fAC22) by *Kpn*I and *Sac*I restriction enzymes. Lane M shows DNA marker. Lane 1 shows insertion cDNA (2,836 bp) excised from pBK-CMV phagemid (3,884 bp).

Expression, purification and immunocharacterization of the recombinant fusion protein

The fAC22 ORF was amplified by PCR and cloned into pET-46 Ek/LIC expression vector. The fAC22 recombinant protein fused with a His6-tag was expressed in *E. coli* [BL21(DE3) strain] after induction with 1mM IPTG. Its size on 13% SDS-PAGE gel stained with Coomassie brilliant blue was approximately 81 kDa and the incubation period yielding optimum expression was 3 hours (Fig 4A). The recombinant His6-tagged fusion protein was heterologously expressed in an insoluble form, and therefore had to be purified under denaturing conditions. Purified recombinant protein was eluted from the Ni-NTA column with 100 mM imidazole as indicated by a single band 13% SDS-PAGE gel stained with Coomassie brilliant blue (Fig 4B) and silver (Fig 4C). The antigenic property of this fusion protein was confirmed by its strong reactivity with pooled positive serum (Fig 4D) compared to the pooled negative serum control (Fig 4E).

```
1     ggc acg agg ggc atc aaa aca gta gtc gtg gac aac acc aat att   45

46    ttc atc cat cac atg caa cag tat gct ttt cat gct gtt aga tac   90
                       M   Q   Q   Y   A   F   H   A   V   R   Y    11

91    tgc tac gaa att ttt gtc gtg gaa cca gag acc acg tgg aaa tac   135
12     C   Y   E   I   F   V   V   E   P   E   T   T   W   K   Y    26

136   aca gtt aga gag tgc ttc aga cgc aac ata cat ggg ata gaa ata   180
27     T   V   R   E   C   F   R   R   N   I   H   G   I   E   I    41

181   tgg aaa att gat tcg atg atg cag tcg ttg ctt gat caa ggg cga   225
42     W   K   I   D   S   M   M   Q   S   L   L   D   Q   G   R    56

226   ccg act tta tct tgt ttg gta ggt gga gac cgt gaa att agg ttg   270
57     P   T   L   S   C   L   V   G   G   D   R   E   I   R   L    71

271   gtg cca cca ctt gaa ttt tcg gaa aaa gag cac gac acg gaa ttt   315
72     V   P   P   L   E   F   S   E   K   E   H   D   T   E   F    86

316   cac tgt ttg aag cta aga agt ctt gtg cta gat act tca cca aat   360
87     H   C   L   K   L   R   S   L   V   L   D   T   S   P   N    101

361   cgc gat gaa atc ttg cat tta aag gat gat gat ggc cct tct cca   405
102    R   D   E   I   L   H   L   K   D   D   D   G   P   S   P    116

406   gag gtc tct att gct gat cct cct ctt gag ctt cct tat ccg aat   450
117    E   V   S   I   A   D   P   P   L   E   L   P   Y   P   N    131

451   gct gca gtt gac cgt tcg tca ttt ctg ctg cct tca gtt gcg ccg   495
132    A   A   V   D   R   S   S   F   L   L   P   S   V   A   P    146

496   act cct gct gct ttt tcg tta gct gtc gat cgt cgt tca cat att   540
147    T   P   A   A   F   S   L   A   V   D   R   R   S   H   I    161

541   aga ata tta gaa gtt cga gaa gta gcg act caa act aat gag atc   585
162    R   I   L   E   V   R   E   V   A   T   Q   T   N   E   I    176

586   gtc gta aca tta gtc tgt gct ggc gtc tat tgt ccg ttt gat gac   630
177    V   V   T   L   V   C   A   G   V   Y   C   P   F   D   D    191

631   gtt tca gaa ggt gtt cca cat agc gtg gaa ata tca aaa gag ttg   675
192    V   S   E   G   V   P   H   S   V   E   I   S   K   E   L    206

676   aag tca caa atg aag gat cgt gta gtc aga atg gag aac att cca   720
207    K   S   Q   M   K   D   R   V   V   R   M   E   N   I   P    221

721   cag ttt tct gag ttg gac gtg ttg gtt gca gtc ttt ccg tac gag   765
222    Q   F   S   E   L   D   V   L   V   A   V   F   P   Y   E    236

766   gag ctt ggg aac ctt tct cat tat tat cag atg ctg ggt ttg gaa   810
237    E   L   G   N   L   S   H   Y   Y   Q   M   L   G   L   E    251

811   gaa tgc atc agg tta ttc gtg gaa ttg gga gct tat gta gac tgg   855
252    E   C   I   R   L   F   V   E   L   G   A   Y   V   D   W    266

856   atg gca cag gtt acg gag aaa ccg ttc gta gag gag ctt gca gca   900
267    M   A   Q   V   T   E   K   P   F   V   E   E   L   A   A    281

901   tct gaa ctc agt ggt act cta ctt cac gct cca gta ccc agg act   945
282    S   E   L   S   G   T   L   L   H   A   P   V   P   R   T    296

946   gac tgg gaa aga ata gca gaa cag gag agt atg aaa gaa tat gtg   990
297    D   W   E   R   I   A   E   Q   E   S   M   K   E   Y   V    311

991   gta act gag cca gtt tat gaa gtt ggt tct tca cga agt gtg gat   1035
312    V   T   E   P   V   Y   E   V   G   S   S   R   S   V   D    326
```

1036 tat tcg gga aat gag ata aca gta aca ctt ggc gtt gat ttg ctg 1080
327 Y S G N E I T V T L G V D L L 341

1081 caa aag atc agt tta ttg ttt ggt gaa gga atc att gtt gaa gaa 1125
342 Q K I S L L F G E G I I V E E 356

1126 gaa tgt agt gta cgt ttg cca ctc tgg ctt cta aag cag ctg tat 1170
357 E C S V R L P L W L L K Q L Y 371

1171 ctt ttt tgg caa aat agt gga act tca ttt cca agc aat aga gaa 1215
372 L F W Q N S G T S F P S N R E 386

1216 gcg ttg aac gat gca gaa ata gct gca gct ctt caa gag gaa gag 1260
387 A L N D A E I A A A L Q E E E 401

1261 gat gcg att gct tcg gcc agt ttt aaa gct agt att cct att gga 1305
402 D A I A S A S F K A S I P I G 416

1306 aga cca gca tcc acc gcc gtc ttg gta cca aac tgg tcg cat ggt 1350
417 R P A S T A V L V P N W S H G 431

1351 ggc aaa tct cct gat cca caa gag cgg caa cat ggt ggt gat gac 1395
432 G K S P D P Q E R Q H G G D D 446

1396 ctg gag cag act ttg gct agg atg acc tcg gga ctc cag aga acc 1440
447 L E Q T L A R M T S G L Q R T 461

1441 aca atg gtt aaa cca gca aga ctc caa aaa att gga aat ttt gct 1485
462 T M V K P A R L Q K I G N F A 476

1486 tgg gcc tgc gca acc aat gag gac ggg cca tcc aag tac cta agt 1530
477 W A C A T N E D G P S K Y L S 491

1531 agg tgt tgg att ttt gtc cgc caa cgg tat att tta tcc cat agg 1575
492 R C W I F V R Q R Y I L S H R 506

1576 tat gat tct act gct aca cgc gct acc ctt cat atc atg ttg aat 1620
507 Y D S T A T R A T L H I M L N 521

1621 cct gaa gca aat agt gtg gag cat tgt cgt aac tca cca gca acg 1665
522 P E A N S V E H C R N S P A T 536

1666 act tca gat acg gtt gta ccc gca caa aaa ccc agc ttt aag cgt 1710
537 T S D T V V P A Q K P S F K R 551

1711 cgg ttt gaa gca aac ctt cct gat gcc caa gaa aaa gca cgt caa 1755
552 R F E A N L P D A Q E K A R Q 566

1756 tat cag aaa caa gct aat gag ttt gct gag aag aaa ttt gca gaa 1800
567 Y Q K Q A N E F A E K K F A E 581

1801 atg cga aaa gtt gag aga tac ctt cag tgc cgt aac ttg ctg gca 1845
582 M R K V E R Y L Q C R N L L A 596

1846 gcg gac tac ttc cgc caa gtg gca cga gaa cat tct ctt cgt gaa 1890
597 A D Y F R Q V A R E H S L R E 611

1891 aag aat ctt cgt aag cag gcc ggt gat att atc ata aaa gca aat 1935
612 K N L R K Q A G D I I I K A N 626

1936 gaa gat tct acc gta ctt gac ctt cat ctt ctt agc cag aag gac 1980
627 E D **S T V L D L H L L S Q K D** 641

1981 gct att atg ttg cta aaa gag cgt ctt tcc gcg ctt gat cgt ccc 2025
642 **A I M L L K E R L S A L D R P** 656

```
2026  gtt tcc atg agg cat ggt cgg tct agt cag cgt ctc cat gtc att   2070
657    V   S   M   R   H   G   R   S   S   Q   R   L   H   V   I    671

2071  acg ggt tac ggt aga agt act ggt gga aga tct gtg ata aaa cca   2115
672    T   G   Y   G   R   S   T   G   G   R   S   V   I   K   P    686

2116  gca gtc gaa ttc tac ctg aaa agg aaa gga tat atc tat tca ttt   2160
687    A   V   E   F   Y   L   K   R   K   G   Y   I   Y   S   F    701

2161  gca aat atg ggt gaa gtt gtg gtt caa tgc aaa tag cgc att cta   2205
702    A   N   M   G   E   V   V   V   Q   C   K   *

2206  tct gtt ctt cga aag ctg att cac aaa tta tac att aga att tgt   2250

2251  gca ggg agt cct aga cac tgt ttg ttt agc aag ata ggt atg aca   2295

2296  agg cct ttc tgt gta ttt ctg agt ctt cac tgc aaa ttt gtg att   2340

2341  tgt tat tgt tct ttg aat aca caa ctc tgt ccc ttt ctt tcg ttg   2385

2386  att ttt gag tgt ttt tga acg tgc ctg ttt att cgt tgt tga ata   2430

2431  gcc tcc cag cat caa aaa ttt atc ctg tga gaa tga ggc ttt ttt   2475

2476  tac ctc agt tta aga gtt ctc gga ttt cgg gtt tgt cga tat caa   2520

2521  tgc act tag ctt tct tct ttt tta tat gta tga gat ttc tct ttg   2565

2566  aac gtg cat att aat cat aag act act gca gat aga tgg tta act   2610

2611  tta gcc aat agg aag atc cgt agt ttt tct ttt act gta gtt aaa   2655

2656  tgt ttc cta tac aca caa ttg tat tta ctt ggg aac act att aaa   2700

2701  atg ttt tgt tat aaa aaa aaa aaa aaa aaa aaa aaa   2736
```

Fig 2–Nucleotide and deduced amino acid sequence of clone fAC22. The polyadenylation signal sequence (attaaa) is underlined. An asterisk (*) indicates stop codon. The highlighted region represents the amino acids in Smr domain.

Application of recombinant fusion protein for immunoblotting

Western immunoblot analysis employing the purified 81-kDa recombinant fusion protein was used to determine the protein's efficacy in detecting *A. cantonensis* infections using individual sera from 'clean' patients or those harboring various parasitic infections, including angiostrongyliasis (Fig 5). All proven angiostrongyliasis sera (4 cases) and 53/57 (93.5%) clinically-suspected angiostrongyliasis sera reacted with the fusion protein. In contrast, none of the 31 sera from the uninfected control group exhibited seroreactivity with this fusion protein. However, cross-reactivity of serum antibodies to the 81 kDa protein was detected in a number of cases of other helminth infections including gnathostomiasis (2/12), bancroftian filariasis (3/8), ascariasis (4/6), echinococcosis (3/5), paragonimiasis (1/10) and opisthorchiasis (2/9). The calculated sensitivity, specificity, and positive and negative predictive value was 93.5, 91.5, 79.0 and 97.5%, respectively.

```
Smr_domain-containing_protein_      MVTPSIHEQCWEPTTNLEHIRKCIQNGHHIMVIMRGIPGSGKSYLASDLI
Clone_fAC22                         ---------------------------------MQQYAFHAVRYCYEIFV
                                                                     *         *

Smr_domain-containing_protein_      SGTNGAVFNTDKYFVQNGVYQFDPTKLDEYHQKNWKEAKDAIQQGIKPII
Clone_fAC22                         VEPETTWKYTVRECFRRNIHGIEIWKIDSMMQS-----------------
                                             *               * *   *

Smr_domain-containing_protein_      IDNTNIFVTHMKPYINLAVKNLYEIYFVEPETEWKKNAKECARRNAHSVP
Clone_fAC22                         ------LLDQGRPTLSCLVGGDREIRLVPPLEFSEK---------EHDTE
                                                *     *    **  * *     *          *

Smr_domain-containing_protein_      EEKIAYMAECFEKVSLSDVIKPTQLRTVP-PLVDINDEDDTYNLLLSKLD
Clone_fAC22                         FHCLKLRSLVLDTSPNRDEILHLKDDDGPSPEVSIADPPLELPYPNAAVD
                                                     * *        * * * * *            *

Smr_domain-containing_protein_      SLPDSLLGKDATDNKKSSEQLISLIPHQIPKNLRTFGCQTSDLIRVLDLS
Clone_fAC22                         --RSSFLLPSVAPTPAAFSLAVDRRSHIRILEVREVATQTNEIVVTLVCA
                                        * *                   *      *    **      *

Smr_domain-containing_protein_      NPSSSVNDEFVCETVCDAPDFXYKKKMKVKATQAGDGNILSDIELLIAFF
Clone_fAC22                         GVYCPFDDVSEGVPHSVEISKELKSQMKDRVVRMENIPQFSELDVLVAVF
                                           *               *  **            *    * * *

Smr_domain-containing_protein_      PDEKPSDLSHILEIAGLKNAMTLLKEMNAHMDICTPVGKNKNIDAESLSQ
Clone_fAC22                         PYEELGNLSHYYQMLGLEECIRLFVELGAYVDWMAQVTEKPFVEELAASE
                                    * *    ***     **     *  *  *  *    *           *

Smr_domain-containing_protein_      TYYWWDKSECEQVDNNIDNNSSPAFVPNFELNRPISEELAPCTYMQCCDP
Clone_fAC22                         LSGTLLHAPVPRTD--WERIAEQESMKEYVVTEPVYEVGSSRSVDYSGNE
                                                 *                   *  *

Smr_domain-containing_protein_      EPVPSGYCRMQISVDMMEQLTQLFGDAESNTFLKTYVDLPIYLWRQIYFH
Clone_fAC22                         ITVTLG-------VDLLQKISLLFG-EGIIVEEECSVRLPLWLLKQLYLF
                                      *  *       **       ***           * **  *  * *

Smr_domain-containing_protein_      WQG-ISTTTTEVAVAVDNAFGSENFDFSALVSSDEELARILQGHELASDE
Clone_fAC22                         WQNSGTSFPSNREALNDAEIAAALQEEEDAIASASFKASIPIGRPASTAV
                                    **              *               *    * *  *

Smr_domain-containing_protein_      FLENGKHMSIAERLQLSALMKDYSGVDRERIAECFRDNKFSAEATRNTLD
Clone_fAC22                         LVPNWSHGGKSPDPQERQHGGDDLEQTLARMTSGLQRTTMVKPARLQKIG
                                       *  *       *      *       *             *

Smr_domain-containing_protein_      LFVNGSENIQTVP-------------------------------------
Clone_fAC22                         NFAWACATNEDGPSKYLSRCWIFVRQRYILSHRYDSTATRATLHIMLNPE
                                     *          *

Smr_domain-containing_protein_      ANPSRIEYRPNQSGNCSVXAASSYEESSVLKPDLELAHKEAFELREQAEW
Clone_fAC22                         ANSVEHCRNSPATTSDTVVPAQKPSFKRRFEANLPDAQEKARQYQKQANE
                                    **               *  *             *  *   *    **

Smr_domain-containing_protein_      YDKQKHELLLRAN---NHRDFGAKMHYFAEAQKLGKKAKDCVAELNERLI
Clone_fAC22                         FAEKKFAEMRKVERYLQCRNLLAADYFRQVAREHSLREKNLRKQAGDIII
                                        *             *   *       *       *          *

Smr_domain-containing_protein_      KANTSTLFIDLHYMNVQSALKLLKAKLNAADRPPEFRRGRSRKKLVVLTG
Clone_fAC22                         KANEDSTVLDLHLLSQKDAIMLLKERLSALDRPVSMRHGRSSQRLHVITG
                                    ***      ***     *  ***    * * ***   * ***   * * **

Smr_domain-containing_protein_      YGKLSDGQAKIKPAVIQWLEQCGYEYYNTSNKGELIVECK
Clone_fAC22                         YGRSTGGRSVIKPAVEFYLKRKGYIYS-FANMGEVVVQCK
                                    **    *   *****   *   ** *    * **  * **
```

Fig 3–Pairwise alignment of deduced amino acid sequence of Smr domain-containing protein of *B. malayi* (accession no. XP_001893874) and deduced amino acid sequence of clone fAC22 by ClustalW. The amino acids that are identical to clone fAC22 are indicated by asterisk and the gap are represented by (--).

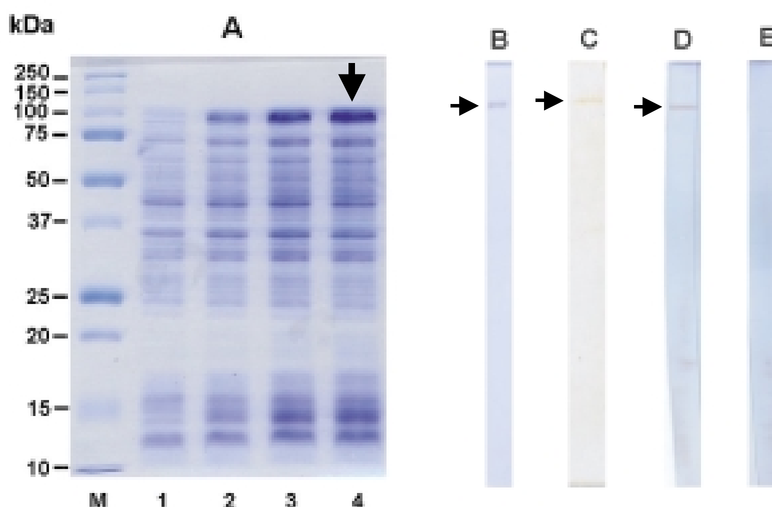

Fig 4–Expression, purification and immuno-characterization of 81-kDa recombinant fusion protein. (A) SDS-PAGE analysis; lane M, protein marker, lane 1, uninduced whole cell lysate, lane 2, 1 hour induced whole cell lysate, lane 3, 2 hours induced whole cell lysate, lane 4, 3 hours induced whole cell lysate. (B) Ni-NTA column-purified 81-kDa purified recombinant fusion protein stained with Coomassie brilliant blue. (C) Isolated 81 kDa recombinant protein visualized by silver staining. (D) Reactivity of the 81-kDa recombinant fusion protein with pooled sera from *A. cantonensis*-positive patients. (E) 81-kDa protein reactivity with pooled negative sera. Arrow indicates 81-kDa recombinant protein.

DISCUSSION

Native antigens of *Angiostrongylus cantonensis* worms have been shown to be useful in the detection of antibodies associated with angiostrongyliasis (Eamsobhana *et al*, 2001, 2004; Intapan *et al*, 2003). However, although many immunological tests have demonstrated satisfactory to excellent sensitivities, there still remains considerable room for improvement of the detection rates (Maleewong *et al*, 2001; Intapan *et al*, 2003). In addition, labor, cost and requirement of animals to generate native antigens remain barriers to develop large scale and consistent assay tests.

In the present study, a recombinant protein antigen, identified and cloned from cDNA library of female *A. cantonensis* worm was successfully expressed in *E. coli* and purified as a potential antigenic assay product. The clone fAC22 was identified by immunoscreening of the cDNA expression library and, following sequence analysis, was identified as an 81-kDa Smr domain-containing protein of *A. cantonensis*. Silver staining of the His6-tagged 81-kDa *A. cantonensis* protein eluted as a single band demonstrated high purity of the recombinant protein preparation, thus providing antigenic material for follow-up serological tests.

The antigenicity of this novel fusion protein was evaluated with sera where infection by *A. cantonensis* worms had been proven, and in cases where the infection criteria were less direct. Using Western blot analysis, the 81-kDa protein specifically reacted with serum antibodies from 57 of the 61 (93.5%) angiostrongyliasis cases,

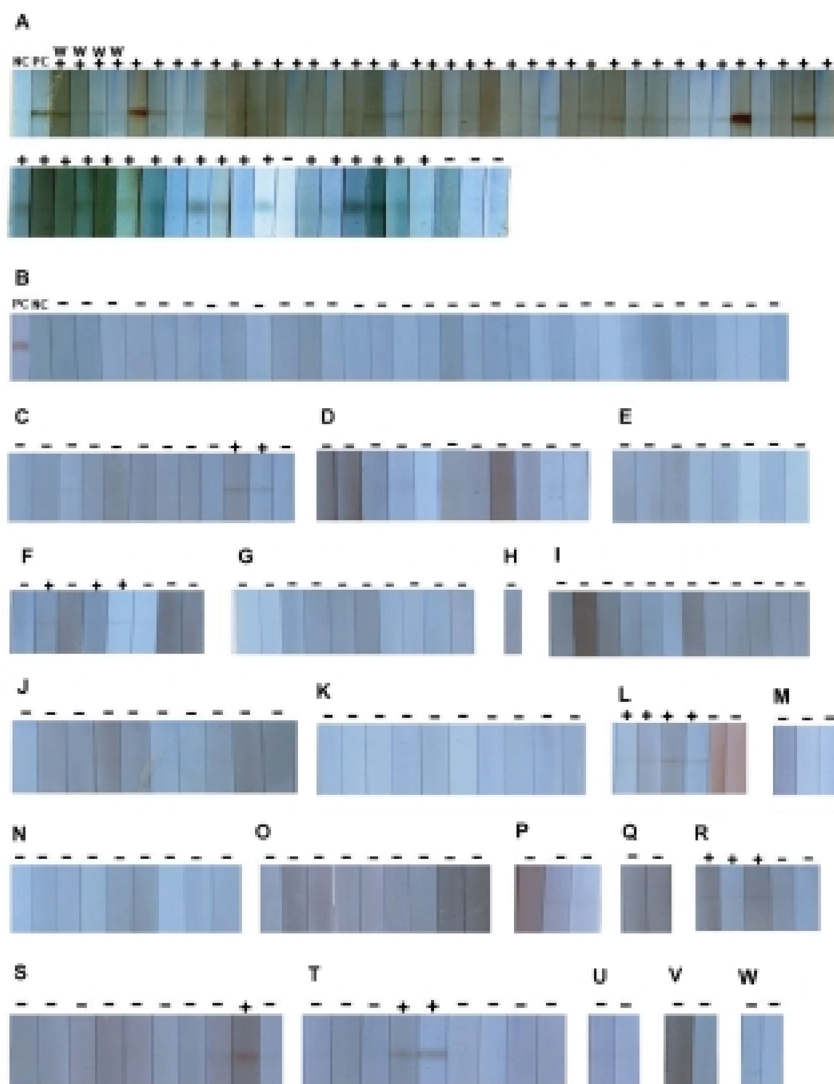

Fig 5–Detection of angiostrongyliasis infection using *A. cantonensis* 81-kDa recombinant fusion protein. (A) Immunoblot reactivity of the recombinant protein with individual sera from patients with proven angiostrongyliasis (n = 4), clinically-suspected angiostrongyliasis (n = 57) and other parasitic helminth infections (n = 144). "NC", "PC" and "w" indicate pooled negative control, pooled positive control and proven angiostrongyliasis, respectively. "-" and "+" indicate negative and positive result, respectively. (B) Negative 81-kDa protein reactivity with sera from healthy control people (n = 31). Immunoblot reactivity with individual sera from patients with proven (C) gnathostomiasis (n = 12), (D) trichinellosis (n =11), (E) brugian filariasis (n = 8), (F) bancroftian filariasis (n = 8), (G) toxocariasis (n = 10), (H) dilofilariasis (n = 1), (I) strongyloidiasis (n = 12), (J) trichuriasis (n =10), (K) hookworm infection (n = 10), (L) ascariasis (n = 6), (M) capillariasis (n = 3), (N) neurocysticercosis (n = 9), (O) taeniasis saginata (n = 9), (P) hymenolepiasis (n = 3), (Q) sparganosis (n = 2), (R) echinococcosis (n =5), (S) paragonimiasis heterotremus (n = 10), (T) opistorchiasis (n =9), (U) schistosomiasis (n = 2), (V) fascioliasis (n = 2), and (W) haplorchiasis (n = 2).

which included samples where actual worm infections were confirmed. However, in several cases (36) weak reactions were seen including one of those having a proven infection. Assuming that the recombinant protein possesses multiple antigenic epitopes, differences in reaction intensities likely are due to differences in either quantitative or qualitative reactivities between the fusion protein and antibodies present within the sera of presumptive angiostrongyliasis patients. Importantly, no detectable reactions were observed with any of the negative control sera (31 samples).

The specificity of the recombinant protein also was evaluated with serum antibodies from patients with heterologous infections. The 81 kDa antigen did not crossreact with sera of patients infected with 14 different parasitic infections, while sera from other infections (gnathostomiasis, bancroftian filariasis, ascariasis, echinococcosis, paragonimiasis and opisthorchiasis) yielded varying numbers of positive reactions. Such false positives may have resulted from previous or concurrent *A. cantonensis* infection since cross reactions were observed in only 1-3 cases of those diseases. Of particular note, the Smr domain-containing 81-kDa protein was immunogenic to angiostrongyliasis, but was not to brugian filariasis, as evidenced by the fact that the recombinant protein did not react with antibodies from brugian filariasis patients. This result may be due to the poor homology (approximately 24%) in amino acids sequence of the 81-kDa protein to the *B. malayi* homolog, and thus is consistent with the reaction specificity of this fusion protein. A question that remains, however, is whether the observed cross-reactivity with other helminth infections may be due to the presence of *A. cantonensis* Smr-like protein homolog in these worm species.

Native proteins isolated from *A. cantonensis* worm extracts have been used as potential antigenic targets in immunoblot-type diagnosis of human angiostrongyliasis. A 31-kDa protein demonstrated highest sensitivity (100%) and specificity (100%) (Eamsobhana *et al*, 2004), but specificity was determined by comparing cross-reactivity using sera representing only 6 other parasitic diseases. Nuamtanong (1996), using the same 31 kDa antigen extract, demonstrated 69.3% sensitivity and 82.4% specificity against serum samples representing 13 different parasitic infections. In this case, the 31-kDa extract showed significant cross-reactivity with sera from patients infected with trichinellosis (8/10), trichuriasis (5/10), and opisthorchiasis (8/10). In addition, 29 kDa antigen from adult worm extracts demonstrated low specificity (47.1%), cross-reacting with most other parasitic infections that were tested (Nuamtanong, 1996). A similar low sensitivity (55.6%) was shown for a 29 kDa native protein from young adult female worms (Maleewong *et al*, 2001). More recently Intapan *et al* (2003), using the 29 kDa antigen of young adult worm and the IgG4 antibody isotype of proven angiostrongyliasis sera, reported 75% sensitivity and 95% specificity, and 85.7% positive and 90.4% negative predictive values. It may be concluded that these assay results involving these two antigenic components should be interpreted with some caution due to the apparent variability in sensitivity, specificity, and their positive and negative predictive values. By comparison, the *A. cantonensis* 81 kDa recombinant protein has demonstrated superior sensitivity (93.5%) and specificity (91.5%) in immunoblot analysis in differentiating active and clinically-presumptive *A. cantonensis* infections.

In summary, a novel 81 kDa protein containing a Smr-like domain was identified by immunoscreening of *A. cantonensis* female adult worm cDNA expression library and was purified from heterologous expression system His-tagged recombinant protein. Western immunoblot analysis indicated its suitability as an antigen for serodiagnosis of human angiostrongyliasis due of its high sensitivity, specificity and negative and positive predictive values. This represents the first recombinant *A. cantonensis* antigenic protein to be produced with demonstrated application to the serodiagnosis of human angiostrongyliasis.

ACKNOWLEDGEMENTS

The authors thank staff of the Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, Thailand and the Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, USA, for providing laboratory facilities and assistance in laboratory techniques. We also thank Dr Norman Scholfield for assistance in editing the manuscript. This research was supported by the Commission on Higher Education, Ministry of Education, Thailand.

REFERENCES

Akao N, Kondo K, Ohyama TA, Chen ER, Sano M. Antigens of adult female worm of *Angiostrongylus cantonensis* recognized by infected humans. *Jpn J Parasitol* 1992; 41: 225-31.

Chen SN. Enzyme-linked immunosorbent assay (ELISA) for detection of antibodies to *Angiostrongylus cantonensis*. *Trans R Soc Trop Med Hyg* 1986; 80: 398-405.

Chen XG, Li H, Lun ZR. Angiostrongyliasis, mainland China. *Emerg Infect Dis* 2005; 11: 1645-7.

Cross JH, Chi JCH. ELISA for the detection of *Angiostrongylus cantonensis* antibodies in patients with eosinophilic meningitis. *Southeast Asian J Trop Med Public Health* 1982; 13: 73-6.

Eamsobhana P, Mak JW, Yong HS. Detection of circulating antigens of *Parastrongylus cantonensis* in human sera by sandwich ELISA with specific monoclonal antibody. *Southeast Asian J Trop Med Public Health* 1995; 26: 712-5.

Eamsobhana P, Mark JW, Yong HS. Detection of circulating antigens of *Parastrongylus cantonensis* in human sera by dot-blot ELISA and sandwich ELISA using monoclonal antibody. *Southeast Asian J Trop Med Public Health* 1997; 28(suppl): 139-42.

Eamsobhana P, Yoolek A, Punthuprapasa P, Suvouttho S. A dot-blot ELISA comparable to immunoblot for the specific diagnosis of human parastrongyliasis. *J Helminthol* 2004; 78: 287-91.

Eamsobhana P, Yoolek A, Suvouttho S, Suvouttho S. Purification of a specific immunodiagnostic *Parastrongylus cantonensis* antigen by electroelution from SDS-polyacrylamide gels. *Southeast Asian J Trop Med Public Health* 2001; 23: 308-13.

Galen RS. Predictive value and efficiency of laboratory testing. *Pediatr Clin North Am* 1980; 27: 816-9.

Garcia LS. Diagnostic medical parasitology. 5th ed. Wahington, DC: ASM Press, 2007.

Intapan PM, Maleewong W, Sawanyawisuth K, Chotmongkol V. Evaluation of human IgG subclass antibodies in the serodiagnosis of angiostrongyliasis. *Parasitol Res* 2003; 89: 425-9.

Maleewong W, Sombatsawat P, Intapan PM, Wongkham C, Chotmongkol V. Immunoblot evaluation of the specificity of the 29-kDa antigen from young adult female worms *Angiostrongylus cantonensis* for immunodiagnosis of human angiostrogyliasis. *Asian Pac J Allergy Immunol* 2001; 19: 267-73.

Nuamtanong S. The evaluation of the 29 and 31 kDa antigents in female *Angiostrongylus cantonensis* for serodiagnosis of human angiostrongyliasis. *Southeast Asian J Trop Med Public Health* 1996; 27: 291-6.

Sawanyawisuth K, Kitthaweesin K, Limpawattana P, *et al.* Intraocular angiostrongyliasis: clinical finding, treatment and outcome. *Trans R Soc Trop Med Hyg* 2007; 101: 497-501.

Sinawat S, Sanguansak T, Angkawinijwong T, *et al*. Ocular angiostrongyliasis: clinical study of three cases. *Eye* 2008; 1-3.

Slom TJ, Cortese MM, Gerber SI, *et al.* An outbreak of eosinophilic meningitis caused by *Angiostrongylus cantonensis* in travelers returning from the Caribbean. *N Engl J Med* 2002; 346: 668-75.

Tsai HC, Liu YC, Kunin CM, *et al.* Eosinophilic meningitis caused by *Angiostrongylus cantonensis*: report of 17 cases. *Am J Med* 2001; 111: 109-14.

Wang QP, Lai DH, Zhu XQ, Chen XG, Lun ZR. Human angiostrongyliasis. *Lancet Infect Dis* 2008; 8: 621-30.

Welch JS, Dobson C, Campbell GR. Immunodiagnosis and seroepidemiology of *Angiostrongylus cantonensis*. *Trans R Soc Trop Med Hyg* 1980; 74:614-23.

Yen CM, Chen ER. Detection of antibodies to *Angiostrongylus cantonensis* in serum and cerebrospinal fluid of patients with eosinophilic meningitis. *Int J Parasitol* 1991; 21: 17-21.