Identification of *Parastrongylus cantonensis* antigens for use in immunodiagnosis

Eamsobhana P, Mak JW [1] and Yong HS [2] *Department of Medical Sciences, Ministry of Public Health, Bangkok, Thailand; [1] Institute for Medical Research, Kuala Lumpur; [2] Department of Zoology, University of Malaya, Malaysia*

Abstract

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis revealed that the somatic extracts of *Parastrongylus cantonensis* were highly heterogeneous, with at least 4 and 25 components of the larval and adult worm antigens respectively being immunogenic. Sera from two patients with parasitologically confirmed parastrongyliasis, 8 patients with presumptive parastrongyliasis, 12 patients with various other parasitic infections and two healthy adults were analysed for their reactivities against the somatic extracts of *P. cantonensis* third-stage larvae and adult worms. Extensive cross-reaction of *P. cantonensis* antigens with the sera from patients with other parasitic infections was observed. However, a 31 kDa component present in the larval and adult worm antigens of *P. cantonensis* reacted with the sera of all the parasitologically proven patients and 4 out of the 8 presumptive patients, but not with those of other parasitic infections or healthy individuals. This 31 kDa *P. cantonensis* antigen is a potential candidate for the specific immunodiagnosis of human parastrongyliasis.

Key Words: *Parastrongylus cantonensis*; parasite antigens; immunodiagnosis

Introduction

Human infection with the rat lung-worm, *Angiostrongylus cantonensis*, now known as *Parastrongylus cantonensis* (Uberlaker, 1986), is still an important public health problem in Southeast Asia and the Pacific islands (Cross, 1987). Definite diagnosis of the infection can only be made if the worm is recovered from the patient but this is very rare.

Using several immunological methods, antibodies to *P. cantonensis* somatic and metabolic antigens have been detected in the serum and cerebrospinal fluid of patients with eosinophilic meningitis and meningoencephalitis (Tharavanij, 1979). However, these antigens cross-reacted with sera from patients with other parasitic infections. Cross-reaction is most likely due to the complex antigenic mosaic of the parasite (Sirisinha *et al.*, 1977). Consequently, there is a need to develop specific immunodiagnostic assays for this parasitic infection. In this srudy, identification of the *P. cantonensis* specific antigen was determined by comparing the serum reactivities between patients wirh parastrongyliasis (angiostrongyliasis) and those with other parasitic infections using rhe immunoblotting technique.

Materials and Methods

Antigen preparation

The Thailand strain of *Parastrongylus cantonensis* was used in the present study. Third-stage larvae were collected from snails (*Biomphalaria glabrata*) infected with first-stage larvae approximately 3 weeks earlier. Adult worms were obtained from the pulmonary arteries of albino rats that were infected with *P. cantonensis* infective larvae for at least 6 weeks.

Male and female adult worms and third-stage larvae of *P. cantonensis* were washed and then homogenised in a small volume of normal saline with a glass tissue grinder. The suspension was then sonicated and left overnight at 4°C to allow elution of antigens. Soluble antigens were obtained as the supernatant after centrifugation at 4,000 rpm at 4°C for 15 minures. Protein content of the extracts was determined using a protein assay kit II (Bio-Rad Labs., U.S.A.).

Sera

Human sera were obtained from two patients with parasitologically confirmed parastrongyliasis (one with cerebral parastrongyliasis from whom a fourth-stage larva was recovered from the cerebrospinal fluid; another had ocular parastrongyliasis from whom an immature male was recovered from anterior chamber of the eye) and 8 patients with a presumptive diagnosis of patastrongyliasis based on history of exposure to infection and clinical symptoms, as well as having high antibody titres as detected with an ELISA.

Antigen cross-reactivity studies were also carried out on sera from 12 patients with other parasitic infections (1 patient each with parasitologically confirmed and clinically diagnosed gnathostomiasis, 2 patients each with malaria, filariasis, paragonimiasis, cysticercosis and toxocariasis). In addition, control sera were obtained from one healthy Malaysian living in Kuala Lumpur and one healthy Thai living in Bangkok.

The confirmed gnathostomiasis serum was from a patient who had a *G. spinigerum* larva recovered from the skin, while the presumptive gnathostomiasis serum was from a patient with intermittent cutaneous migratory swelling. All patients with paragonimiasis, filariasis

and malaria were diagnosed parasitologically. The two cases of toxocariasis were serologically positive on ELISA using excretory-secretory antigens of the second-stage larvae of *T. canis*, while the two patients with cysticercosis were diagnosed pathologically as subcutaneous cysticerci.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

The adult worm and third-stage larval antigens of *P. cantonensis* were separated on SDS-polyacrylamide slab gels using the discontinuous system of Laemmli (1970). A 4% acrylamide stacking gel and 12% acrylamide separating gel were used. Approximately 10 (g protein samples were boiled at 100°C for 5 minutes in Laemmli sample buffer before loading onto the gel. The separated protein bands were visualized by staining with Coomassie brilliant blue R. The molecular weights (MWs) of the separated proteins of *P. cantonensis* were estimated by comparing their electrophoretic mobilities with those of known standard molecular weight markers (Sigma) after electrophoresis in the same gel. For transblotting, the SDS-PAGE separated antigens from the gel were electroblotted onto a 0.45 µm nitrocellulose membrane (Toyo Roshi Co. Ltd.) by the method of Towbin et al. (1979). The non-specific binding sites on the membrane were blocked by soaking in a solution of 5% skimmed milk in PBS, pH 7.4 for one hour. The membrane was then incubated with test serum, which was diluted 1: 200 in 1% BSA in PBS, pH 7.4 overnight at 4°C. After washing thoroughly the membrane was then reacted with horseradish peroxidase conjugated rabbit anti-human immunoglobulins (DAKO, Denmark) at a dilution of 1: 1,000 in PBS, pH 7.4 for one hour at room temperature followed by washing. The bound antigen-antibody complexes were visualized by addition of the chromogenic substrate solution containing 60 mg of 4-chloro-1-naphthol (Bio-Rad Labs.) in 20 ml of cold absolute methanol mixed with 60 µl of 30% H_2O_2 in 100 ml of PBS, pH 7.4. The blots were rinsed in distilled water, air-dried and photographed.

Results

The polypeptide patterns of the somatic extracts from infective third-stage larva, and adult male and female adult worms of *P. cantonensis* were highly complex and showed many common proteineous components after Coomassie brilliant blue staining (Fig. 1). Human sera with confirmed parastrongyliasis reacted strongly with the 31 kDa polypeptide of the third-stage larval, and male and female worm antigens of *P. cantonensis* (Fig.2). In addition, a less reactive 32 kDa band was almost always recognized, especially in the adult male and femaleworm components. Approximately 18 polypeptide bands of adult worm extracts were recognized by the sera, with predominant bands having MW 205, 110, 80, 70.8, 64, 60, 53, 48, 43, 39.5, 31 and 29 kDa. The

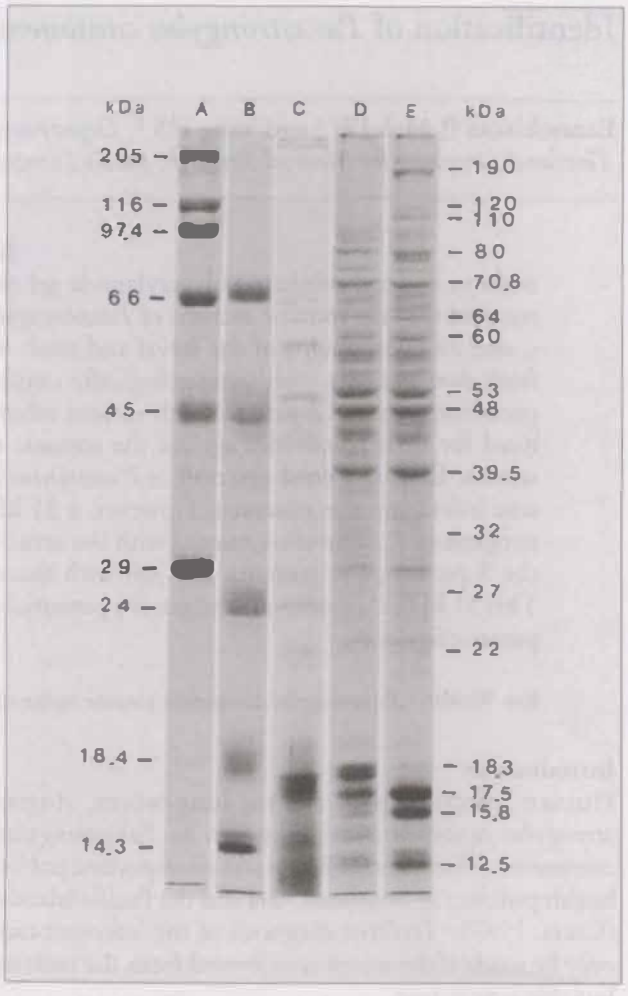

Fig. 1. SDS-PAGE patterns of *Parastrongylus cantonenesis* third-stage larva (C), and adult male (D) and female (E) worm antigens, stained with Coomassie blue. A, B: High and low molecular weight markers.

bands of 205 and 15.8 kDa were more intense in the male worm components. Polypeptides of third-stage larva recognized were at 80, 66, 32 and 31 kDa.

The reactivity patterns of the sera from 8 patients with presumptive parastrongyliasis were similar to those obtained with confirmed parastrongyliasis sera. Among the 8 sera tested, two of them reacted slightly with the larval and adult worm antigens. Only 4 sera reacted strongly with the 31 kDa component of the third-stage larval and adult worm antigens. Both the sera from normal healthy Thai and Malaysian did not react with *P. cantonensis* antigens.

The antigenic polypeptides of various *P. cantonensis* antigens recognized by sera of patients with gnathostomiasis, toxocariasis, filariasis, paragonimiasis, cysticercosis and malaria are shown in Figs. 3-5. Gnathostomiasis, toxocariasis and filariasis sera reacted with polypeptides ranging from 29 to 116 kDa but the cross-reactive antigenic components were variable. Sera from patients with paragonimiasis, cysticercosis and malaria showed weak reactivity against *P. cantonensis*

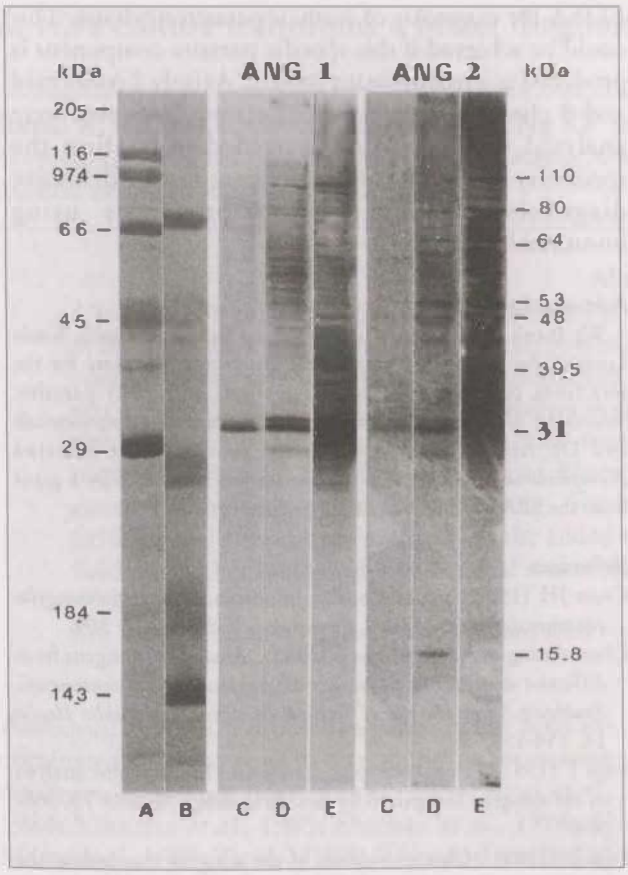

Fig. 2. Immunoblot analysis of sera from 2 confirmed parastrongyliasis patients (ANG 1,2) against *P. cantonensis* third stage larva (C), and adult male (D) and female (E) worm antigens. A, B: High and low molecular weight markers.

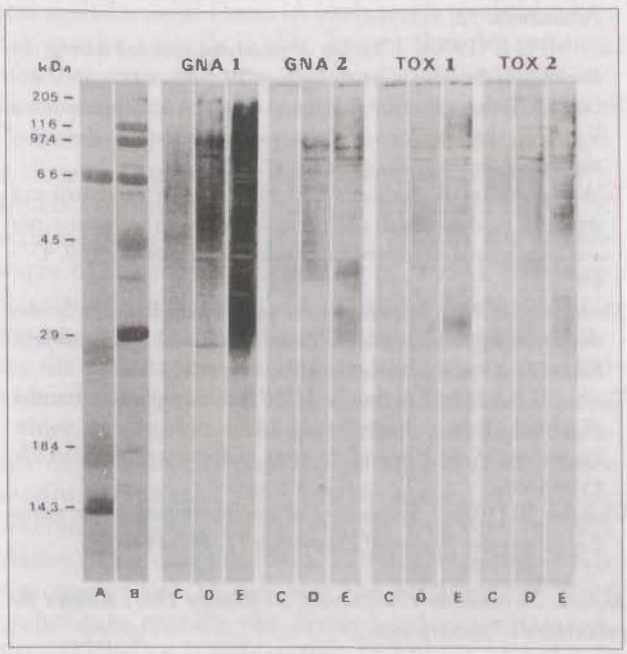

Fig. 3. Immunoblot analysis of sera from 2 gnathostomiasis (GNA 1,2) and 2 toxocariasis (TOX 1,2) patients against *P. cantonensis* third-stage larva(C), and adult male (D) and female (E) worm antigens. A, B: High and low molecular weight markers.

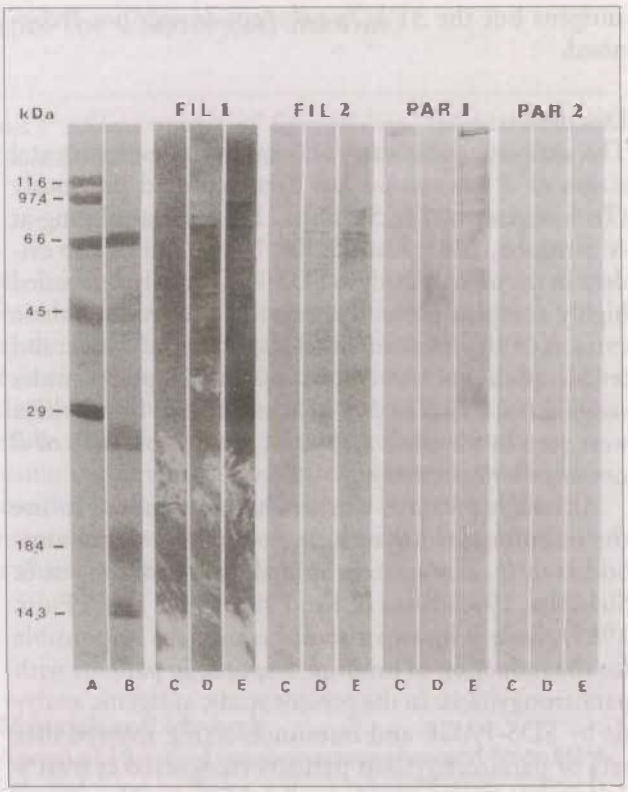

Fig. 4. Immunoblot analysis of sera from 2 filariasis (FIL 1,2) and 2 paragonimiasis (PAR 1,2) patients against *P. cantonensis* third-stage larva (C), and adult male (D) and female (E) worm antigens. A, B: High and low molecular weight markers.

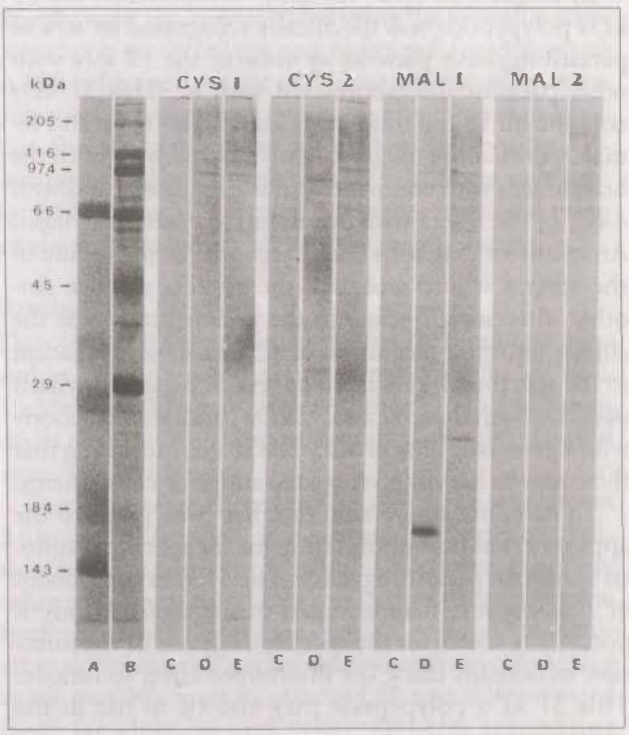

Fig. 5. Immunoblot analysis of sera from 2 cysticercosis (CYS 1,2) and 2 malaria (MAL 1,2) patients against *P. cantonensis* third-stage larva (C), and adult male (D) and female (E) worm antigens. A, B: High and low molecular weight markers.

antigens but the 31 kDa polypeptide was not recognized.

Discussion

The antigenic complexity of the various developmental stages of *P. cantonensis* has been reported previously (Techasoponmani & Sirisinha, 1980; Dharmkrong-at & Sirisinha, 1983; Kum & Ko, 1985). This is also evident in the present study as SDS-PAGE analysis revealed highly complex protein patterns of the crude somatic extracts of *P. cantonensis* third-stage larva, and male and female adult worms. At least 40 and 20 polypeptides ranging from 12.5 to 205 kDa and from 11 to 66 kDa were seen in the adult and larval somatic proteins of *P. cantonensis* respectively.

Although previous workers have attempted to use the immunoblotting technique to detect serum antibodies to *P. cantonensis* in animals (Dharmkrong-at & Sirisinha, 1983; Kum & Ko, 1986; Fujii, 1987; Fujii, 1989), little is known about the antigens responsible for the induction of immune response in patients with parastrongyliasis. In the present study, antigenic analysis by SDS-PAGE and immunoblotting showed that sera of parastrongyliasis patients recognized at least 4 antigenic polypeptides from the third-stage larval and 25 bands from adult worm antigens. There are also common antigens in the somatic extracts of *P. cantonensis* which reacted strongly with sera from patients with parastrongyliasis and various other parasitic infections.

In contrast to other antigenic components, the 31 kDa polypeptide was specifically recognized by sera of parastrongyliasis patients as none of the 12 sera with other parasitic diseases reacted with it. This 31 kDa component in the third-stage larval, and male and female worm antigens consistently reacted with both the human sera with confirmed parastrongyliasis, and with 4 out of the 8 sera with presumptive parastrongyliasis. An incorrect diagnosis could account for the failure of the other 4 sera to recognize the 31 kDa antigen. Another interesting finding in the present study was the almost universal recgnition of a 32 kDa band adjacant to the specific 31 kDa band by sera of parastrongyliasis patients. Both these 31 and 32 kDa bands stained poorly with Coomassie blue in SDS-PAGE gel, indicating that these may be heavily glycosylared antigenic components.

To date, not much attention has been given to the application of immunoblotting for the specific diagnosis of human parastrongyliasis. The 31 kDa component of *P. cantonensis* demonstrated in the present study is potentially useful for the specific diagnosis of the infection in humans using the immunoblotting technique. This 31 kDa polypeptide may also be of use in the ELISA for diagnosis of human parastrongyliasis. This could be achieved if this specific parasite component is produced as a recombinant protein. As only 2 confirmed and 8 clinically diagnosed parastrongyliasis sera were analysed, a larger study is needed to confirm the specificity of the 31 kDa component for the definitive diagnosis of human parastrongyliasis using immunoblotting.

Acknowledgements

We thank the Director, Institute for Medical Research, Kuala Lumpur, for permission to publish; Dr. Nimit Morakote for the sera from patients with parastrongyliasis and other parasitic infections; Mr. Paron Dekumyoy for the sera with paragonimiasis and Dr. Araya Dharmkrong-at for providing the infected *Biomphalaria glabrata* snails. This work was supported by a grant from the SEAMEO-TROPMED project.

References

Cross JH (1987). Public health importance of *Angiostrongylus cantonensis* and its relative. *Parasitology Today* 3, 367-369.

Dharmkrong-at A & Sirisinha S (1983). Analysis of antigens from different developmental stages of *Angiostrongylus cantonensis*. *Southeast Asian Journal of Tropical Medicine and Public Health* 14, 154-159.

Fujii T (1987). *Angiostrongylus cantonensis*: Immunoblot analysis of the antigens recognized by rats. *Parasitology Research* 73, 366-374.

Fujii T (1989). Characterization of the antigens that induce the production of antibodies in mice immediately after infection with *Angiostrongylus cantonensis*. *Parasitology Research* 75, 636-640.

Kum WS & Ko RC (1985). Isolation of specific antigens from *Angiostrongylus cantonensis*. 1. By preparative isoelectric focusing. *Zeitschrift für Parasitkunde* 71, 789-800.

Kum WS & Ko RC (1986). Isolation of specific antigens from *Angiostrongylus cantonensis* 2. By affinity chromatography. *Z. Parasitkunde* 72, 511-516.

Laemmli U K (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

Sirisinha S, Techasoponmani R, Dharmkrong-at A & Uahkowithchai V (1977). Immunology of *Angiostrongylus* infection. *Journal of the Science Society of Thailand* 3, 157-161.

Techasoponmani R, Sirisinha S (1980). Use of excretory and secretory products from adult female worms to immunize rats and mice against *Angiostrongylus cantonensis*. *Parasitology* 80, 457-461.

Tharavanij S (1978). Immunology of angiostrongyliasis, In: *Studies on Angiostrongyliasis in Eastern Asia and Australia*. (ed. Cross JH), NAMRU-2 Special Publication No. 44, pp. 151-164.

Towbin H, Staehelin T & Bordon J (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. *Proceedings of the National Academy of Sciences of the USA* 76, 4350-4354.

Ubelaker JE (1986). Systematics of species referred to the genus *Angiostrongylus*. *Journal of Parasitology* 72, 237-244.

Received 24 November 1996; revised 10 January 1997; accepted for publication 17 January 1997.