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Immunoblot Evaluation of the Specificity of the 29-kDa Antigen from Young Adult Female Worms *Angiostrongylus cantonensis* for Immunodiagnosis of Human Angiostrongyliasis

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SUMMARY **The antigenic components of *Angiostrongylus cantonensis* young adult female worm somatic extract (FSE) were revealed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. The sera tested were from patients with proven angiostrongyliasis, other parasitic diseases, and healthy adults. Both the sera and cerebrospinal fluid (CSF) were tested from patients with clinical angiostrongyliasis. The CSF from patients with other neurological diseases were also included. Using SDS-PAGE, we found that the FSE comprised more than 30 polypeptides. Immunoblot analysis revealed at least 12 or 13 antigenic bands in patients with proven or clinical angiostrongyliasis, respectively. The patterns of reactivity recognized by the serum and CSF antibodies against FSE were similar. These antigenic components had molecular masses ranging from less than 14.4 to more than 94 kDa. The prominent antigenic band of 29-kDa might serve as a reliable marker for the diagnosis of angiostrongyliasis. The sensitivity, specificity, positive and negative predictive values of immunoblot analysis in this antigenic band were 55.6%, 99.4%, 83.3% and 97.4%, respectively.**

Angiostrongylus cantonensis, the rat lungworm, is an important etiological agent causing human eosinophilic meningitis in the Asia and Pacific regions.[1-5] Definitive diagnosis requires the worm(s) to be found in the cerebrospinal fluid (CSF) or eyes of the patients, or in the brains of autopsied cases. However, recovery of the parasite from an infected host is rarely successful. Immunodiagnostic tests for the detection of antibodies against *A. cantonensis* are needed to supplement parasitological methods. Attempts to detect antibody responses to *A. cantonensis* in serum and CSF have been unsatisfactory. The tests used either crude or partially purified adult worm antigens,[6-7] young adult worm antigen[8-9] or female adult antigen.[9-10]

To enhance the information on the serology of angiostrongyliasis, we used immunoblot analysis to reveal the antigenic components of young adult female worms recognized by infected human sera and CSF. Furthermore, we investigated the potential for using this method to diagnose human angiostrongyliasis.

MATERIALS AND METHODS

Human sera

Proven angiostrongyliasis sera were obtained from 9 parasitologically confirmed patients. Among these 9 patients, 4 cases were parasitologically confirmed by lumbar punctures and the other 5 cases by recovery of the worms from an eyeball. Eighty-five cases of clinically suspected angiostrongyliasis patients with eosinophilic meningitis or meningoencephalitis were obtained from Srinagkarind Hospital, Faculty of Medicine, Khon Kaen University. All the cases had a history of eating raw snails within

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11 to 40 days before the onset of disease. Among these patients, both serum and CSF were sampled from 20 patients, while only serum specimens were taken from the other 65 patients. Serum samples from 123 patients with opisthorchiasis, opisthorchiasis concordant with other parasitic infections, trichinellosis, strongyloidiasis, gnathostomiasis, hookworm infection, cysticercosis, capillariasis philippinensis, paragonimiasis and fascioliasis were obtained from parasitologically confirmed cases. Thirty serum specimens were obtained from healthy adults whose stool examination at the time of blood collections gave no evidence of any intestinal parasitic infection to serve as negative controls. Ten CSF specimens from hospitalized patients with other neurological diseases without eosinophilic meningitis or eosinophilic meningoencephalitis (i.e. syphilis, bacterial meningitis, headache, and third nerve palsy syndrome), were also employed as control cases. The study protocol was approved by the Scientific-Ethics Committee of Khon Kaen University. Informed consent was obtained from the study subjects using a standard approved procedure.

Animals

Female rats (*Rattus norvegicus*), aged 8 weeks, were obtained from the Animal Section, Faculty of Medicine, Khon Kaen University. Rats were housed in-groups of 4 or 5 in plastic box cages and provided with rodent chow and water *ad libitum*.

Parasite and antigen

The life cycle of a Thai strain of *A. cantonensis* has been maintained in our laboratory in *Achatina fulica* snails and rats. Third-stage *A. cantonensis* larvae were obtained by digestion of infected snails with pepsin, and these larvae were transferred to rats by stomach intubation. Rats infected for 3 weeks were killed by ether euthanesia and young adult worms were removed from their brains. The young adult female worms were thoroughly washed with normal saline (0.85 % NaCl in distilled water). Young adult female worm somatic extract (FSE) was prepared as previously described with some modifications.[11] Briefly, the worms were homogenized with a tissue grinder in a small volume of normal saline containing 0.1 mM of phenyl-methyl sulfonyl fluoride, 0.1 mM of tosylamide-2-phenyl-ethyl-chloromethylketone, and 1 μM of N-(N-[L-3-trans carboxyoxiran-2-carbonyl]-L-leucyl)-agmatine. The preparation was then sonicated with an ultrasonic disintegrator and centrifuged at 10,000 × *g* for 30 minutes at 4°C. The protein concentration of the supernatant was determined by the method of Lowry *et al.*[12]

SDS-PAGE and immunoblotting technique

The protein components of the FSE were separately resolved by SDS-PAGE under reducing conditions on a 10% to 18% gradient gel prepared by the method of Laemmli.[13] Antigen containing 40 μg protein per lane of 0.5 cm width, or 560 μg protein per lane of 7 cm width, was loaded onto the gel. After electrophoresis, the resolved polypeptides were either revealed by silver staining[14] or electrophoretically transferred to nitrocellulose membranes for immunoblotting.[15] The antigen-blotted nitrocellulose membrane was immersed in a blocking solution (1% skim milk and 0.1% Tween 20 in 100 mM PBS pH 7.4) for 30 minutes at room temperature and cut vertically into strip of 0.5 x 5.5 cm. One strip was incubated with one serum sample (diluted 1:100 in the blocking solution) or one CSF sample (undiluted) for 2 hours with gentle rocking, washed 5 times and then incubated for 2 hours with peroxidase conjugated goat anti-human immunoglobulin G (Cappel Laboratories, USA) in a blocking solution with a dilution of 1:1,000. After washing, the strips were then incubated in diaminobenzidine (Sigma Chemical Co, USA) in 50 mM Tris pH 7.6. The blot was developed at room temperature with agitation until the dark brown bands appeared. The strips were then washed with distilled water and air dried. Following that, the experimental results were calculated for diagnostic sensitivity, specificity and predictive values using the method of Galen.[16]

RESULTS

SDS-PAGE and protein staining of FSE revealed at least 30 bands with approximate molecular masses ranging from less than 14.4 to more than 94 kDa (Fig. 1, lane B). Sera of patients with proven and clinical angiostrongyliasis reacted to at least 12 and 13 antigenic bands, respectively, with the molecular masses scattering from less than 14.4 to more than 94 kDa (Fig. 2, lanes C to F). The frequency of reactivity against an individual antigenic component from FSE with serum samples from the different patients and normal healthy controls is summarized in Table 1.

The prominent antigenic band at an approximate molecular mass of 29-kDa was found to react with 5 out of 9 and 28 out of 85 sera from patients with parasi-

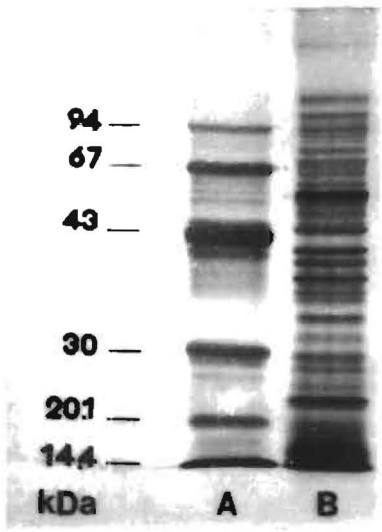

Fig. 1 Silver staining for young adult female worm somatic extract (FSE) of *Angiostrongylus cantonensis* after sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Lane A, molecular mass standards in kilodaltons (kDa), lane B, FSE.

tologically confirmed and clinical angiostrongyliasis, respectively. The specificity of the 29-kDa band was defined further by comparing the serum reactivities of the angiostrongyliasis patients, with those of healthy controls and patients with other parasitic infections (Table 1; Fig. 2, lanes C to Q). The diagnostic sensitivity and specificity of the test using the presence of 29-kDa band as the marker for human angiostrongyliasis were 55.6% and 99.4%, respectively. The positive and negative predictive values were 83.3% and 97.4%, respectively.

The antigenic polypeptides recognized by pairs of serum and CSF from individual cases of clinically diagnosed angiostrongyliasis are shown in Fig. 3 and Table 2.

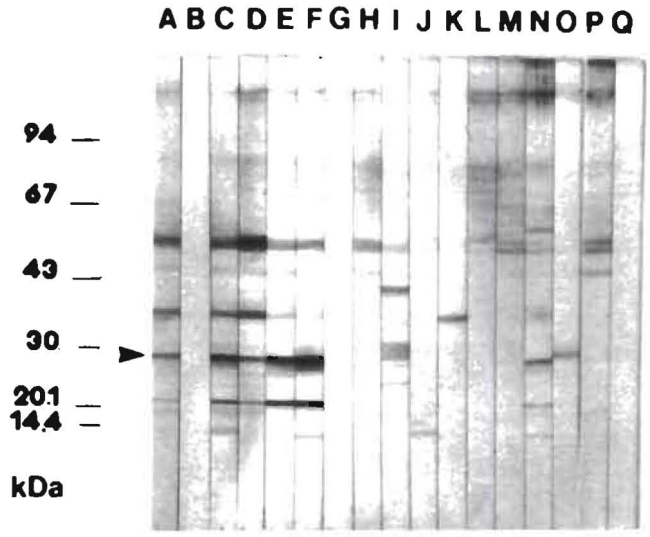

Fig. 2 The representative patterns of immunoblot analysis of *Angiostrongylus cantonensis* young adult female worm somatic extract after reaction with individual sera from pooled positive references (A), pooled negative references (B), proven diagnosed angiostrongyliasis (C), clinically diagnosed angiostrongyliasis (D to F), gnathostomiasis (G), hookworm infection (H), strongyloidiasis (I), trichinellosis (J), capillariasis (K), cysticercosis (L), paragonimiasis (M), fascioliasis (N), opisthorchiasis (O), mixed infections with *Opisthorchis viverrini*, hookworm and *Strongyloides stercoralis* (P) and healthy controls (Q). The arrow indicates the band at the approximate molecular mass of 29 kDa.

Table 1 Number of sera that recognized individual antigenic components from young adult female worm somatic extract of *A. cantonensis* as demonstrated by SDS-PAGE and immunoblotting

Serum type	Number	No. (%) reacting with the component (kDa)													
		>94	87	83	77	65	54	52	46	41	36	29	27	21	<14.4
Proven angio-strongyliasis	9	8 (88.9)	3 (33.33)	3 (33.3)	1 (11.1)	5 (55.6)	6 (66.7)	0 (0)	0 (0)	2 (22.2)	3 (33.3)	5 (55.6)	1 (11.1)	2 (22.2)	2 (22.2)
Clinical angi-ostrongyliasis[a]	85	81 (95.3)	34 (40)	47 (55.3)	43 (50.6)	48 (56.5)	52 (61.2)	48 (56.5)	45 (52.9)	0 (0)	46 (54.1)	28 (32.9)	6 (7.1)	27 (31.8)	38 (44.7)
Trichinellosis	27	11 (40.7)	0 (0)	0 (0)	1 (3.7)	2 (7.4)	4 (14.8)	0 (0)	0 (0)	1 (3.7)	0 (0)	0 (0)	0 (0)	1 (3.7)	0 (0)
Strongyloidiasis	12	8 (66.7)	0 (0)	3 (25)	2 (16.7)	1 (8.3)	2 (16.7)	5 (41.7)	2 (16.7)	2 (16.7)	1 (8.3)	0 (0)	1 (8.3)	1 (8.3)	1 (8.3)
Gnathostomiasis	8	7 (87.5)	0 (0)	3 (37.5)	0 (0)	0 (0)	1 (12.5)	3 (37.5)	1 (12.5)	1 (12.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Hookworm infection	4	3 (75)	0 (0)	0 (0)	0 (0)	2 (50)	0 (0)	0 (0)	0 (0)	2 (50)	2 (50)	0 (0)	0 (0)	0 (0)	1 (25)
Cysticercosis	5	1 (20)	0 (0)	1 (20)	0 (0)	1 (20)	0 (0)	1 (20)	1 (20)	0 (0)	0 (0)	0 (0)	1 (20)	0 (0)	0 (0)
Capillariasis	7	1 (14.3)	0 (0)	0 (0)	0 (0)	0 (0)	1 (14.3)	1 (14.3)	0 (0)	0 (0)	1 (14.3)	0 (0)	0 (0)	0 (0)	0 (0)
Paragonimiasis	15	13 (86.7)	2 (13.3)	7 (46.7)	0 (0)	0 (0)	10 (66.7)	4 (26.7)	4 (26.7)	5 (33.3)	5 (33.3)	0 (0)	4 (26.7)	0 (0)	0 (0)
Fascioliasis	15	11 (73.3)	5 (33.3)	8 (53.3)	1 (6.7)	1 (6.7)	3 (20)	6 (40)	2 (13.3)	6 (40)	5 (33.3)	1 (6.7)	2 (13.3)	3 (20)	4 (26.7)
Opisthorchiasis	21	15 (71.4)	1 (4.8)	1 (4.8)	3 (14.3)	2 (9.5)	5 (23.8)	3 (14.3)	2 (9.5)	5 (23.8)	5 (23.8)	0 (0)	0 (0)	0 (0)	0 (0)
Other parasitosis[b]	9	4 (44.4)	1 (11.1)	1 (11.1)	1 (11.1)	0 (0)	1 (11.1)	2 (22.2)	1 (11.1)	1 (11.1)	1 (11.1)	0 (0)	0 (0)	0 (0)	1 (11.1)
Healthy control	30	7 (23.3)	1 (3.3)	1 (3.3)	1 (3.3)	5 (16.7)	1 (3.3)	0 (0)	5 (16.7)	2 (6.7)	3 (10)	0 (0)	1 (3.3)	1 (3.3)	0 (0)

[a]Clinically diagnosed

[b]Total of 9 cases, 4 were infected with *Opisthorchis viverrini*, hookworm and *Strongyloides stercoralis*, 4 were infected with *O. viverrini*, hookworm and minute intestinal flukes and 1 was infected with *O. viverrini* and hookworm

Table 2 Number of sera and CSF that recognized individual antigenic components from young adult female worm somatic extract of *A. cantonensis* as demonstrated by SDS-PAGE and immunoblotting

Type of specimen[a]	Number	No (%) reacting with the component (kDa)												
		>94	87	83	77	65	54	52	46	36	29	27	21	<14.4
Sera	20	17 (85)	9 (45)	15 (75)	9 (45)	13 (65)	14 (70)	9 (45)	12 (60)	14 (70)	10 (50)	6 (30)	7 (35)	7 (35)
CSF	20	17 (85)	13 (65)	17 (85)	10 (50)	12 (60)	13 (65)	13 (65)	14 (70)	15 (75)	10 (50)	7 (35)	10 (50)	8 (40)

[a]Clinically diagnosed angiostrongyliasis

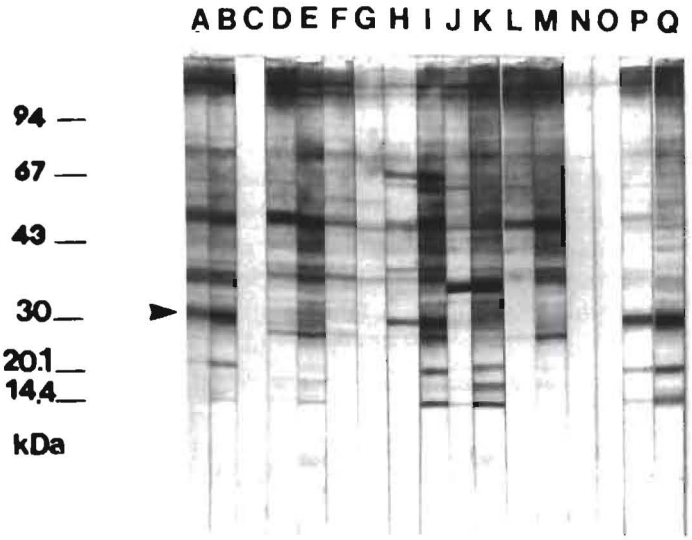

Fig. 3 Immunoblot analysis of *Angiostrongylus cantonensis* young adult female worm somatic extract after reaction with individual sera from proven diagnosed angiostrongyliasis (A), pooled positive references (B) and pooled negative references (C). Lanes D-E, F-G, H-I, J-K, L-M, N-O, and P-Q show the representative patterns of immunoblot analysis after reaction with pairs of serum and CSF from individual cases of clinically diagnosed angiostrongyliasis. The arrow indicates the band at approximate molecular mass of 29 kDa.

The patterns of reactivity recognized by antibodies against FSE in serum and CSF were similar. When looking at the specific band of 29-kDa, 10 out of 20 (50%) of the pair specimens reacted while this reaction did not occur in the CSF obtained from other neurological diseases (data not shown).

DISCUSSION

The diagnosis of angiostrongyliasis from the presentation of clinical symptoms of patients is difficult since symptoms are often confused with other neurological disorders caused by other parasitic diseases, such as cerebral gnathostomiasis, cerebral cysticercosis and cerebral paragonimiasis.[17] Thus, differential diagnosis is very important because the sequelae and treatment may be quite different. Computerized tomography is particularly useful in diagnosis as well as in monitoring response to therapy but it is frequently unavailable

in the poor, developing areas, where parasitic infections are the most common cause of health problems.[17] It is therefore necessary to develop a specific and practice serological test for supportive diagnosis of human angiostrongyliasis.

The present investigation indicated that the soluble somatic extracts of young adult female *A. cantonensis* were highly heterogeneous and contained at least 12 to 13 antigenic components. However, the differences in the reactivity patterns with angiostrongyliasis sera were probably due to different time schedule of blood collection during the course of infection among these patients. This problem could be eliminated if blood collection was done serially. Other reasons for the different reactivity patterns were the intensity of infection and the degree of host response. The FSE antigen contained a specific 29-kDa antigen which reacted with 55.6% and 32.9% of proven and clinical angiostrongyliasis sera, respectively. The 29-kDa antigenic band was not demonstrated in sera of healthy control subjects nor in sera of patients with other parasitic infections and similar neurological symptoms and signs (*i.e.* gnathostomiasis, cysticercosis and paragonimiasis), except in one case of fascioliasis. In conclusion, this specific band is possibly useful for immunodiagnosis of eosinophilic meningitis or meningoencephalitis related to angiostrongyliasis. In addition, this result is also in agreement with those of previous reports regarding the specificity of the 29-kDa and 31-kDa antigenic components of adult female *A. cantonensis*.[10,18] Nevertheless, it was considered that the low reactivity (55.6% and 32.9% of proven and clinical angiostrongyliasis sera reacted with the specific 29-kDa antigen; Table 1) was probably due to sera collection done too early in the course of the infection. This may explain the low response of the specific IgG antibodies. To demonstrate whether IgM or total immunoglobulin antibodies were specifically related to *A. cantonensis* requires further study.

In our study, the antigens from young adult worms may be an important means of serodiagnosis for *A. cantonensis* infection because the incubation period between infection and symptoms of disease in humans is generally more than two weeks.[3] During that time, the larvae develop to a juvenile stage in the central nervous system prior to the onset of illness. Even though Yen and Chen[9] reported that sensitivity and specificity in the detection of antibodies against adult worm antigens appeared very similar to those against young adult worm antigens, some reports concluded that juvenile worm antigens gave better results for the immunodiagnosis of angiostrongyliasis.[2,8] Other reports prefered adult worm antigens.[7,10,18,19]

We compared the patterns of antigenic components reacting with the pair of serum and CSF from eosinophilic meningitis patients. The reactivity patterns of CSF antibodies specific to worm antigenic polypeptides possibly corresponded with the reactivity patterns of the serum antibodies (Fig. 3). Specific IgG antibodies in the CSF might also have come via the blood by changes in the blood brain barrier. Nevertheless, some of the evidence indicates that local immunoglobulin synthesis is involved in the central nervous system in response to an infection with *A. cantonensis*.[20] These specific IgG antibodies in the CSF may be used as markers for serodiagnosis of human angiostrongyliasis.

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