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Antigenic Components of *Gnathostoma spinigerum* Recognized by Infected Human Sera by Two-Dimensional Polyacrylamide Gel Electrophoresis and Immunoblotting

C. Wongkham[1], W. Maleewong[2], K. Ieamviteevanich[3], P.M. Intapan[2] and N. Morakote[4]

SUMMARY Antigenic components of *Gnathostoma spinigerum* larval extract were revealed by two-dimensional gel electrophoresis (2-DE) and immunoblot analysis using sera from patients with 6 proven cases of gnathostomiasis, 5 presumptive cases of gnathostomiasis, 3 proven cases of angiostrongyliasis, 3 proven cases of cysticercosis, and pooled sera from healthy adults. By the 2-DE, the larval extract was highly complex and consisted of more than 75 polypeptides. Immunoblotting analysis of this larval extract after reaction with each of 6 proven gnathostomiasis sera revealed various numbers of antigenic spots ranging from 30 to 70 spots at the approximate molecular masses of less than 14.4 to more than 94 kDa with isoelectric points (pI) of less than 4.65 to 9.6. Antigenic spots at the approximate molecular mass of more than 30 kDa were recognized with the proven angiostrongyliasis, proven cysticercosis and healthy control sera but these sera did not react with the spots at approximate molecular masses of 23-25 kDa with pI of 8.3-8.5. The reacted spots, which consisted of at least 1 to 2 spots, were unique for the recognition of gnathostomiasis sera. Five out of 6 (83.3%) proven and 4 out of 5 (80%) presumptive gnathostomiasis sera reacted with these specific spots.

Gnathostoma spinigerum causes human gnathostomiasis in Thailand and many Asian countries.[1-2] Humans acquire the disease mainly by consuming raw or half-cooked foods containing infective larvae of the parasite. The worm usually migrates in subcutaneous tissue, causing an intermittent migratory swelling. Sometimes it reaches the central nervous system, producing various signs and symptoms, and the disease becomes life threatening.[3-5] Definitive diagnosis relies on identification of worms removed by surgery or after it spontaneously leaves the host, but this occurs in only a small number of infected patients. Attempts have been made to diagnose this disease by immunological methods which detect antibodies against larval somatic and/or metabolic products.[6-9] However, these tests have been hampered by the complex and cross-reactive nature of the antigens used. Furthermore, the antigen analysis of this parasite had been revealed on the basis of one-dimensional polyacrylamide gel electrophoresis using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent immunoblotting, which aim to identify the specific polypeptide for use as the antigen in immunodiagnosis of this disease.[10-12] A 24 kDa glycoprotein of larval extract has been shown to be the diagnostic band.[12] However, the standard discontinuous SDS-PAGE as described above separates polypeptides into bands of different molecular weights. In one band, there may be a number of different proteins with the same molecular

From the [1]Department of Biochemistry and [2]Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen, [3]Loei Hospital, Ministry of Public Health, Loei, [4]Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.
Correspondence: W. Maleewong

weight but which have different amino acid compositions. Such proteins have different isoelectric points (pIs). The resolving power of SDS-PAGE can thus be greatly enhanced if the proteins are first separated according to their pIs and subsequently separated according to their molecular weights under two-dimensional gel electrophoresis (2-DE). In addition, little is known about the antigenic components of *G. spinigerum* recognized by infected human sera as shown by the 2-DE and immunoblotting technique. The purpose of the present study is to characterize the parasite antigens in more detail using the powerful 2-DE, followed by immunoblotting with sera from individuals with human gnathostomiasis, other parasitic infections, and normal healthy adults. The results should be useful for the development of immunodiagnostic methods of human gnathostomiasis.

MATERIALS AND METHODS

G. spinigerum antigen

Advanced third-stage larvae of *G. spinigerum* (al3) were obtained from mice orally inoculated with early third-stage larvae from infected copepods.[13] The al3 somatic extract was prepared by homogenization and extraction as described previously.[14] Briefly, the worms were homogenized with a tissue grinder in a small volume of 0.1 M phosphate buffered saline, pH 7.4 containing 0.1 mM of phenylmethylsulfonylfluoride, 0.1 mM of tosylamide-2-phenyl-ethylchloromethylketone, and 1 μM of L- trans-3-carboxyoxiran-2-carbonyl-6-leucylagmatine. The preparations were then sonicated with an ultrasonic disintegrator and centrifuged at 10,000 *x g* for 30 minutes at 4°C. The supernatant was dialyzed against distilled water containing the same proteinase inhibitors and kept at -20°C until further analysis. The protein content of the antigen was determined by the Folin phenol method.[15]

Serum samples

Sera were obtained from six parasitologically confirmed cases of adult human gnathostomiasis. Of these, five cases recovered *G. spinigerum* advanced third-stage larvae; three from the eye balls (two males and one female, 40, 22 and 25 years old), one from the skin of the hand (male, 45 years old) and one from the skin of the chest (female, 50 years old). The last one recovered a sexually immature adult from the skin of the abdomen (female, 41 years old). Sera were also obtained from five presumptive patients with signs and symptoms of cerebral gnathostomiasis (three males and two females adults 30-54 years old) and a demonstration of antibodies against somatic larval antigens in their sera and cerebrospinal fluid as detected by ELISA.[16] Three sera were collected from parasitologically confirmed cases of *Angiostrongylus* infection (three males, 30, 22, 45 years old); all of these individuals had eosinophilic meningitis with the presence of antibodies against *Angiostrongylus cantonensis* young adult antigen in their sera as previously described.[17] Three sera of patients with cysticercosis were also obtained from parasitologically confirmed cases (three males, 19, 25 and 40 years old). Control negative serum was obtained from the pooled sera of 30 healthy Thai adults who had no history of intermittent cutaneous migratory swelling and were negative for any intestinal parasitic infection at the time of blood collection. The serum collections were started several years before the present investigation began, though a study was envisioned, and therefore informed consent was obtained from the study subjects using standard approved procedure. The study protocol was approved by the Scientific-Ethical Committee of Khon Kaen University.

2-DE and immunoblotting technique.

2-DE test was performed by the combination of isoelectric focusing (first dimension) and SDS-PAGE (second dimension), as developed by O'Farrell,[18] with some modifications. Ultrathin gels (0.6 mm thick) contained 7.3% acrylamide, 0.225% N, N'-methylenebisacrylamide (Pharmacia Biotech, Sweden), and 8% v/v carrier ampholytes (Biolyte pH 3-10 [Bio-Rad Laboratories, USA]) were prepared on the Gel Bond films (FMC, USA) and the isoelectric focusing was performed in the LKB 2117 Multiphor II Electrophoresis System (Pharmacia Biotech). Each antigen sample (200 μg of protein per sample) was applied to the anodic side of each gel and focused at 2,000 Vh with cooling. After isoelectric focusing, the gels were equilibrated for 15 minutes in a sample buffer (0.062 M Tris-HCl, pH 6.8, 10% glycerol, 5% 2-mercaptoethanol, 2% SDS and 0.001% bromphenol blue). Each of the isoelectrically focused gels was further applied onto SDS-PAGE containing a linear gradient

between 10% and 18% acrylamide.[19] After electrophoresis, the gels were stained by silver staining (Silver Stain Plus kit, Bio-Rad Laboratories). Another gel containing the same batch of the antigen sample was then blotted onto nylon membranes[20] under semidry blotting conditions (Bio-Rad Laboratories), according to the manufacturer's instructions. The blot was blocked for 30 minutes with blocking solution (50 mM Tris buffered saline [TBS], pH 7.4 containing 1% Blotto [Boehringer Mannheim, Germany] with 0.05% Tween-20). The blot was incubated with serum sample (diluted 1:100) in blocking buffer for 2 hours. It was then washed 5 times with TBS, pH 7.5 containing 0.05% Tween-20, followed by an incubation for 2 hours with alkaline phosphatase-conjugated rabbit anti-human immunoglobulin (Dako, Denmark) in the blocking solution. For visualization of antibody reactions, nitroblue tetrazolium and 5-bromo-4 chloro-3-indolyl phosphate were used as the chromogenic substrates. To ensure the reproducibility of tests, each experiment was done in triplicate, and subsequent tests produced uniform results.

RESULTS

When using 2-DE, the separation patterns of *G. spinigerum* larval extract were highly complex and consisted of more than 75 visible protein spots (Fig. 1). Most protein spots were presented in the acidic and neutral areas. Immunoblotting was performed using individual serum from 6 proven cases of gnathostomiasis, 5 cases of clinical presumptive gnathostomiasis, 3 cases of proven cysticercosis, 3 cases of proven angiostrongyliasis, and a pool of sera from healthy controls, as shown in Fig. 2. Analysis of blots reacting with each of the proven gnathostomiasis sera showed various numbers of major antigenic peptides, ranging from 30 to 70 spots (Fig. 2A-2B) at the approximate molecular masses of less than 14.4 to 94 kDa with pI of between 4.65 and 9.6. All proven gnathostomiasis sera reacted with the similar immunodominant antigenic patterns at the approximate molecular masses of between 30 and 43 kDa with pI of between 4.65 and 6.5. However, the number of antigenic spots which reacted with sera from individuals with proven gnathostomiasis varied from 3 to 16 spots at the approximate molecular masses of 20 to 30 kDa with pI of between 4.65 and 9.6. The number of antigenic spots which reacted with sera from individuals with clinical presumptive gnathostomiasis, ranged from 26 to 93 major antigenic spots at approximate molecular masses of less than 14.4 to more than 94 kDa with pI of between 4.65 and 9.6 (Fig 2 C). Sera from 3 cases of proven angiostrongyliasis, 3 cases of proven cysticercosis and the pool of sera from healthy controls reacted the gnathostome larval antigen at least 4 to 25, none to 13, and 23 major spots, respectively, at the approximate molecular masses of less than 14.4 to more than 94 kDa with pI of between 4.65 to 9.6 (Fig. 2D-2F). At least 4 to 24 antigenic spots at the approximate molecular masses of more than 30 kDa were recognized with proven angiostrongyliasis, proven cysticercosis and the pool of healthy control sera. However, the healthy

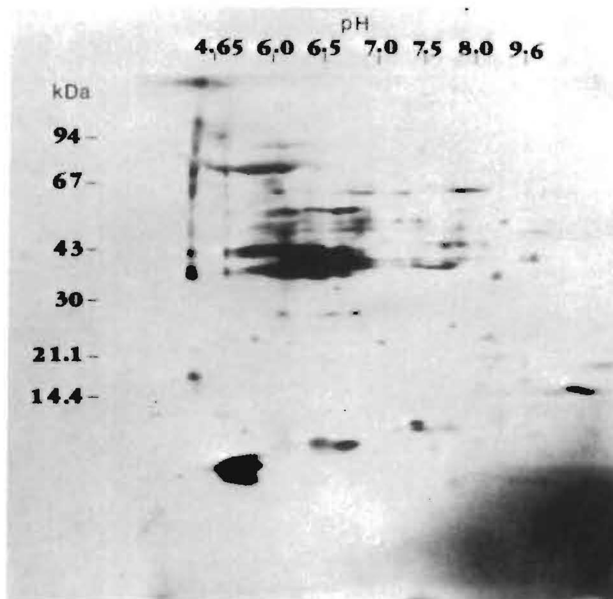

Fig. 1 Silver staining of protein spot pattern from 2-DE of *Gnathostoma spinigerum* advanced third-stage larvae. The pH markers are indicated at top. The molecular weight markers are indicated at left in kilodaltons (kDa).

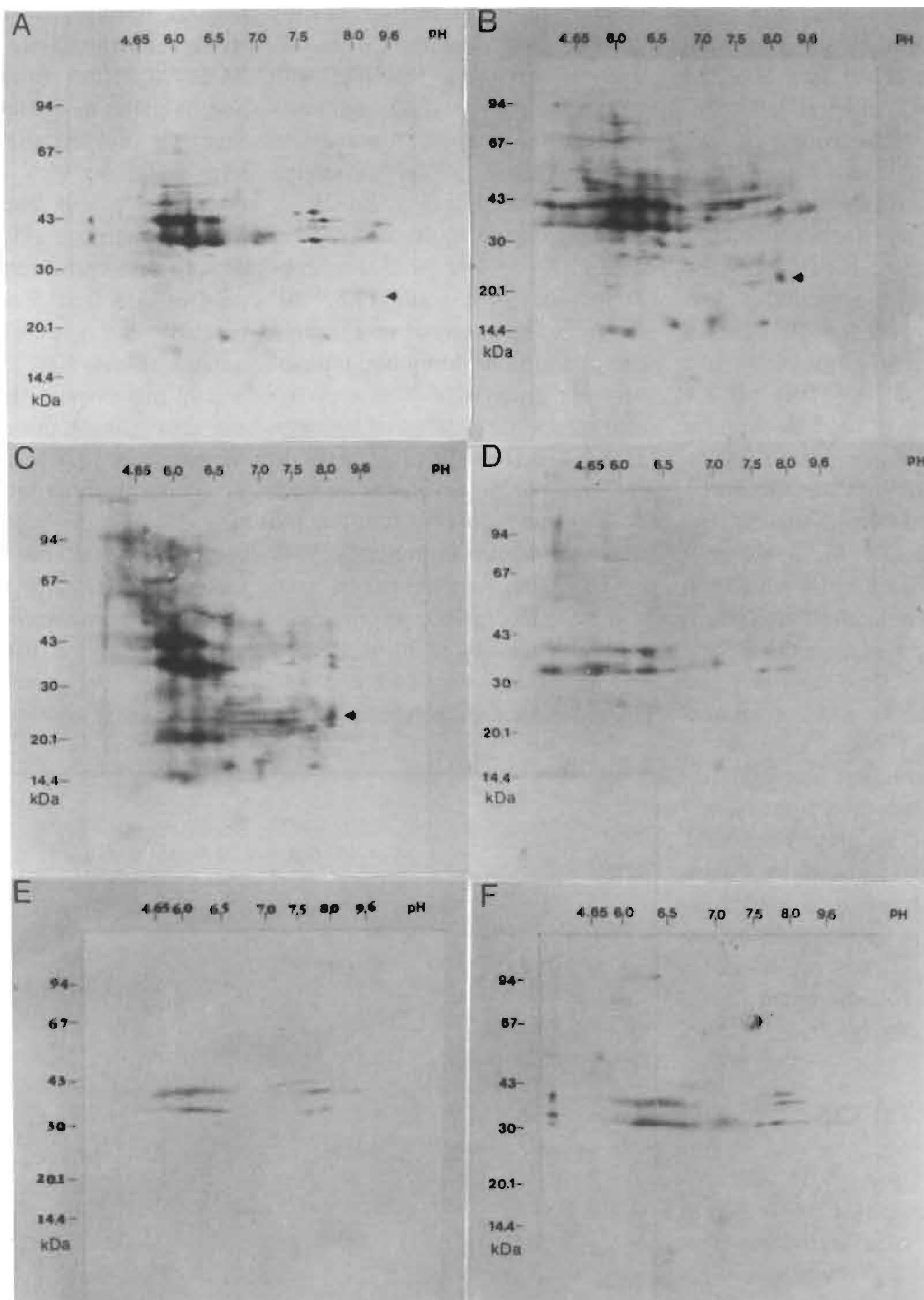

Fig. 2 The representative 2-DE patterns after immunoblotting analysis of proven (A-B), and presumptive gnathostomiasis (C), angiostrongyliasis (D), cysticercosis (E), and a pool of healthy control sera (F). The pH markers are indicated at top. The molecular mass markers are indicated at left in kilodaltons (kDa). The arrowheads indicate the reacted spots at the approximate molecular masses of between 23 to 25 kDa with pI of between 8.3 to 8.5.

control pool did not react with the antigenic spots at the molecular masses of 23 to 25 kDa with pI of 8.3 to 8.5 (Fig. 2D-2F). When looking specifically for diagnostic antigens related to 24 kDa protein, 1 to 2 reacted spots were seen at the approximate molecular mass of 23-25 kDa with pI of 8.3-8.5. These reacted spots were specific for the recognition of human gnathostomiasis sera. However, only 5 of 6 (83.3%) proven and 4 of 5 (80%) presumptive gnathostomiasis sera reacted with these specific spots.

DISCUSSION

Previous studies have characterized the 2-DE protein database of parasites, characterized subsets of worm proteins and human immunogen released by adult *Schistosoma mansoni*,[21] identified the stage-specific proteins associated with the molting process of *Dirofilaria immitis*,[22] characterized various genetically-controlled differences in *Plasmodium falciparum*,[23] classified of *Babesia* spp.,[24] and differentiated the stage proteins of *Strongyloides venezuelensis*.[25] However, little is known about the 2-DE databases and antigenic characterization of *G. spinigerum*.

In the present study, the protein extract from *G. spinigerum* al3 was separated by 2-DE and subsequent immunoblotting. The *G. spinigerum* antigens were recognized by the sera obtained from patients with parasitologically proven and clinical presumptive gnathostomiasis and compared with sera from patients with other parasitic infections and pooled sera from healthy controls. The results revealed the new data on the molecular weights and isoelectric points of *G. spinigerum* larval somatic antigen recognized by infected human sera. High resolution 2-DE, as shown in our study, has been useful as an analytical tool for the separation and quantification of protein species from complex mixtures.[18] It was also shown that the *G. spinigerum* al3 extract was highly complex, as detected by sensitive silver staining. This protocol was sufficiently adequate to establish a protein map. In addition to this, interestingly, immunoblotting revealed that all sera from proven gnathostomiasis and presumptive patients gave similarly positive reactivities against the larval somatic antigenic components at approximate molecular masses of 30 and above. It suggests that the parasite is highly immunogenic and that the antibody responses are systemic and characteristic for the infection, since the worms were recovered from different patient tissues.[11] Several proteins were identified, but of particular interest, this study has demonstrated specific spots at an approximate molecular mass of between 23 and 25 kDa with pI of between 8.3 and 8.5, which for the most part reacted with sera from gnathostomiasis (Fig. 2A-2B) but not with those from angiostrongyliasis (Fig. 2D), cysticercosis (Fig 2E), and healthy control sera (Fig 2F). The recognition in the present study was unique for gnathostomiasis sera. This data confirms the previous report that the *G. spinigerum* specific component with the approximate molecular mass of 24 kDa is revealed by SDS-PAGE and immunoblotting.[10] The specific components revealed by 2-DE and immunoblotting might be useful for diagnosis of human gnathostomiasis because this method did not require complex methods such as involved column chromatography for purification of the specific components use as antigens. In addition, this method gave more detail on the isoelectric points and molecular weights of their antigenic components. Notwithstanding, only 5 out of 6 proven and 4 out of 5 clinical presumptive gnathostomiasis sera reacted with these spots. The explanation for the negativity might be due to the samples collected at the different times during the infection course among these patients. This could be resolved by a serial blood sample collection.

In conclusion, the information in the present study provides a framework for further analyses of the biochemistry and immunology of gnathostomiasis. Further studies are necessary to elucidate each of these specific spots and definition of the amino acid sequencing process is still needed. The protein database was used to design the degenerated oligonucleotide probe for identification of clones that produce specific antigen from *G. spinigerum* cDNA libraries and for use in serodiagnosis of human gnathostomiasis.

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