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Immunodiagnosis of Trichinellosis : Efficacy of Somatic Antigen in Early Detection of Human Trichinellosis

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SUMMARY **Crude antigens prepared from the infective stage larvae of *Trichinella spiralis* were used for antibody detection by indirect ELISA and Western blotting in serum samples taken from trichinellosis patients and from normal, parasite-free controls. The serum specimens were collected from acute ill, symptomatic patients on the first day of treatment (Day 0), and then two months (M2) and 4 months (M4) later. The sensitivities of the indirect ELISA and Western blotting on Day 0 were 81% and 92%, respectively. Both tests were 100% sensitive for M2 and M4 serum samples. Every serum sample from the parasite-free controls tested negative by both immunological assays, indicating 100% specificity. Crude somatic antigens can therefore be used for the early detection of human trichinellosis (acute trichinellosis).**

Both somatic and excretory-secretory (ES) antigens have been commonly used in immunodiagnostic assays for trichinellosis. Somatic and ES antigens can be obtained from either the adult worms of infective larvae of *Trichinella spiralis*. Antigens prepared from the infective larvae are more often used because it is easier to recover the infective larvae from muscles of experimentally infected animals than it is to detach adult worms from the small intestinal mucosa. In addition, ES antigen from adult worms was found to be poorly immunogenic.[1] Immunological tests using infective larvae ES antigens can not only diagnose human trichinellosis but also can serve as a test of cure following chemotherapy.[2] ES antigens had higher specificity than crude somatic antigens (CLE) both in human and porcine trichinelloses by indirect ELISA.[2-5] However, CLE offers certain advantages over ES in the immunodiagnosis of trichinellosis. CLE is easier to prepare than ES antigens and produces higher yields. Some CLE components cross-react with antibodies elicited by other parasites, eg *Capillaria philippinensis, Opisthorchis viverrini, Gnasthostoma spinigerum, Strongyloides stercoralis* and hookworms. This problem can be avoided by using Western blotting, which gives a specific antigen reactive band (M_r 109), instead of indirect ELISA.[6] The present communication report an additional advantage of CLE over ES-the early detection of human trichinellosis by indirect ELISA and Western blotting.

MATERIALS AND METHODS

Antigens

Crude somatic antigens (CLE) were prepared as previously described.[2] Infective larvae of *T.spiralis* were originally isolated from muscle biopsies of patients suffering from trichinellosis during the outbreak in 1986 in Mae Hong Son province, Thailand. They were identified as *T.spiralis* S strain.[8] The life cycle of the parasite was maintained in laboratory mice by feeding each mouse with 350 infective larvae

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using a gastric tube connected to a syringe. The infected mice were sacrificed one month after the infection. The infective larvae were recovered by digesting mouse muscles with 1% HCl-1% pepsin and collecting larvae by a modified Baermann's technique. The larvae were washed thoroughly with sterilized normal saline solution and homogenized in the same solution which contained protease inhibitors, ie 0.1 mM phenyl methyl sulphonyl fluoride (PMSF; Sigma Chemical Company, USA) and 0.1 mM tosyl-amide-2-phenyl-ehyl-chloromethyl ketone (TPCK; Sigma Chemical Company, USA) using a glass tissue grinder. The preparation was then subjected to ultrasonication at 20 kHz for 10 minutes, stirred at 4°C for 2 days and centrifuged at 1,000 × g for 30 minutes. The supernate was collected and used as the CLE. The protein content was determined by the method of Lowry *et al.*[9] and the preparation was kept at -70°C in small aliquots until use.

Sera

Sera were obtained from 37 patients aged 17-66 years who were enrolled in a treatment trial of trichinellosis conducted during an outbreak of the disease in Ban Pasak village, Chiang Saen district, Chiang Rai province, Northern Thailand. Trichinellosis was diagnosed in individuals presenting to the village health center during the outbreak with signs, symptoms and laboratory findings indicating acute, active muscle stage disease. Specific clinical requirements were fever and myositis-(myalgia and/or muscle tenderness). Laboratory criteria were eosinophilia-and/or elevated serum phosphokinase (CPK). Most study patients gave a clear history of having ingested raw pork 1-2 weeks earlier. Muscle biopsies from 5 randomly selected patients provided parasitologic proof of trichinellosis. All patients were initially treated as out-patients at the village health center, but several severely ill individuals subsequently requried hospitalization and one died. Serum was obtained on the first day of treatment (day 0) and then 2 months (M2) and 4 months (M4) later. Sera of 22 healthy, parasite-free counterparts were collected as controls.

Indirect ELISA, SDS-PAGE and Western blot analysis

The tests were performed as previously described.[2,6] For the ELISA, each well of a microtitre ELISA plate (Greiner, Germany) was coated with 100 µl of 5 µg/ml of CLE in carbonate-bicarbonate buffer pH 9.6 and incubated at 37°C for 1 hour then at 4°C overnight. The excess antigen was then washed away using a washing solution containing 0.05% Tween-20 in PBS (PBST). The unoccupied sites in the antigen-sensitized wells were blocked by incubating each well with 200 µl of a blocking solution containing 0.5% BSA in the PBST at 37°C for 1 hour. After the excess BSA was removed by washing as above, 100 µl of diluted serum samples (1:200 in PBST) to be tested was added to appropriate well. The last well of each plate received serum-free PBST and served as negative control or blank well. The plates were reincubated at 37°C for another 1 hour then washed several times with PBST. Peroxidase-labelled rabbit anti-human IgG (Dakopatt, Denmark) diluted 1:1000 in PBST was added to all wells (100 µl per well) and the plates were reincubated for 1 hour. After thorough washing the substrate (0.05% para-phenylenediamine dihydrochloride in 0.05 M citrate buffer pH 7.4 containing 0.003% H_2O_2) was added to each well (100 µl per well) and the enzyme-substrate reaction was allowed to occur at room temperature in the dark for 30 minutes. The reaction was stopped by adding 50 µl of 1 M NaOH to each well. The OD of the content of each well was measured at 492 nm against the blank using Uniskan II ELISA reader (Labsystems, Finland).

The precision of the assay was determined by testing, on different occasions, the same positive serum (pool of five parasite-confirmed trichinellosis serum samples) more than 20 times. The OD were determined and percent coefficient of variation (% CV) was calculated from the formula:

$$\% \text{ CV} = \frac{\text{SD} \times 100}{\bar{X} \text{ of OD}}$$

The % CV was found to range from 5 to 6 when the test was performed on different occasions indicating high reproducibility of the assay.[10]

The SDS-PAGE was carried out in a vertical slab gel apparatus (Bio-Rad Laboratories, USA) according to the system of Laemmli.[11] A 4% acrylamide stacking gel and 7.5% acrylamide separating gel were used in the process. Western blotting was performed by trans-blotting the SDS-PAGE separated antigens from the gel to nitrocellulose paper (NC).[12] The unoccupied sites on the NC were blocked by soaking in a solution of 2% BSA and 0.2% gelatin at 26°C for 1 hour. After washing thoroughly, the NC was put in a solution of peroxidase-labelled rabbit anti-human immunoglobulins (Dakopatt, Denmark) at a dilution of 1:500 in PBS pH 7.4 containing 1% BSA and 1% gelatin for 30 minutes at 26°C. The NC was washed four times with PBST. Finally it was washed with phosphate buffer pH 7.6 before placing the paper into a substrate solution containing 0.2 g of 2,6-dichlorophenol indophenol (Sigma Chemical Company, USA) and 0.01% H_2O_2 in 100 ml phosphate buffer pH 7.6 until the brown bands appeared, washed thoroughly with distilled water, then air-dried.

The sensitivity and specificity of the immunological assays were calculated using the method of Galen.[13]

RESULTS

With CLE as antigen for indirect ELISA it was found that sera of all of the healthy, parasite free individuals had OD < 0.163 (range = 0.000-0.163; $\bar{X} = 0.043$; SD = 0.038). Using an OD of 0.233 ($\bar{X}$ + 5 SD) as a cut-off, 30 of 37 serum samples from the trichinellosis patients at day 0 and all of the M2 and M4 specimens tested positive (Table 1). Then sensitivity and specificity of the assay at day 0 was 81.1% and 100%, respectively, while the sensitivity and specificity for sera collected at M2 and M4 was 100%.[13]

Patient sera collected at day 0 were subjected to Western blot analysis against SDS-PAGE fractionated CLE. All samples which had tested positive by indirect ELISA were also positive by Western blotting ie a specific band at M_r 109 was present.[6] In addition, 4 day 0 specimens which were ELISA negative tested positive by Western blotting (92% sensitivity). Representative positive and negative patterns are shown in Figure 1. All M2 and M4 patient samples were positive by Western blot analysis and all negative control samples were negative. For day 0 specimens, therefore, Western blot analysis was 91.9% sensitive and 100% specific. Sensitivity and specificity of sera at M2 and M4 was 100%.

DISCUSSION

The sensitivity and specificity of immunodiagnostic tests for antibody detection depend on the antigens used. ES and crude somatic antigens (CLE) prepared from infective stage larvae have been commonly used to diagnose trichinellosis. In 1992, we reported using ES antigen obtained from *in vitro* culture of larvae in an indirect ELISA to diagnose human trichinellosis.[2] This assay could not detect acute infection; symptomatic patients tested negative on admission to the hospital. However, the ELISA was 100% sensitive and specific

Table 1. OD of the indirect ELISA performed on sera of trichinellosis patients using CLE as the antigen.

Patient No.	Age (yr)	Sex	OD of serum samples taken at day 0	M_2	M_4
1	60	F	0.602	0.791	0.731
2	39	F	0.346	0.496	0.499
3	43	M	0.865	0.865	0.808
4	51	M	0.280	0.833	0.700
5	31	M	0.434	0.778	0.820
6	25	F	0.433	0.677	0.600
7	63	M	0.560	0.627	0.626
8	23	M	0.542	0.625	0.634
9	51	M	0.312	0.604	0.528
10	53	M	0.398	0.705	0.604
11	36	M	0.779	0.782	0.713
12	52	M	0.342	0.450	0.382
13	25	M	0.506	0.429	-
*14	29	F	0.108	0.529	0.629
15	30	M	0.364	0.441	0.506
16	25	M	0.400	0.680	0.596
17	48	F	0.506	0.704	0.602
18	37	M	0.404	0.654	0.697
19	19	M	0.486	0.648	0.546
20	18	M	0.470	0.736	0.699
*21	41	F	0.059	0.512	0.699
22	19	M	0.594	0.647	0.673
*23	43	F	0.053	0.565	0.497
24	17	M	0.427	0.747	0.747
25	66	M	0.300	0.470	0.464
26	45	M	0.377	0.525	0.483
*27	43	M	0.196	0.371	0.322
28	40	M	0.285	0.607	0.665
29	25	M	0.883	-	0.783
*30	66	F	0.095	0.552	0.252
31	35	M	0.408	0.578	0.561
*32	22	F	0.027	0.653	0.612
33	26	M	0.323	0.694	0.684
*34	30	F	0.012	0.544	0.583
35	30	F	0.282	-	-
36	29	M	0.593	-	-
37	29	M	0.235	-	-

- = serum sample was not available
* = patient whose day 0 serum sample was classified as indirect ELISA negative
F = female
M = male

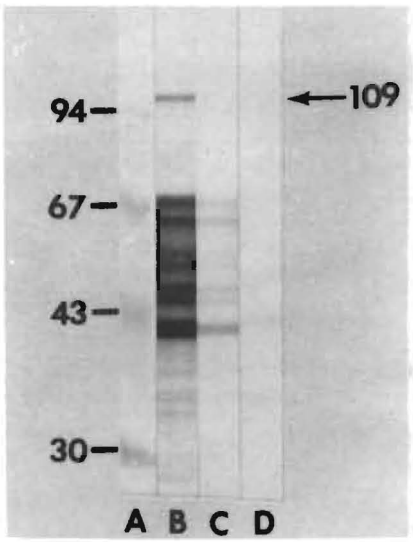

Fig. 1 Representatives of positive (lane B) and negative (lanes C and D) patterns of Western blot analysis. Note the presence of specific band (M_r 109) in the positive pattern and the absence of the band in lane C (patient with opisthorchiasis and strongyloidiasis) and lane D (parasite free normal control). A = molecular weight markers. Numbers indicate M_r ($\times 10^3$).

when performed on convalescent sera obtained 57 days later. In addition, ELISA using ES antigen was a useful test of cure; two years after treatment, patients in whom treatment was successful tested negative.

CLE has several advantages over ES antigen. First, CLE is easier to prepare, cheaper and gives a higher yield. Second, as presented in this communication and as has been previously reported.[14] CLE can detect early, acute infections. The problem of CLE cross-reacting with serum antibodies from other parasitic infections can be resolved by Western blot analysis looking for the trichinella-specific 109 kDa component.[6] Thus, CLE is the most appropriate antigen for the diagnosis of early disease and ES the antigen of choice for evaluating the success of chemotherapy. Both antiens are 100% specific when used in Western blot analysis.

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