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Trichinella spiralis-Specific Monoclonal Antibodies and Affinity-Purified Antigen-Based Diagnosis

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SUMMARY **Hybridomas secreting monoclonal antibodies (MAbs) to *Trichinella spiralis* were produced. Myeloma cells were fused with splenocytes of a mouse immunized with excretory-secretory (E-S) antigen of infective larvae. A large percentage of growing hybrids secreted antibodies cross-reactive to many of 23 heterologous parasites tested. Only 6 monoclones (designated 3F2, 5D1, 10F6, 11E4, 13D6 and 14D11) secreted MAbs specific to the E-S antigen and/or a crude extract (CE) of *T. spiralis* infective larvae. The 6 monoclones secreted IgM, IgG_3, IgM, IgG_3, IgG_3 and IgG_3, respectively. Clone 5D1 was selected to mass produce MAbs which were then coupled to CNBr-activated Sepharose CL-4B to prepare an affinity-purified antigen. Dot-blot ELISA with either purified antigen or CE was evaluated. There were 17 patients with acute trichinellosis and 76 individuals convalescing from *T. spiralis* infection (group 1). Controls were 170 patients with parasitic infections other than trichinellosis (group 2) and 35 healthy parasite-free controls (group 3). CE-ELISA was positive in all group 1 patients. However, sera from many group 2 patients also were reactive (opisthorchiasis-44.2%, schistosomiasis-44%, gnathostomiasis-30%, paragonimiasis-28.6%, taeniasis-27.3%, strongyloidiasis-23.1% and hookworm infections-20%). Affinity-purified antigen was 100% specific, all sera from group 2 and group 3 individuals tested negative. Although 74 of 76 patients (97.4%) with convalescing trichinellosis tested positive, sera from only 3 of 17 patients (17.6%) with acute *T. spiralis* were reactive. Thus, CE antigen is appropriate when sensitivity is needed, while purified antigen should be used when specificity is required. Dot-blot ELISA is easier to perform, more rapid and less expensive than indirect ELISA. Many samples can be assayed simultaneously, special equipment is not required, and results can be preserved for retrospective analysis. Dot-blot ELISA is therefore the method of choice for the rapid diagnosis of trichinellosis, particularly when more complex laboratory tests are unavailable.**

Both excretory-secretory (E-S) and crude somatic (CE) antigens have been used for the immunodiagnosis of trichinellosis. These antigens can be obtained from either adult worms or infective larvae of *T. spiralis*. Larval antigens are more often used because large numbers of parasites can be recovered from the muscles of animals such as laboratory mice. Adult worms, however, must be detached individually from the mucosa of the small intestine. E-S antigen from adult parasites is sometimes poorly immunogenic.[1] An antibody detection assay using E-S antigens of infective larvae is not only useful for diagnosis but may also serve as a test of cure.[2]

E-S antigen is more specific than CE antigen for the immunodiagnosis of human and porcine trichinellosis.[3-7] CE cross-reacts with antibodies elicited by other parasites, including *Schistosoma* spp.,[8] *Gnathostoma spinigerum*, *Opisthorchis viverrini*, *Capillaria philippinensis*, *Strongyloides stercoralis* and others.[9,10] CE-ELISA cross-reactivity can be reduced by either using a more specific immunoassay like Western blot[9] or by using purified antigen.

Purified antigen can be prepared by passing crude antigen through a specific monoclonal antibody-affinity column and then eluting out the bound antigen. In

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this study, specific monoclonal antibodies (MAbs) to *T. spiralis* E-S antigen were produced and then used to prepare an affinity column for antigen purification. Affinity-purified antigen specific to *T. spiralis* was then used in a dot-blot ELISA for the immunodiagnosis of human trichinellosis.

MATERIALS AND METHODS

Serum samples

Sera were obtained from three groups of individuals. Group 1 patient sera were obtained on admission from 17 individuals with acute trichinellosis. Sera from 76 convalescing patients were also tested. Group 1 patients were infected during 2 outbreaks of trichinellosis in Chiang Rai province, Northern Thailand. The first occurred in May 1989 in Mae Chan district, when 117 villagers ate under-cooked pork from a wild pig. The second outbreak was in 1992 at Ban Pasak, Chiang Saen district. Muscle biopsies taken the same day that convalescent sera were obtained revealed *T. spiralis* encysted larvae. The 170 patients in a group 2 had a variety of non-trichinella parasitic infections (Table 1). Group 3 sera were from 35 healthy individuals with no detectable parasitic infection.

Trichinella spiralis larvae and antigens

E-S antigen from a Thai isolate of *Trichinella spiralis* was prepared as previously described.[2] Individual mice were given 350 infective larvae orally and were sacrificed one month after infection. Muscle digestion was performed using 1% HCl-2% pepsin and larvae were collected by the Baerman's technique[11] with slight modifications.

Larvae were then washed thoroughly with sterile RPMI-1640 medium and cultivated in serum-free RPMI-1640 medium containing 2 mM L-glutamine, 10 mM HEPES, 50 μg/ml gentamycin and protease inhibitors: 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 mM N-tosyl-L-phenylalanine-chloromethyl ketone (TPCK). There were 5,000 larvae/ml of medium and cultivation was performed for 18 hours at 37°C in a 5% CO_2 incubator.[2] More than 95% of the larvae remained alive after culture, as shown by motility. The culture fluid was collected, centrifuged at 200 × *g* at 4°C for 5 minutes and dialyzed thoroughly against excess phosphate buffered saline (PBS), pH 7.2 at 4°C. The

Table 1 Diagnoses of patients of group 2 by parasitology and/or serological method

Amount of patients	Infection(s)/disease	Diagnostic method(s)
16	Schistosomiasis mekongi	*Schistosoma mekongi* eggs in stool
9	Schistosomiasis japonicum	*Schistosoma japonicum* eggs in stool
20	Gnathostomiasis	Western blot analysis with the presence of band at 24 kDa[20]
7	Paragonimiasis heterotremus	*Paragonimus heterotremus* eggs in stool and/or sputum and positive bands in Western blot analysis[21]
43	Opisthorchiasis viverrini	*Opisthorchis viverrini* eggs in stool and/or *Opisthorchis viverrini* adult worms in stool after Praziquantel treatment and purgation
26	Strongyloidiasis	Stool cultures were positive for filariform larvae of *Strongyloides stercoralis*[22]
11	Taeniasis	Mature segments of *Taenia* spp. in stool
10	Hookworm infection(s)	Hookworm eggs in stool
18	Malaria	*Plasmodium falciparum* in blood smears

preparation was concentrated by Amicon ultrafiltration through a PM10 membrane and protein content was determined[12] using bovine serum albumin as standard. E-S antigen was obtained.

CE antigens was prepared from larvae as previously described.[6] Larvae were homogenized by a glass tissue grinder in PBS containing protease inhibitors at pH 7.2. The homogenate was subjected to a MSE sonicator at 20 kHz in an ice bath for 10 minutes twice and the preparation then centrifuged at 10,000 × *g* at 4°C for 30 minutes. The supernatant was collected and its protein content determined.[12] E-S and CE antigens were kept in small aliquots at -70°C until use.

The heterologous antigens used for cross-reactivity testing of trichinella MAbs are shown in Table 2.

Mouse immunization

Five 7 week-old BALB/c mice were immunized intraperitoneally with 50 μg of *T. spiralis* E-S antigen in 0.1 ml of normal saline solution (NSS) mixed with equal volume of Freund's complete adjuvant. The mice were reimmunized intraperitoneally twice with 100 μg of the same immunogen mixed with Freund's incomplete adjuvant. Each mouse was bled from the retro-orbital plexus 14 days after the third immunization. Serum antibody titers were determined by indirect ELISA against the homologous antigen 10 μg/ml of coating buffer.[13] The mouse with the highest ELISA titer was selected as a splenocyte donor for hybridoma production. Sera of the remaining mice were pooled to be used as positive control serum (PS).

Hybridoma and MAb production

The donor mouse was reimmunized with 50 μg of the immunogen in 0.2 ml of NSS intravenously 3 days before cell fusion. On the day of cell fusion, serum was collected for use as immune serum (IS). The mouse was sacrificed, the spleen was dissected aseptically, washed several times with serum-free RPMI-1640 medium and placed on a fine nylon mesh in a small petri dish containing RPMI-1640 medium. The spleen was homogenized with a sterile glass syringe plunger. Single spleen cells were collected from the medium outside the mesh into a sterile plastic centrifuge tube and washed once with the same medium by centrifugation at 200 × *g* for 10 minutes at room temperature. Viability of the cells was checked by trypan blue exclusion. Immune splenocytes with more than 98% viability were fused with P3x-63-Ag8.653 myeloma cells non-immunoglobulin secreters with more than 98% viability, using polyethylene glycol 4,000 as a fusogen at a ratio of 10 spleen cells to 1 myeloma cell. Fused cells were suspended in hypoxanthine-azaserine selective medium to 1 × 10^6 cells/ml and aliquots of 200 μl were distributed into 96-well tissue culture plates. All plates were placed in a humidified 5% CO_2 incubator at 37°C and media was changed periodically. Supernatants from wells containing growing hybrids were collected and screened for antibodies against the homologous antigen by indirect ELISA. Cells from the antibody positive wells were cloned by limiting dilution using spleen cells of normal non-immune BALB/c mice as feeder cells. Culture supernatants from these clones (hybridomas) were retested against the homologous antigen as well as against the panel of the heterologous antigens listed in Table 2 for cross-reactivity by indirect ELISA. MAb antigenic specificities were determined by Western blotting against SDS-PAGE-separated homologous antigen and *T. spiralis* CE antigen.[9] Immunoglobulin isotyping was performed with Bio-Rad mouse immunoglobulin isotyping kits.[14]

Immuno-alkaline phosphatase staining of *T. spiralis* tissue sections

Frozen sections of *T. spiralis* larvae were reacted with monoclonal antibodies from hybridoma clone 5D1 (Fig. 2) and stained by immuno-alkaline phosphatase (Dakopatt, Denmark) to determine the anatomical localization of worm tissue recognized by MAbs.

Preparation of affinity-purified *T. spiralis* antigen

Hybridoma-secreting MAbs to *T. spiralis* (clone 5D1) with IgG_3 isotype were grown in large scale for bulk production of MAb 5D1. MAbs were attached to Sepharose CL-4B (Pharmacia, Uppsala, Sweden) to prepare an immunosorbent affinity column. Amicon concentrated MAb was repeatedly precipitated with saturated ammonium sulfate and MAb IgG was then coupled to CNBr-activated Sepharose CL-4B according to manufacturer's instructions. *T. spiralis* larvae CE was applied to the affinity column and adsorbed antigen was eluted out.[15] Affinity-

Table 2 Parasites used for preparing heterologous antigens

Name	Developmental stage and source
Angiostrongylus cantonensis	Male and female adult worms from lungs of infected rats
Ascaris lumbricoides	Adult worms from stool of a patient after purgation
Ascaris suum Cysticercus cellulosae	Kind gift from Dr. P. Setasuban, Department of Helminthology, Faculty of Tropical Medicine, Mahidol University
Dirofilaria immitis	Adult worms collected from hearts of infected dogs
Echinococcus granulosus	Hydatid fluid
Echinostoma ilocanum *Echinostoma malayanum* *Echinostoma revolutum* *Haplorchis taichui* *Hymenolepis nana* *Opisthorchis viverrini*	Adult worms from stools of infected individuals after Praziquantel treatment and purgation[17]
Opisthorchis felineus	Adult worm extract, a gift from Dr. Grenady, Martsinovski Institute, Moscow, Russia
Gnathostoma spinigerum	Infective larvae from livers of naturally infected eels[22]
Entamoeba histolytica	Trophozoites from axenic culture
Paragonimus heterotremus	Adult worms collected from cats infected two months previously with metacercariae collected from crabs[21]
Paragonimus westermani	A gift from Department of Social Medicine and Environment, Faculty of Tropical Medicine, Mahidol University
Plasmodium falciparum	Blood stages from *in vitro* culture
Schistosoma mansoni	Male and female adult worms were kind gift from Dr. H. Sugiyama, National Institute of Infectious Diseases, Tokyo, Japan
Schistosoma japonicum *Schistosoma mekongi*	Adult worms from mice infected with the respective cercariae
Strongyloides stercoralis	Filariform larvae[23]
Toxoplasma gondii	Tachyzoites; a kind gift from Dr. Vanna Mahakittikun, Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok

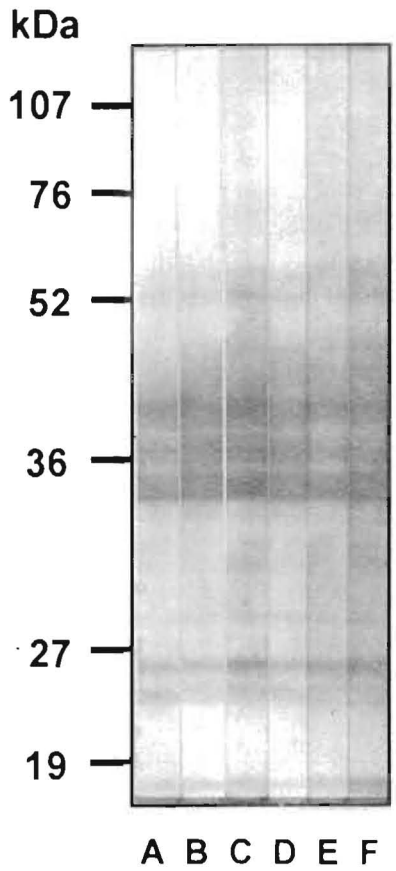

Fig. 1. Western blot patterns of MAbs from the six specific monoclones against SDS-PAGE-separated crude extract (CE) of *Trichinella spiralis*: Lanes A through F, Western blot patterns of immune mouse serum and MAbs from clones 3F2, 5D1, 10F6, 11E4, 10D6 and 14D1, respectively. Numbers at left are relative molecular masses x 10^{-3}.

purified antigen was used in the dot-blot ELISA to detect specific antibodies in serum samples from the 3 groups of individuals.

Dot-blot ELISA

Fifty microliter aliquots of either CE or affinity-purified antigen at a concentration of 1 μg/ml in PBS, pH 7.4 were dotted onto a nitrocellulose membrane (NCM) using a 96-well slot-blot device (Bio-Rad, USA) and air dried. The NCM was submerged in 3% bovine serum albumin (BSA) for 1 hour to block unoccupied sites. The NCM was cut into individual squares; each square accommodated one spot of the dotted antigen; all squares were then labeled and placed into ELISA wells containing individually diluted serum samples (1:200). The antigen-antibody reaction was allowed to take place for 30 minutes; the NCM pieces were washed individually with PBS at pH 7.4 and then placed in a container of goat anti-human IgG-horseradish peroxidase conjugate (Dakopatt, Denmark; diluted 1:1,000 in substrate buffer) fol-

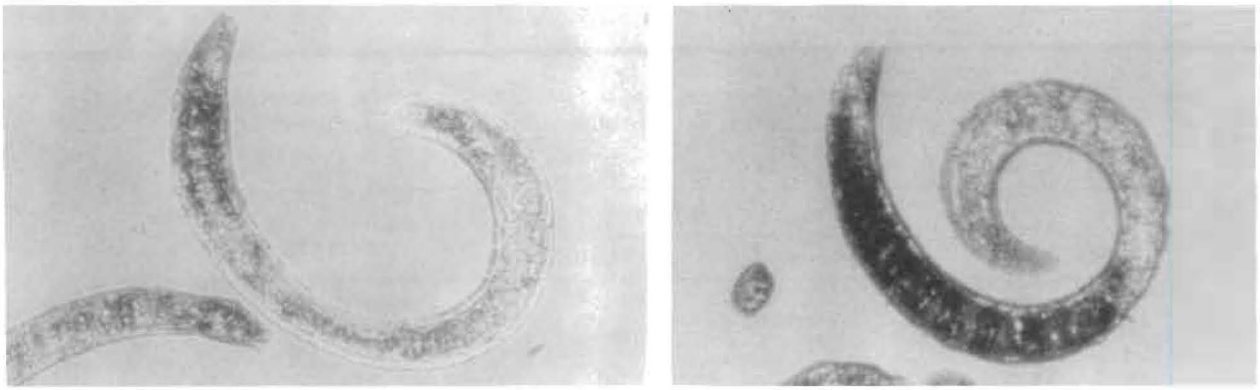

Fig. 2. Specificity of monoclonal antibodies from clone 5D1 to tissue of infective larvae of *Trichinella spiralis* as revealed by indirect immuno-alkaline phosphatase staining: A = negative control; B = MAbs reacted to the intestinal contents of the larva.

lowed by substrate, respectively. Positive and negative controls (a pool of five sera from patients with convalescing trichinellosis [diluted 1:200] and PBS, respectively) were included in the test. A positive dot-blot ELISA was seen as a brownish-red spot on the NCM square, where the antigen had been dotted and was clearly distinguishable from the negative control (NCM square to which PBS but no antibody had been added). Sensitivity, specificity and accuracy of the dot-blot ELISA for trichinellosis diagnosis were calculated according to Galen's method.[16]

RESULTS

Indirect ELISA antibody titers against homologous antigen were measured 14 days after mice received the last intraperitoneal injection. Mice 1, 2 and 3 had titers of 1:102,400. The fourth mouse had a titer of 1:51,200 and the fifth mouse had a titer of 1:25,600. Pooled sera from mice 2-5 were PS and mouse 1 was chosen as the splenocyte donor. Five hundred and thirteen of 2,640 wells contained growing hybrid cells (19.4%). Supernatants from 50 of 513 wells (1.9%) contained *T. spiralis* E-S antigen by indirect ELISA. Antibodies from these 50 wells were tested against crude extract of the infective stage of *Gnathostoma spinigerum* obtained from the livers of naturally infected eels.[17] Culture supernatants of 28 wells (56%) were positive by indirect ELISA, and only the cells from the 22 wells which did not cross react with *Gnathostoma spinigerum* antigen were cloned.

Six hybridomas whose culture supernatant MAbs did not react to any of the heterologous antigens listed in Table 2 were finally obtained. These monoclones were designated 3F2, 5D1, 10F6, 11E4, 13D6 and 14D11. All 6 clones secreted kappa light chains. Clones 3F2 and 10F6 produced IgM heavy chains. 5D1, 11E4, 13D6 and 14D1 produced IgG_3 (Table 3). Supernatant reciprocal indirect ELISA titers ranged from 256 to 1,024 (Table 3). Fig. 1 illustrates the antigenic specificities of MAbs to SDS-PAGE-separated *T. spiralis* CE. Clone 5D1 IgG_3 MAbs were used to prepare affinity-purified antigen. Tissue specificity of clone 5D1 MAb as determined by immuno-alkaline phosphatase staining is shown in Fig. 2. The MAb reacted to intestinal content of the parasite. Fig. 3 illustrates SDS-PAGE-separated patterns of purified antigen stained by silver and Coomassie brilliant blue dye. No sera from group 2 or 3 had positive dot-blot ELISA results with purified antigen. Three of 17 acute group 1 sera (17.6%) and 74 (including the 14 samples which were negative at the acute phase of illness) of 76 convalescent samples (97.4%) were positive using purified antigen. The sensitivity, specificity and accuracy of diagnostic assays using purified antigen were 17.6%, 100% and 92.1%, respectively, in acute trichinellosis and 97.4%, 100% and 99.2%, respectively, in convalescent trichinellosis. CE antigen-based dot-blot ELISA was positive in 100% of group 1 sera but was also positive in sera from as many as 44% of patients infected by parasites other than *T. spiralis* (Table 4).

Table 3 Specific hybridomas, their secreted immunoglobulins and the reciprocal indirect ELISA titers of their culture supernatants at stationary phase of growth

Hybridoma	Ig isotype		Reciprocal Indirect ELISA titer
	H chain	L chain	
3F2	μ	κ	512
5D1*	γ_3	κ	1,024
10F6	μ	κ	512
11E4	γ_3	κ	256
13D6	γ_3	κ	512
14D11	γ_3	κ	1,024

*The hybridoma the culture supernatant of which was used in the affinity chromatography for purification of *T. spiralis* specific antigen

Table 4 Results of dot-blot ELISA using affinity-purified antigen and crude somatic extract (CE) of *T. spiralis* infective larvae to detect antibodies in serum samples of all groups of individuals

Group infection/disease	No. positive cases/total (%) when the antigen used in the dot-blot ELISA was	
	Purified antigen	CE
1. Acute trichinellosis	3/17 (17.6)	17/17 (100)
Convalescing trichinellosis	74/76 (97.4)	76/76 (100)
2. Opisthorchiasis viverrini	0/43 (0)	19/43 (44.2)
Schistosomiasis	0/25 (0)	11/25 (44)
Schistosomiasis japonicum	0/9 (0)	7/9 (77.8)
Schistosomiasis mekongi	0/16 (0)	4/16 (25)
Gnathostomiasis	0/20 (0)	6/20 (30)
Paragonimiasis heterotremus	0/7 (0)	2/7 (28.6)
Taeniasis	0/11 (0)	3/11 (27.3)
Strongyloidiasis	0/26 (0)	6/26 (23.1)
Hookworm infections	0/20 (0)	2/10 (20)
Malaria	0/18 (0)	0/18 (0)
3. Normal controls	0/35 (0)	0/35 (0)

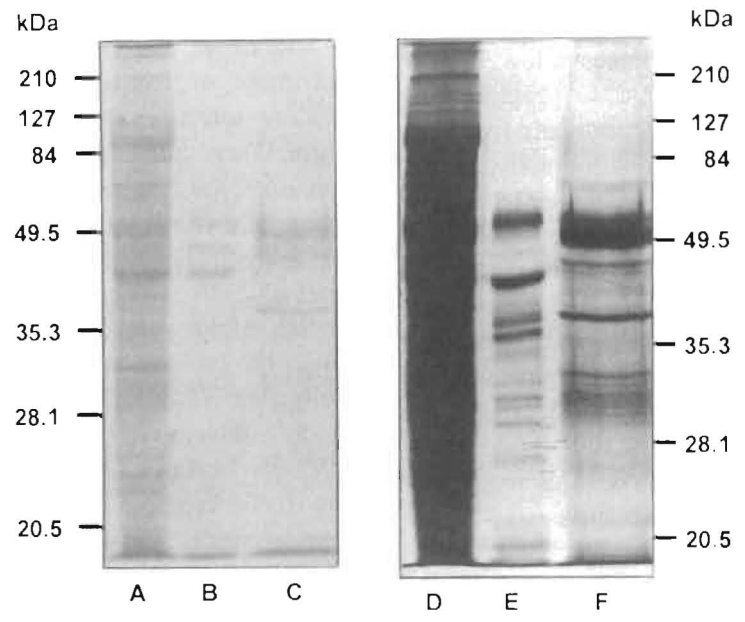

Fig. 3. SDS-PAGE-separated patterns of CE, E-S and affinity-purified antigen of *T. spiralis* stained with Coomassie brilliant blue (left; lanes A, B and C, respectively) and Silver stain (right; lanes D, E and F, respectively).

DISCUSSION

Potential applications of hybridoma technology to parasitic disease were outlined by Nabholz and Lambert in 1979.[8] MAbs of the desired immunoglobulin isotype directed against a specific epitope can be used to detect specific parasite antigen in clinical specimens. IgG isotype MAbs are appropriate for high antigen binding affinity while IgM isotypes provide strong antigen agglutination. Specific antibodies avoid the problems of cross-reactivity between parasite antigens and the non-specificity conventional polyclonal antisera. The detection of parasite antigen(s) in the host can be used to diagnose current infection, to test treatment efficacy, and can provide prevalence data for epidemiological surveys. Monoclonal antibodies

are ideal reagents for diagnosis, for strain typing, and for the detection of parasite variants such as African trypanosomes, *Trichinella* spp., *Gnathostoma* spp. and *Schistosoma* spp. Established hybridoma clones can produce an unlimited supply, stable, defined monoclonal antibodies. MAbs can be used to standardize parasite antigens and for the quality control of vaccine preparations. Monoclonal antibodies are excellent tools for purification of parasite antigens by affinity chromatography. Purified antigens, in turn, can be used in immunodiagnosis and in characterizing the host response to infection as antibody or cell-mediated or both.

Trichinellosis has been diagnosed in both humans and pigs by the detection of serum antibodies.[5,19] The sensitivity and specificity of these assays depends on the nature of the antigen and the timing of serum samples.[2,9,10] During symptomatic, acute illness, indirect ELISA using *T. spiralis* larval CE antigen was 100% sensitive in biopsy-confirmed trichinellosis.[6] However, cross-reactivity was observed with sera from patients infected with other parasites.[6] Dot-blot ELISA using CE as the antigen in this study confirmed that CE contains antigenic components shared by a broad range of parasites (see Table 4). E-S antigen prepared from infective larvae, on the other hand, yielded test results which could differentiate patients with trichinellosis from those infected with other parasites.[2] However, indirect ELISA results using E-S antigen require a high cut-off optical density to differentiate trichinellosis from other parasitic infections.[2] This suggests that E-S antigen preparations contains some components shared by other parasites. In this study, this suggestion is confirmed. Twenty-eight of 50 hybrids produced antibodies cross-reactive with somatic extract of infective larvae of *Gnathostoma spinigerum*. Clones of the remaining 22 hybrids still yielded MAbs which cross-reacted with parasites such as *Angiostrongylus cantonensis, Ascaris suum,* cysticercus, *Dirofilaria immitis, Echinostoma ilocanum, Echinostoma malayanum, Echinostoma revolutum, Fasciola gigantica, Haplorchis taichui, Opisthorchis viverrini, Paragonimus heterotremus, Paragonimus westermani, Plasmodium falciparum* and *Schistosoma* spp. (data not shown). Only 6 monoclones secreted MAbs which were specifically directed against *T. spiralis* E-S and CE antigens.

Preparing crude somatic parasite extracts is less laborious than preparing E-S antigen and CE yields are higher. Specific *T. spiralis* hybridomas allowed their secreted MAbs to capture. *T. spiralis*-specific antigens from CE by affinity chromatography. Eluted specific antigen can then be used to detect diagnostic antibody. We used MAbs secreted by clone 5D1 for affinity chromatography of CE extract; these MAbs were the IgG_3 isotype, which has high binding affinity. Dot-blot ELISA using this pure antigen was 100% specific for both acute and convalescing trichinellosis. It is sensitive (93.4%) for diagnosis of convalescing trichinellosis but less so (17.7%) during acute illness. The dot-blot ELISA offers several advantages over the indirect ELISA and Western blot assays previously described for serodiagnosis of trichinellosis.[2,6,9] The dot-blot ELISA is quicker, simpler and allows testing of multiple samples at a single time. Results can be seen without a microscope and no special equipment is required. We therefore recommend the CE-dot-blot ELISA during acute trichinellosis and affinity-purified antigen for confirmation during convalescence.

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