USE OF DELIPIDIZED ANTIGENS OF *TAENIA SOLIUM* METACESTODES IN IgG-ELISA FOR DETECTION OF NEUROCYSTICERCOSIS

Paron Dekumyoy[1], Sirivan Vanijanonta[2], Jitra Waikagul[1], Surapol Sa-nguankiat[1] and Martin Danis[3]

[1]Department of Helminthology and [2]Department of Clinical Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand; [3]Service de Parasitologie et Mycologie, Groupe Hospitalier Pitié-Salpêtrière, 47-83 Boulevard de l'Hôpital, 75651 Paris Cedex 13, France

Abstract. The development of IgG-ELISA for detecting neurocysticercosis is aimed at the routine laboratory, and requires a particular antigen preparation, an acceptable number of serum samples to be tested (both homologous and heterologous) and patients with a diversity of helminthic infections to rule out cross-reactions. This study characterizes IgG-antibodies from cases of neurocysticercosis by assaying the sera against ether-delipidized antigens (5 µg/ml) prepared from metacestodes of *Taenia solium*. The test had a sensitivity of 90% and a specificity of 83%. IgG-antibodies from heterologous serum samples elicited a number of false positives (25/147) from six different helminthic infections, *ie* paragonimiasis, echinococcosis, opisthorchiasis, ascariasis, taeniasis and fascioliasis. In additional tests to detect antibody levels to these stage-related antigens, one of three serum samples from *T. solium*-infected cases gave negative at OD value of 0.187 while the others yielded 0.472 and 0.576. Conversely, assays of all serum samples from neurocysticercosis cases reacted against antigens from *Echinococcus granulosus* cystic fluid, *Paragonimus heterotremus* and *Opisthorchis viverrini* adult worms. In comparison, the antigens from these three species yielded higher mean OD values when assayed against the corresponding infected serum samples. Furthermore, neurocysticercosis cases yielded OD values that are separate and distinct from those of paragonimiasis cases.

INTRODUCTION

Various diagnostic strategies for neurocysticercosis have involved conventional parasitological and immunodiagnostic technics, clinical manifestations and a whole range of special procedures: such as radiological examination, computer tomography and magnetic resonance imaging. These technics are often unavailable in rural areas as well as in cities of some developing countries. Furthermore, the high costs of such services also pose a problem to the financially deprived. However, immunodiagnosis of this disease is regularly required to prove infection, and many aspects of the process will be dependent on antigens used, availability of homologous serum samples, and the serological technics carried out. Different degrees of cross-reaction are also observed with a number of diverse heterologous serum samples. Several kinds of antigens are derived from adult worms and metacestodes of *Taenia solium* and from other species of *Taenia* to obtain an effective antigen for particular infections. Diagnosis of human neurocysticercosis has been done using antigens of adult worms and entire/parts of *Taenia solium* metacestodes as well as by using recombinant products from cDNA of *T. solium* (Diwan *et al,* 1982; Coker-Vann *et al,* 1984; Choromanski *et al,* 1990; Kalinna and McManus, 1996); antigens from other species, *eg T. saginata* extract from adult worms (Morakote *et al,* 1992), and the synthesized peptides from cDNA of *T. crassiceps* to detect the infection (Gevorkian *et al,* 1996).

Among immunological tests, ELISA and the immunoblot technic are the most commonly used in the routine diagnosis of various parasitic infections. ELISA systems are able to detect both antibodies (AB-ELISA) and circulating antigens (AG-ELISA) from serum and cerebrospinal fluid (Diwan *et al,* 1982; Estrada and Kuhn, 1985; Choromanski *et al,* 1990). ELISA is suitable for routine laboratory work due to the economy and simplicity of the method. The technic requires only a few micrograms of antigen and is therefore also possible even when only small amounts of test materials are available. It is a useful tool when other sophisticated diagnostic procedures are not available. Although the enzyme-linked immunoelectrotransferred blot (EITB) is reported to be a better technic than ELISA in detecting this disease, it requires a larger amount of antigen and is inconclusive with regards to the specific band(s) for detecting the infection (Gottstein

et al, 1986; Tsang *et al,* 1989; Plancarte *et al,* 1994; Simac *et al,* 1995).

It is important to evaluate the sensitivity and the specificity of the tests with the controls and the other parasitic infections of each geographical region due to the variety of parasites endemic in different areas, especially in the tropics. In this study, sera of Thai and French patients were tested by ELISA using an antigen preparation from ether-extracted whole cysts to characterize IgG-antibodies to neurocysticercosis and to various heterologous infections.

MATERIALS AND METHODS

Preparation of *Taenia solium* metacestodes antigens

Taenia solium metacestodes were excised from the muscles of naturally infected pigs, placed in 0.02% NaN_3-PBS (pH 7.4) and kept on ice. The cysts were washed thoroughly in PBS, and then manually ground in a mortar and pestle with the gradual addition of PBS containing enzyme inhibitors [0.1mM phenylmethylsulfonylfluoride (PMSF), 0.1mM N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 10 mM ethylene diamine tetraacetic acid (EDTA)] and alumina paste. The alumina paste was then separated from the mixture by low speed centrifugation at 100*g* for 15 minutes. The homogenate was further sonicated by 1 minute intervals for 30 minutes. The supernatant was collected after centrifuging at 45,000*g* for 45 minutes at 4°C, and then delipidized with an equal volume of cold ether. The ether was allowed to evaporate by simply airing the mixture, and the suspension was recentrifuged as above. The delipidized extract was collected and the protein content was determined by the method of Lowry *et al* (1951).

Human serum samples

The serum samples were collected from various sources and divided into three groups as follows:

Group 1. Cestode infections;

neurocysticercosis (France and Thailand)	20 cases
taeniasis	20 cases
echinococcosis	8 cases
sparganosis	2 cases

Group 2. Trematode infections;

paragonimiasis	18 cases
opisthorchiasis	11 cases
schistosomiasis	5 cases
fascioliasis	5 cases

Group 3. Nematode infections;

gnathostomiasis	8 cases
capillariasis	2 cases
hookworm infections	12 cases
strongyloidiasis	11 cases
trichinelliasis	8 cases
toxocariasis	4 cases
ascariasis	6 cases
filariasis	2 cases
trichuriasis	8 cases
angiostrongyliasis	7 cases

Ten positive HIV cases comprised of 5 Thais (kindly provided by Professor Prapon Pranupak and Miss Urai Chaisri, Chulalongkorn Hospital) and 5 from France. Twenty-two normal controls (16 Thais, 5 Australians and 1 German) were included in the test.

Antibody detection by indirect ELISA

The standard procedure of indirect ELISA was performed with some modifications. MicroELISA-wells were sensitized with 100 µl of antigen diluted in a carbonated buffer pH 9.6, at 37°C for 1 hour and followed by an overnight incubation at 4°C. The unbound antigens were eliminated with a washing solution (0.05% Tween20-PBS) for 3 times; 1 minute each by using a microshaker; after which 150 µl of 1% bovine serum albumin were added to each well, incubated for 1 hour at 37°C, and washed as above. Test serum samples were diluted with washing solution and 100 µl each of the diluted serum were put in triplicate wells, incubated at 37°C for 1 hour and then washed as above. The immune complexes were then combined with 100 µl of peroxidase-conjugated rabbit anti-human immunoglobulin G (gamma chain, Dakopatts) diluted in washing solution, incubated at 37°C for 1 hour. After washing, the reactions were then visualized with 100 µl of substrate (p-phenylenediamine dihydrochloride) at a 30-minute incubation. Optical density values were measured at 492 nm after

the addition of 1N NaOH to stop the reaction.

Consideration of positivity : The cut off value was established by the mean and the standard deviation of the optical density of 22 normal controls.

RESULTS

Determination of ELISA standardization

The optimal antigen concentration, conjugate and serum dilutions were found to be 5 µg/ml, 1:1,000 and 1:200 respectively as determined by the ELISA procedure mentioned above as performed during preparatory experiments. The reactivity and potential non-specificity were evaluated by testing 22 serum samples from negative controls. By using 5 µg/ml of ether-delipidized antigen, the mean value ($\bar{X}$) and standard deviation (SD) were determined to be 0.123 and 0.038 respectively. The negative-positive discriminating threshold was calculated by $\bar{X}$ + 7SD = 0.389.

Sensitivity and specificity

Based on the threshold value of 0.389, two of the 20 neurocysticercosis cases gave low absorbance values, *ie* a false negative result. Evaluation of the diagnostic sensitivity yielded 90 % while the specificity was at 83 % (Table 1). Additional tests for antibody levels to the related stage of this parasite, serum samples from three *Taenia solium*-infected cases yielded their respective OD's at 0.187, 0.472 and 0.576 which only one case showed negative.

Cross-reactivity among heterologous serum samples (n = 147) was tested and calculated to be 83% of the negatives (25/147) at the threshold value. This reference value resulted in a number of false positives among six different types of helminthic infections, *ie* taeniasis (n = 5/20; 25%), echinococcosis (n = 8/8; 100%), ascariasis (n = 1/6; 16.67%), paragonimiasis (n = 6/18; 33.33%), opisthorchiasis (n = 4/11; 36.36%) and fascioliasis (n = 1/5; 20%) (Tables 1, 2; Fig1). Conversely, the same technic was used to observe absorbance results of serum samples from neurocysticercosis cases as against *Echinococcus granulosus* cystic fluid antigens, (provided by the Institut de Medicine Tropicale Prince Leopold, Antwerp, Belgium),

Table 1

Demonstration of specificities and positivities at the threshold value of 0.389.

Infections	No.	Positive abs = 0.389	% Positive
Neurocysticercosis	20	18	90
Taeniasis	20	5	25
Echinococcosis	8	8	100
Sparganosis	2	-	-
Gnathostomiasis	8	-	-
Strongyloidiasis	11	-	-
Hookworm infection	12	-	-
Trichinelliasis	8	-	-
Capillariasis	2	-	-
Toxocariasis	4	-	-
Angiostrongyliasis	7	-	-
Ascariasis	6	1	16.67
Trichuriasis	8	-	-
Filariasis	2	-	-
Paragonimiasis	18	6	33.33
Opisthorchiasis	11	4	36.36
Schistosomiasis	5	-	-
Fascioliasis	5	1	20
HIV	10	-	-
Normal controls	22	-	-

and with antigens derived from *Paragonimus heterotremus* and *Opisthorchis viverrini* adult worms. By following each ELISA-diagnosis of these infections, all 20 neurocysticercosis cases showed absorbance values within the range of 0.043 - 0.570 ($\bar{X}$ = 0.186) against *E. granulosus* cystic fluid antigens while serum antibodies of eight echinococcosis cases fell between 0.222 - 0.988 ($\bar{X}$ = 0.568). Based on this mean value, only one neurocysticercosis case (from a Thai, OD = 0.570) showed a higher absorbance value. When against *Paragonimus* antigens, IgG-antibodies of neurocysticercosis cases gave absorbance values from 0.048 to 0.442 ($\bar{X}$ = 0.198) and those of paragonimiasis cases showed higher values from 0.676 to 0.886 ($\bar{X}$ = 0.812). No sera from neurocysticercosis cases revealed an OD over the value (0.812). Neurocysticercosis cases showed absorbance values from 0.054 to 0.715 ($\bar{X}$ = 0.258) and the values of opisthorchiasis cases ranged from 0.216 to 0.776 ($\bar{X}$ = 0.593) when assayed against

Table 2

Demonstration of absorbance and mean values of other helminthiasis cases with higher ODs than the threshold (0.389).

Infections	No. abs = 0.389	Absorbance range	$\bar{X} \pm SD$
Neurocysticercosis	20	0.216 - 1.101	0.705 ± 0.273
Taeniasis	20	0.113 - 0.460	0.285 ± 0.115
Echinococcosis	8	0.460 - 0.705	0.591 ± 0.095
Ascariasis	6	0.106 - 0.407	0.251 ± 0.107
Paragonimiasis	18	0.252 - 0.535	0.372 ± 0.091
Opisthorchiasis	11	0.015 - 0.519	0.315 ± 0.156
Fascioliasis	5	0.101 - 0.483	0.240 ± 0.147

Opisthorchis antigens. Two neurocysticercosis cases had readings over this mean, while two opisthorchiasis cases had low OD values (Fig 2).

In an attempt to observe reactivity of the antigen used, all sera of neurocysticercosis and normal controls were tested against PBS-extracted antigen with and without ether delipidization. The ether-extract showed higher reactive than PBS-extract by mean OD values; for neurocysticercosis cases, 0.705 and 0.630, and for normal controls, 0.123 and 0.107 respectively.

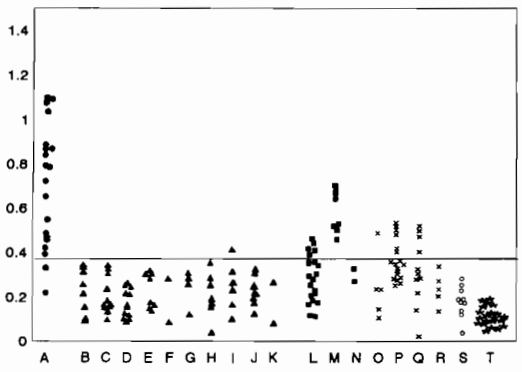

Fig 1–Scatter patterns of ELISA absorbance values using serum samples from neurocysticercosis cases and other infections. The following columns indicate as follows : A = neurocysticercosis, B = gnathostomiasis, C = strongyloidiasis, D = hookworm infection, E = trichinelliasis, F = capillariasis, G = toxocariasis, H = angiostrongyliasis, I = ascariasis, J = trichuriasis, K = filariasis, L = taeniasis, M = echinococcosis, N = sparganosis, O = fascioliasis, P = paragonimiasis, Q = opisthorchiasis, R = schistosomiasis, S = HIV, T = normal controls.

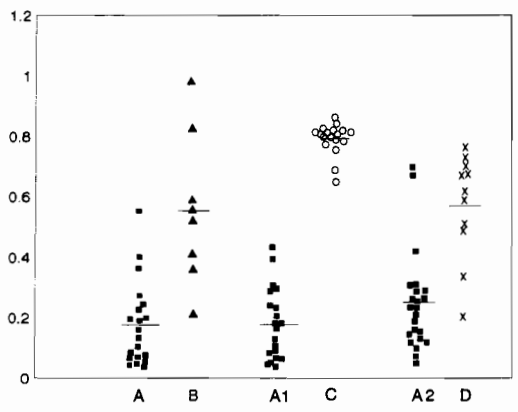

Fig 2–Alternative ELISA using *Echinococcus granulosus* cystic fluid antigens (A-B), *Paragonimus heterotremus* (A1-C) and *Opisthorchis viverrini* (A2-D) antigens with antibodies from both neurocysticercosis (A, A1, A2) and others; echinococcosis (B), paragonimiasis (C) and opisthorchiasis (D) cases to obtain the respective absorbance values. (The horizontal bars denote mean values.)

DISCUSSION

In this study, only two of neurocysticercosis serum samples gave false negatives (ODs = 0.338 and 0.216). It may be presumed that these patients have low level of antibodies which may be attributed to the anatomical position of cysts and calcified cysts (Zini *et al,* 1990; Wilson *et al,* 1991). Upon evaluation of the assays, antibodies from both serum and CSF of neurocysticercosis patients recognized antigenic molecules from the crude extract of *T. solium* metacestodes by ELISA. The interpretations of ELISA result by various sensitivities as follows; 79% and 61% sensitivities with PBS-extracted metacestodes and sera from patients from Mexico and from Irian Jaya respectively (Diwan *et al,*1982), 70% sensitivity (Coker-Vann *et al,* 1984) and 65% sensitivity for Diaz *et al* (1992). The other buffers are capable to extract the antigenic molecules which produced a range of sensitivities of the tests such as antigens from *T. solium* metacestodes; 96.6% by urea-soluble antigens and 86.6% by water-soluble antigens (Wang *et al,* 1993), 85.1% sensitive (Retamal *et al,* 1995), sensitivity 98.2% and specificity 94.1% when using saline-extract (Pialarissi and Nitrini, 1995). The antigens from Tris-HCl buffer-extracted *T. solium* scolices gave only 82.1% positive (Yong *et al,*1993). The purified antigens from the metacestodes also gave vary results such as 73% by using purified antigen B (Espinoza *et al,* 1982) and 80% from partially purified chromatofocused antigen (Coker-Vann *et al,* 1984). Other workers have used other species as sources of antigens, *eg* Gracia *et al* (1995) used *T. crassiceps* cysticerci instead of *T. solium* and detected antibodies by ELISA giving 99% precision rate. Our results showed sensitivity and specificity at 90% and 83% respectively with the PBS-ether-extracted antigens, twenty cases of homologous sera and many kinds of heterologous serum samples yielding results which were both slightly higher and lower than the results obtained from previous authors.

Various helminthic infections also contributed to a small number of false positives, 10 genera of nematode infections, only one out of sixty eight cases which was ascariasis case yielded false positive at slightly higher absorbance value (0.407). It might be high level of antibody against *Ascaris* worm itself, undetectable parasitic agents by fecal examinations and/or any previous parasitic infections. The group of cestode infections consisting of two genera also gave false positives; the OD values of five out of 20 cases of taeniasis were not much higher than the threshold value where all of these serum samples estimated their $\bar{X} \pm SD$ to be about 0.285 ± 0.115. All cases of echinococcosis gave a definite 100% false positives and produced rather high absorbance values. It seems difficult to eliminate the fact that cross-reactions with both crude and purified cysticercus antigens frequently occur with antisera from echinococcosis cases as proven in many other studies (Coker-Vann *et al,* 1984; Moro *et al,* 1992; McManus and Leggatt, 1993; Retamal *et al,* 1995). However, only one case of echinococcosis was officially reported in Thailand, and this patient never travelled out of his native land. Another Thai was infected from the Middle East during working there. The *T. solium* metacestodes are cross-reactive with antibodies from other parasitoses, *eg* fascioliasis and filariasis (Michault *et al,* 1988), schistosomiasis (Pammenter *et al,* 1992) which fascioliasis, including paragonimiasis and opisthorchiasis also led to false positives in this study. In the Fig 2, cases of neurocysticercosis and echinococcosis against *Echinococcus* antigens and cases of neurocysticercosis and opisthorchiasis against *Opisthorchis* antigens could not alternatively differentiate for diagnosis due to a number of their OD values were in the ranges of their mean values of 0.186-0.568 and 0.258-0.593 respectively. For *Echinococcus* antigens, almost all neurocysticercosis serum samples gave very low reactions. However, this point seems to be unsatisfactory. The situation is different with *Paragonimus* antigens, the results being quite good with OD values of neurocysticercosis cases distinctly separate from those of paragonimiasis cases. Cases of neurocysticercosis were not checked against *Ascaris lumbricoides, T. saginata* and *Fasciola* sp because of unavailable antigens and only one case each of ascariasis and fascioliasis gave false positive results, and except for the five cases of taeniasis.

We conclude that the ether-delipidized antigen is more reactive than PBS-extracted antigen alone since ether does not damage the antigenic molecules. If the ELISA system of our study is used to detect neurocysticercosis, any unknown suspected serum should be assayed against *E. granulosus* cystic fluid antigens. However, the results were not good in illustrating this point because the overlapping of OD values was observed in some cases of

neurocysticercosis. It may be suggested that one should therefore bear in mind the rarity of echinococcosis in Thailand and patients' history when interpreting results of such tests. Diagnosis for other parasitic diseases may be carried out as mentioned, including other parasitological procedures. Secondly, different kinds of antigen preparations including purified antigens should be used collectively to achieve a specific diagnosis of the infection. Besides these problems of cross-reactivity, the presence of brain tumors and rheumatoid factors also contribute to the problems of diagnosing neurocysticercoses accurately (Urarte *et al,* 1994) so it should be further considered.

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