ELISA FOR IMMUNODIAGNOSIS OF HUMAN GNATHOSTOMIASIS

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INTRODUCTION

Human gnathostomiasis caused by *Gnathostoma spinigerum* is an endemic disease in Thailand (Daengsvang, 1980). The diagnosis is usually presumptive on the basis of clinical features, with laboratory findings of eosinophilia in the peripheral blood and by exclusion of other diseases (Swanson, 1971). A confirmed or parasitologic diagnosis is rare since the parasite is recovered from only a small percentage of the patients by surgical removal of the worm or spontaneous emergence of the worm through skin, gingiva or in the urine.

A number of immunological tests have been applied to the diagnosis of gnathostomiasis (Cross, 1975), but the results have been unsatisfactory due to insensitivity or non-specificity i.e. cross reaction with other parasitic diseases (Tada *et al.*, 1966; Morisita *et al.*, 1969; Punyagupta and Pacheco, 1961; Kasemsuth *et al.*, 1981). Recently, the enzyme-linked immunosorbent assay (ELISA) (Engvall and Perlmann, 1972) have been used to detect antibodies associated with various helminthic infection such as filariasis, schistosomiasis, toxocariasis (Kagan, 1984), and angiostrongyliasis (Jaroonvesama *et al.*, 1985). Since the results appeared to be encouraging, the ELISA might be useful in the immunodiagnosis of gnathostomiasis. In this study, an ELISA system for gnathostomal antibodies in human sera was developed using larval antigens. The specificity and sensitivity of the test were evaluated.

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MATERIALS AND METHODS

Antigens were prepared from *G. spinigerum* third-stage larvae recovered from liver, stomach, intestine and body muscles of mice after one month of experimental infection with oral administration of second-stage larvae in infected cyclops. The larvae were cleaned by several washes with normal saline and finally suspended in distilled water. A crude water-extract of the third-stage larvae was prepared according to the method previously described by Sawada and co-workers, (1965). Aliquots of 5 ml of the extract were kept at –20°C after lyophilization.

Sera were collected from patients with cutaneous migratory swelling presumably due to gnathostomiasis, patients with a clinical diagnosis of eosinophilic meningo-myelo-encephalitis due to *G. spinigerum* (Punyagupta *et al.*, 1968; Boongird *et al.*, 1977), patients with eosinophilic meningo-encephalitis due to *Angiostrongylus cantonensis* based on clinical criteria (Punyagupta *et al.*, 1970) and a history of consuming poorly cooked *Pila* snails that resulted in an outbreak of the illness, and patients with other parasitic infections diagnosed by stool or sputum examinations. The other negative control sera were from healthy blood donors in Bangkok. Sera from parasitological confirmed cases of cutaneous gnathostomiasis

without evidence of other parasitic infections as revealed by stool examination served as positive controls.

In preparing the enzyme-labelled antihuman IgG, goat antihuman IgG (Kallestad Lot. 204 G20999) was labelled with alkaline phosphatase (Type VII, Sigma Chemical company, St. Louis, Mo; U.S.A.: specific activity 1090 units/mg protein) by the method described by Engvall and Perlmann, (1971).

The ELISA used was essentially that of Engvall and Perlmann, (1972) employing disposable polystyrene plates (Cooke Microtiter M220-29A, Dynatech Laboratories). The titre was determined visually and confirmed spectrophotometrically at 405 nm against the reading for control negative serum. Same positive serum from a parasitoligical confirmed case of cutaneous migratory swelling and negative serum from one single individual was used throughout the period of study for quality control purpose.

By checkerboard titration with reference positive (a parasitological confirmed case of gnathostomiasis) and control negative serum, the optimum dilution of enzyme-labelled antihuman IgG used was 1 : 500, and the optimum N-concentration of antigen used was 5 μg/ml.

RESULTS

Sera from 160 patients with cutaneous migratory swelling (CMS) presumably due to gnathostomiasis, 10 patients with a clinical diagnosis of gnathostomal eosinophilic-meningo-myeloencephalitis (EME-Gs) and 4 parasitological confirmed cases of gnathostomiasis were tested for *G. spinigerum* antibodies by ELISA. The results are shown in Table 1. Sera from all the parasitological confirmed patients and patients with clinical EME-Gs showed an ELISA titre of 1 : 400 and above, whilst only 56% of the CMS cases tested gave this result. Thus, at the cut-point titre of 1 : 400, the sensitivity of the test was reduced from 100% to 59% when the CMS cases were included (Table 3).

Sera from 67 healthy blood donors, 24 patients with eosinophilic meningo-encephalitis due to angiostrongyliasis (EME-Ac) and 92 patients that were positive for other parasitoses were tested to determine the specificity of the test. The results are shown in Table 2. At the titre of 1 : 400 and above, positive results were observed in 1.5% of blood donors, 33% of EME-Ac and 23% of other parasitic infections including hookworm, *Strongyloides stercolaris*, *Enterobius vermicularis*, *Ascaris lumbricoides*, *Trichuris trichiu-*

Table 1
Frequency distribution of ELISA titres in sera from cases of cutaneous migratory swelling (CMS), eosinophilic meningo-myeloencephalitis, typically due to gnathostomiasis (EME-Gs) and CMS with parasitological proof (PCMS).

Sources of sera	Reciprocal ELISA Titre						
	neg	25	100	400	1600	6400	Total
PCMS	-	-	-	2	2	-	4
EME-Gs	-	-	-	4	6	-	10
CMS	31	12	28	55	33	1	160
Total	31	12	28	61	41	1	174

Table 2

Frequency distribution of ELISA titres in sera from healthy blood donors, cases of eosinophilic meningo-encephalitis due to *A. cantonensis* (EME-Ac) and cases of other parasitic infections tested against *G. spinigerum* antigen.

Sources of sera	Reciprocal ELISA titre					
	neg	25	100	400	1600	Total
EME-Ac	2	6	8	8	-	24
Infection with						
Hookworm	1	1	1	1	-	4
S. stercolaris	-	-	-	1	-	1
E. vermicularis	1	-	-	-	-	1
O. viverrini	7	3	4	2	1	17
Lung-fluke	-	1	5	4	-	10
Taenia spp.	-	-	1	-	-	1
W. bancrofti	-	1	1	-	-	2
B. malayi & hookworm	-	-	1	1	-	2
P. falciparum	3	2	1	1	-	7
Mixed*	8	11	18	8	2	47
Blood donors	49	8	9	1	-	67
Total	71	33	49	27	3	183

* including: hookworm, *S. stercolaris*, *A. lumbricoides*, *T. trichiura*, *O. viverrini*, lung-fluke, *Taenia* spp., *F. buski*, *Echinostoma* spp., *P. falciparum*.

Table 3

Sensitivity and specificity of ELISA in sera from patients with and without gnathostomiasis.

ELISA ≥ 1 : 400	Gnathostomal infection	
	Confirmed or presumptive (n=174)	Negative (n = 183)
Positive (n = 133)	103	30
Negative (n = 244)	71	153
Sensitivity	(103/174) = 59.0%	
Specificity	(153/183) = 84.0%	
Predictive value	(103/133) = 77.0%	

ra, *Opisthorchis viverrini*, lungfluke, *Taenia* spp., *Trichinella spiralis*, *Fasciolopsis buski*, *Echinostoma* spp., *Wuchereria bancrofti*, *Brugia malayi* and *Plasmodium falciparum*. The specificity of the test was 84% at the titre of 1 : 400 and above (Table 3) when all samples other than CMS and EME-Gs were considered negative. In contrast, the specificity of the test for EME-Ac against EME-Gs was 67%. Table 3 shows that when the proportion of gnathostomiasis (174) in the sample (357) was 0.49, the predictive value at the titre of 1 : 400 or above was 77%. However in EME samples where the proportion of EME-Gs was 0.29, the predictive value was 56% at this titre.

DISCUSSION

The present study evaluated the sensitivity and specificity of the ELISA employing crude extract of the third-stage larvae of *G. spinigerum* in immunodiagnosis of the gnathostomiasis. In most cases of human gnathosto-

miasis the worms obtained surgically or from spontaneous emergence rarely reach maturity; only third-stage larvae or immature adults are recovered from patients (Daengsvang, 1980). It is generally believed that antigens used in a serodiagnostic test should be prepared from the same stage of parasite as that which is recovered from patients; that was the rationale in the selection of third-stage larvae for antigen preparation in this study.

Sensitivity of the ELISA was evaluated from parasitologically confirmed cases (PC MS) as well as presumptive cases i.e. CMS and EME-Gs. The overall sensitivity of the test was 59%, which might be an underestimation since the criteria for CMS cases depended on exclusion of other helminth-associated or allergic swelling based only on clinical features and geographical distribution. Some of the false negatives might actually be non-gnathostomal. On the other hand, it was also possible that antibody levels in CMS, if they were exclusively due to the gnathostomes, may be lower than those in EME-Gs where the worm migrates from the interstitial tissue to an enclosed CNS space or in PCMS where the worm sought to leave the host through the skin.

In determining the specificity, ELISA titre of sera from healthy blood donors, EME-Ac patients and patients with other parasitosis were used to detect nonspecific reaction; at the titre of 1 : 400 and above the specificity of the test was 84%. When only sera from patients with EME-Ac were tested against EME-Gs, the specificity was reduced to 67%. As in the case of sensitivity, the specificity might be an underestimation; the non-gnathostomal controls may have subclinical or latent infections with gnathostomes since patients with gnathostomiasis and those with angiostrongyliasis, opisthorchiasis and paragonimiasis share the habit of eating raw or half-cooked meat (Manson-Bahr and Apted, 1982). Hence some of the false-positive may actually be true-positives.

The ELISA for gnathostomiasis as performed in this study is not yet applicable for general diagnostic purposes. In the majority of patients, a reliable clinical diagnosis can be made from history and clinico-pathological features and the place of domicile of the patient. The ELISA would be helpful when such clinical evidence is lacking. On rare occasions, the serological test may be used in the differential diagnosis of the intestinal mass (Sirikulchayanonta and Chongchitnant, 1979; Laohapand *et al.*, 1981). A change in ELISA titre might also be useful as an indicator of diseases activity, especially when specific treatment for gnathostomiasis become available.

In conclusion the ELISA technique is potentially useful for the immunodiagnosis of gnathostomiasis. Nevertheless improvement of sensitivity and specificity is needed. The specificity might be improved by purification and fractionation of antigens. Simultaneous assay of patients' sera against a panel of antigens derived from *G. spinigerum*, *A. cantonensis*, *Paragonimus heterotremus* etc. might help in making an immunodiagnosis of helminthic infection in that homologous titre should be higher than heterologous titre. Other immunodiagnostic approach includes the detection of circulating antigen or antigens in blood or CSF using highly specific antibodies such as monoclonal antibodies.

SUMMARY

An ELISA for immunodiagnosis of human gnathostomiasis using a crude water extract of third-stage larvae of *G. spinigerum* as antigen, and alkaline phosphatase labelled goat antihuman IgG in the indicator system was developed and evaluated. At the titre of 1 : 400 and above positive results were observed in 100% of 4 parasitological confirmed

and 10 eosinophilic meningo-encephalitis (EME) typical of gnathostomiasis cases, 56% of 160 cutaneous migratory swelling cases, 33% of 24 cases with EME typical of *A. cantonensis* infections, 23% of 92 cases with other parasitic infections and 1.5% of blood donors. The overall sensitivity was 59% and specificity 84%. The predictive value was 77%. The results indicated that ELISA is potentially useful for immunodiagnosis of gnathostomiasis but improvement of sensitivity and specificity is needed.

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