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ORIGINAL PAPER

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Study of specific IgG subclass antibodies for diagnosis of *Gnathostoma spinigerum*

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Abstract *Gnathostoma spinigerum* infection is endemic in Thailand and many Asian countries. Current diagnosis is the skin test and enzyme-linked immunosorbent assay (ELISA) for IgG antibody against the *G. spinigerum* third-stage larvae (L3), but cross-reactivity is common. We evaluated the sensitivity and specificity of anti-*G. spinigerum* L3 IgG subclass antibodies for diagnosis of 43 patients with gnathostomiasis. The majority of patients with gnathostomiasis (91%) had eosinophilia. While the anti-*G. spinigerum* L3 IgG1 antibody provided the highest sensitivity (98%), the anti-*G. spinigerum* L3 IgG2 antibody had the highest specificity (88%). The ELISA that detected anti-*G. spinigerum* L3 IgG1 antibody could be a reliable laboratory screening test, while anti-*G. spinigerum* L3 IgG2 antibody could be used to confirm the diagnosis.

Introduction

The infection caused by *Gnathostoma spinigerum* is common in Southeast Asia, mainly in Thailand, Japan, China, and India (Miyazaki 1960; Daengsvang 1980; Rusnak and Lucey 1993), with a few reported cases in Australia (Moorhouse et al. 1970), Africa (Chhuon et al. 1976), Mexico and Ecuador (Ollague et al. 1984; Ogata et al. 1998). The life cycle, which has dogs and various felines as the definitive hosts (Miyazaki 1960; Daengsvang 1981), involves two intermediate hosts. The first intermediate hosts are crustaceans and copepods. The second intermediate hosts are fresh-water fishes and snakes harbouring the infective third-stage larvae (L3).

Humans are accidental hosts, acquiring infection by eating L3 encapsulated in raw or partially cooked fresh-water fishes, especially *Monopterus alba* (swamp eel), *Fluta alba* (eel), *Charias batrachus* (catfish), and *Channa striatus* (snake-headed fish) (Daengsvang 1981; Rojekittikhun et al. 1989; Saksirisampant et al. 2002). After ingestion, the larvae migrate through various tissues and cause various symptoms of ''larva migrans''. The most common clinical manifestation is subcutaneous or cutaneous intermittent migratory swelling. Most importantly, L3 may migrate to the central nervous system (CNS) and cause serious effects such as myeloencephalitis and meningitis with long-term sequelae or even death (Chitanondh and Rosen 1967; Punyagupta and Juttijudata 1968; Boongird et al. 1977; Schmutzhard et al. 1988). Gnathostomiasis is one of the three most common parasitic infections that involve the CNS in Thailand (Vejjajiva 1978; Tuntipopipat et al. 1989).

The definitive diagnosis of gnathostomiasis can be made by recovery of the migrating larvae from skin lesions. Probable cases of gnathostomiasis are diagnosed by a history of eating raw or partially cooked fishes, subcutaneous or cutaneous intermittent migratory swelling, and eosinophilia. The intradermal test using crude parasite extract has been used as a confirmatory test with high sensitivity but with cross-reactivity (Daengsvang 1980; Chanthachume et al. 1988). High levels of specific IgG (Suntharasamai et al. 1985; Dharmkrong et al. 1986) and IgE antibodies (Soesatyo et al. 1987; Saksirisampant et al. 2001) using *G. spinigerum* L3 crude extract in enzyme-linked immunosorbent assay (ELISA)

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have been shown with high sensitivity and reasonable specificity, but cross-reactivity with other parasites has not been eliminated. Specific IgG subclass antibodies have been shown to provide improved specificity over the total IgG antibody for the diagnosis of many parasitic infections, such as ascariasis (Chatterjee et al. 1996), echinococcosis (Grimm et al. 1998), leishmaniasis (Khairul et al. 1999), and filariasis (Rahmah et al. 1994; Triteeraprapab et al. 1998, 2001a, b; Nuchprayoon et al. 2001). Such studies have not been carried out for gnathostomiasis. We reported here the specific IgG subclass antibodies in patients with gnathostomiasis.

Materials and methods

Samples

Patients

Sera were collected from 43 patients who were referred to the Parasitology Clinic, King Chulalongkorn Memorial Hospital, Bangkok, Thailand. Seventeen (40%) patients were parasitologically confirmed as gnathostomiasis and 26 (60%) patients were diagnosed with gnathostomiasis by the following clinical criteria: (1) subcutaneous or cutaneous intermittent migratory swelling, itching, and pain, (2) a history of eating raw or partially cooked fresh-water fishes, and (3) positive for the skin test using somatic crude extract of *G. spinigerum* L3 (Daengsvang 1986). There was no evidence of other parasitic infections in stool or blood.

Controls

Sera were obtained from 100 healthy medical students with no previous history of subcutaneous or cutaneous intermittent migratory swelling, and no other parasitic infections detected from stool or blood at the time of specimen collection. Sera were also collected from 26 individuals infected with other parasites including hookworms (2), *Trichuris trichiura* (1), *Strongyloides stercoralis* (6), *Capillaria philippinensis* (1), *Parastrongylus* (*Angiostrongylus*) *cantonensis* (6), *Wuchereria bancrofti* (1), *Brugia malayi* (1), *Taenia* spp. (3), *Opisthorchis viverrini* (3), and *Blastocystis hominis* (2). There were no clinical manifestations of gnathostomiasis in patients with other parasitic infections.

This study was approved by the Ethics Committee of the Faculty of Medicine at Chulalongkorn University, Bangkok, Thailand. Informed consent was obtained from each participant. All individuals were informed about the potential danger of gnathostomiasis, and how to prevent it. Those who had parasitic infections were treated with appropriate treatments (Kraivichian et al. 1992).

Detection of other parasites

Diagnosis of other parasitic infections was obtained by stool examination using the formalin–ether concentration method (Nuchprayoon et al. 2002) and Giemsa stain of thin and thick blood films as previously described (Triteeraprapab et al. 2000).

Blood sample collection and complete blood count

Approximately 5 ml of venous blood was obtained from each individual under a sterile technique. Two milliliters of EDTA blood were used for an automated complete blood count (CBC) with differentials (Technicon H*3, Bayer, New York, USA), from which the absolute eosinophil count (AEC) was obtained (Triteeraprapab and Nuchprayoon 1998). Sera were separated from 3 ml clotted blood and stored at −70°C until tested by ELISA for specific total IgG and IgG subclass antibodies.

Preparation of *G. spinigerum* antigens

The infective L3 of *G. spinigerum* were dissected from livers of naturally infected eels. The crude antigen extract of L3 s was prepared as previously described (Saksirisampant et al. 2002). Briefly, the liver samples from swamp eels (*Monopterus alba*) were pooled and digested at 37°C with 1.5% pepsin pH 2.0 for 4 h. The encysted *G. spinigerum* L3 were isolated from the digested liver tissue under a dissecting microscope and washed several times with sterile normal saline. Then, the *G. spinigerum* L3 were homogenized in a ground-glass tissue grinder followed by sonication under an ultrasonic disintegrator (Soniprep 150, MSE Scientific Instruments, UK) at an amplitude of 10 μm for 1 min. The sonication step was repeated until most of the intact cells were broken as judged by microscopic examination. The sonicated suspension was centrifuged at 11,090 *g* at 4°C for 25 min. The aqueous supernatant of the L3 extract was assayed for its protein concentration by a micromodification of the Folin-Ciocalteau method (Lowry et al. 1951).

ELISA for anti-*G. spinigerum* L3 total IgG and IgG subclass antibodies

Antigen concentrations and serum and conjugate dilutions were optimized by checkerboard titration using representative sera from healthy controls, patients with gnathostomiasis, and those with other parasitic infections. The ELISAs for anti-*G. spinigerum* L3 total IgG and IgG subclass antibodies were performed using the procedure previously described (Triteeraprapab et al. 1998) with minor modifications. Briefly, 100 μl of *G. spinigerum* L3 extract in 0.05 M carbonate buffer pH 9.6 [8 μg ml^{-1} (for total IgG and IgG3 antibody tests), 4 μg ml^{-1} (IgG2), 2 μg ml^{-1} (IgG1), or 1 μg ml^{-1} (IgG4)] were coated onto each well of microtiter plates and incubated overnight at 4°C. After three washes with 0.01 M phosphate-buffered saline/0.05% Tween 20 (PBS/T20) pH 7.4, each well was blocked with 100 μl of 2% non-fat dried milk (Carnation) in PBS/T20 at 37°C for 30 min. A 100-μl volume of each serum sample was added to each well at 1:200 (total IgG), 1:200 (IgG1), 1:400 (IgG2), 1:100 (IgG3), or 1:1,600 (IgG4) dilution, then incubated for 1 h at 37°C or overnight at 4°C. After five washes, anti-human total IgG-, IgG1-, IgG2-, IgG3- or IgG4-horseradish peroxidase conjugates (Zymed, South San Francisco, Calif.) diluted in PBS/T20 (1:1,000 for total IgG and IgG4, 1:500 for IgG1, IgG2, and IgG3) were added to each well and incubated at 37°C for 30 min. After another ten washes, 100 μl of a substrate mixture of 3,3′,5,5′-tetramethylbenzidine (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) was added to each well and kept in the dark at room temperature for 30 min. The reactions were stopped by adding 50 μl of 4 N H_2SO_4. The optical density (OD) was read at 450 nm.

Statistical analysis

Data were recorded and analyzed by using Microsoft Excel 6.0. Blood counts were expressed as mean ± standard deviation (SD). Chi-square tests were used to compare binary data. Unpaired *t*-tests were used to evaluate statistically significant differences of the absolute number of white blood cell and eosinophil counts. The anti-*G. spinigerum* L3 antibody levels were analyzed using the unpaired *t*-test of the OD values. Mean + 3 SD of OD values from healthy controls were used as the cut-off points for analysis of the sensitivity, specificity, positive predictive value (PPV; probability that a person who has a positive test result really has the disease), negative predictive value (NPV; probability that a person who has

a negative test result really is healthy), and accuracy (proportion of all tests that are correct) of each ELISA test. Predictive values are useful for physicians to aid diagnosis of the disease. High NPV is useful to exclude the disease and high PPV is useful to confirm the disease.

Results

Patient characteristics

Forty-three patients with gnathostomiasis, aged 16–60 years (mean ± SD , 33.1 ± 10.6) participated in the study. Thirteen were males and 30 were females. All but one (97%) had a history of consumption of raw or partially cooked fishes. All patients had subcutaneous or cutaneous intermittent migratory swelling, pain, and itching, and a positive skin test. The skin involvement was found at upper extremities (51%), face (31%), lower extremities (11%), abdomen (5%), and buttock (2%).

Eosinophilia in patients with gnathostomiasis

Forty-one patients had available CBC data and of these patients those with gnathostomiasis had a significantly higher total white blood cell count (mean ± SD, 7,677 ± 2,149 cells μl^{-1}) than those in the healthy control group (6,561 ± 1,735 cells μl^{-1}, $P = 0.002$). This was mainly due to a higher AEC in the patient group (1,360 ± 830 cells μl^{-1}) compared with the control group (158 ± 141 cells μl^{-1}, $P < 0.001$). The majority of patients with gnathostomiasis (37 of 41; 91%) had eosinophilia (AEC > 500 cells μl^{-1}, Table 1). Eosinophil counts from patients with other parasites were not available.

Anti-*G. spinigerum* L3 total IgG antibody levels in patients with gnathostomiasis

The levels of total IgG antibody against *G. spinigerum* L3 were significantly higher in patients with gnathostomiasis (mean OD 0.371) than the healthy control group (mean OD 0.004, $P < 0.001$) and the group with other parasitic infections (mean OD 0.150, $P < 0.001$, Fig. 1). The specific total IgG antibody levels among patients with parasitologically confirmed gnathostomiasis (mean OD 0.249) were different from patients with clinically diagnosed gnathostomiasis (mean OD 0.451, $P = 0.006$, data not shown).

However, significant cross-reactivity was demonstrated when using ELISAs for total IgG antibody. Forty-one of 43 patients with gnathostomiasis were positive (sensitivity 95%), while 24 of 26 patients with other parasitic infections were also positive for anti-*G. spinigerum* L3 total IgG antibody (specificity 8%) (Table 2).

Anti-*G. spinigerum* L3 IgG1 antibody levels in patients with gnathostomiasis

The levels of IgG1 antibody against *G. spinigerum* L3 were much higher in patients with gnathostomiasis (mean OD 1.663) than the control group (mean OD 0.106, $P < 0.001$) and patients with other parasitic infections (mean OD 0.307, $P < 0.001$, Fig. 1). The specific IgG1 antibody levels among patients with parasitologically confirmed gnathostomiasis (mean OD 1.436) were not statistically different from patients with clinically diagnosed gnathostomiasis (mean OD 1.785, $P = 0.169$).

Except for one, all patients ($n = 43$) with gnathostomiasis were positive (sensitivity 98%), while 10 of 26 patients with other parasitic infections were positive for anti-*G. spinigerum* L3 IgG1 antibody (specificity 62%). The NPV of IgG1 ELISA was high (94%) (Table 2).

Anti-*G. spinigerum* L3 IgG2 antibody levels in diagnosis of gnathostomiasis

Similar to the total IgG, the levels of IgG2 antibody against *G. spinigerum* L3 were significantly higher in patients with gnathostomiasis (mean OD 0.310) than the healthy control group (mean OD 0.010, $P < 0.001$) and patients with other parasitic infections (mean OD 0.024, $P < 0.001$, Fig. 1). The specific IgG2 antibody levels among patients with parasitologically confirmed gnathostomiasis (mean OD 0.254) were not different from patients with clinically diagnosed gnathostomiasis (mean OD 0.346, $P = 0.168$, data not shown).

The majority of patients (37 of 43) with gnathostomiasis were positive (sensitivity 86%), while only 3 of

Table 1 White blood cell counts (*WBC*) and absolute eosinophil counts (*AEC*) in patients with gnathostomiasis compared with healthy volunteers (*Control*)

		Patients ($n = 43$)	Control ($n = 100$)	*P* value
Sex	male	13 (30%)	34 (34%)	0.66[b]
	female	30 (70%)	66 (66%)	
Age	range	16–60	18–28	< 0.001[b]
	mean ± SD	33.1 ± 10.6	20 ± 2	
WBC[a]	range	3,500–15,150	37,40–11,520	0.002[c]
	mean ± SD	7,677 ± 2,149	6561 ± 1,735	
AEC[a]	range	255–3,870	19–906	< 0.001[c]
	mean ± SD	1,360 ± 830	158 ± 141	
	AEC < 500 μl^{-1}	4 (9%)	97 (97%)	< 0.001[b]
	AEC > 500 μl^{-1}	37 (91%)	3 (3%)	

[a]Data from 41 patients
[b]Using the chi-square test
[c]Using the unpaired *t*-test

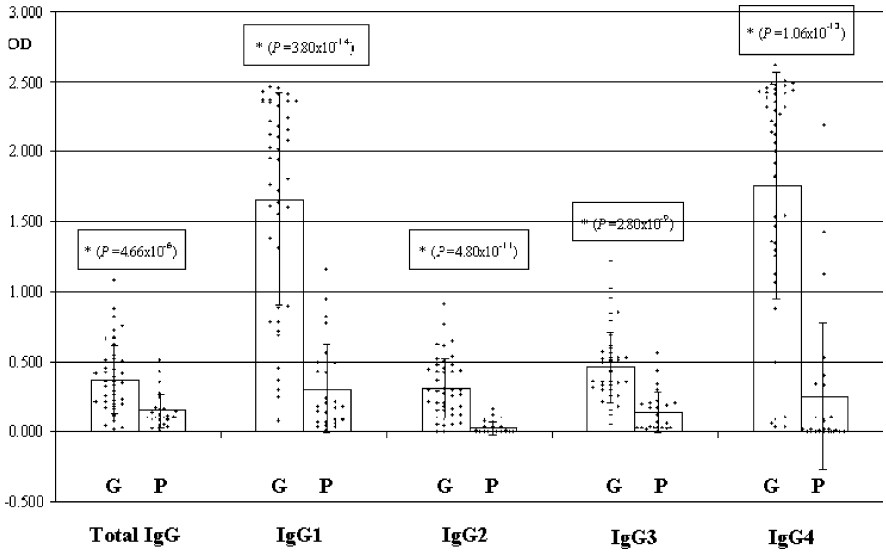

Fig. 1 Opical density values (mean ± SD) of the ELISA reactions for IgG and IgG subclass antibodies in patients with gnathostomiasis (G) compared to those with other parasitic infections (P).*P value obtained using unpaired t-test

Table 2 Sensitivity, specificity, accuracy, positive predictive value (*PPV*), and negative predictive value (*NPV*) of ELISA for diagnosis of human gnathostomiasis

	ELISA for specific anti-*Gnathostoma spinigerum* third-stage larvae				
	Total IgG (%)	IgG1 (%)	IgG2 (%)	IgG3 (%)	IgG4 (%)
Sensitivity	95	98	86	95	93
Specificity	8	62	88	54	65
Accuracy	62	84	87	80	83
PPV	63	81	93	77	82
NPV	50	94	79	88	85

26 patients with other parasitic infections were positive for anti-*G. spinigerum* L3 IgG2 antibody (specificity 88%). The PPV of IgG2 ELISA in this study was high (93%) (Table 2).

Anti-*G. spinigerum* L3 IgG3 antibody levels in patients with gnathostomiasis

Similar to the total IgG, the levels of IgG3 antibody against *G. spinigerum* L3 were significantly higher in patients with gnathostomiasis (mean OD 0.462) than the healthy control group (mean OD 0.061, $P < 0.001$) and cases with other parasitic infections (mean OD 0.140, $P < 0.001$, Fig. 1). The specific IgG3 antibody levels among patients with parasitologically confirmed gnathostomiasis (mean OD 0.453) were not different from patients with clinically diagnosed gnathostomiasis (mean OD 0.468, $P = 0.844$).

Most patients (41 of 43) with gnathostomiasis (sensitivity 95%), and almost half (12 of 26) of the patients with other parasitic infections were positive for anti-*G. spinigerum* L3 IgG3 antibody (specificity 54%) (Table 2).

Anti-*G. spinigerum* L3 IgG4 antibody levels in patients with gnathostomiasis

Similar to the IgG1 antibody, the levels of IgG4 antibody against *G. spinigerum* L3 were much higher in patients with gnathostomiasis (mean OD 1.762) than the healthy control group (mean OD 0.011, $P < 0.001$) and the group with other parasitic infections (mean OD 0.255, $P < 0.001$, Fig. 1). The specific IgG4 antibody levels among patients with parasitologically confirmed gnathostomiasis (mean OD 1.471) were not different from patients with clinically diagnosed gnathostomiasis (mean OD 1.952, $P = 0.057$).

Most patients (40 of 43) with gnathostomiasis were positive (sensitivity 93%), while 9 of 26 patients with other parasitic infections were positive for anti-*G. spinigerum* L3 IgG4 antibody (specificity 65%) (Table 2).

Cross-reactivity of the total IgG and IgG subclass antibodies with other parasitic infections

The IgG antibody against *G. spinigerum* L3 crude extract cross-reacted with many parasitic infections, particularly helminth parasites. Among 26 patients with other parasitic infections, the total IgG ELISAs for gnathostomiasis were positive in all patients infected with hookworms (2), *T. triciura* (1), *S. stercoralis* (6), *C. philippinensis* (1), *P. cantonensis* (6), and *W. bancrofti* (1) as well as some flat worms including *O. viverrini* (all of 3), *Taenia* spp. (1 of 3), and the protozoan *B. hominis* (all of 2) (data not shown). The specificity of ELISA test can be improved by detection of IgG subclass antibodies (Table 2). However, for all subclasses except IgG2, the most significant cross-reactivity was still observed in

patients infected with helminth parasites such as *P. cantonensis* (67%), hookworms (50%), *S. stercoralis* (33%), as well as *O. viverrini* (50–100%). The IgG2 antibody has the least cross-reactivity; cross-reaction was observed in only one of six patients with *P. cantonensis*, and cross-reactivity with other parasites including *T. trichiura*, *S. stercoralis*, *C. philippinensis*, *B. malayi*, *Taenia* spp., *O. viverrini*, and *B. hominis* was excluded

Discussion

Because humans are not the definitive host of *G. spinigerum*, the worms are unable to reach adulthood in the human body. However, the parasite migrates to various tissues, resulting in migratory swelling. Recurring swelling develops randomly, but is reported mainly in the upper and lower extremities. This is not because of the lodging of the parasite, but probably because it is more easily detected by patients. Similar to previous reports (Daengsvang 1980; Rojas-Molina et al. 1999), subcutaneous or cutaneous intermittent migratory swelling of upper extremities was the most common clinical manifestation (51%) in this study (data not shown).

Eosinophilia is a common finding in tissue parasitic infections including gnathostomiasis (Daengsvang 1980; Ollague et al. 1984; Pirompakdi 1984; Migasena et al. 1991). Eosinophilia is observed in more than 90% of patients with gnathostomiasis, ranging from 3% to 96% of the total white blood cell count (Daengsvang 1980; Rusnak and Lucey 1993). However, previous studies have not reported data on AEC, which is a more appropriate assessment for eosinophilia (Triteeraprapab and Nuchprayoon 1998). In this study, we reported eosinophilia as an AEC >500 cells μl^{-1} in gnathostomiasis patients (91%, Table 1).

G. spinigerum-specific IgG and IgE antibodies have been previously shown to be elevated in patients with gnathostomiasis as determined by ELISA (Suntharasamai et al. 1985; Dharmkrong-at et al. 1986; Soesatyo et al. 1987; Saksirisampant et al. 2001), suggesting that the method would be useful with a high degree of reliability in confirming a clinical diagnosis of gnathostomiasis in humans. However, specificities of specific IgG and IgE ELISAs are impaired by cross-reactivity with other parasites, especially *P. cantonensis*. The use of *G. spinigerum* p24 protein as the antigen in ELISA may provide a high sensitivity and specificity to gnathostomiasis (Nopparatana et al. 1991; Tapchaisri et al. 1991). Molecular cloning of the protein would be useful, but the difficulty of purifying the p24 protein is not practical for field application. Thus ELISAs using *G. spinigerum* L3 crude extract as the antigen should be more applicable for general diagnostic purposes.

In this study, using ELISA with *G. spinigerum* L3 crude extract as the test antigens, we confirmed that the mean OD values of total IgG antibodies, as well as all IgG subclass antibodies (especially IgG1 and IgG4), were significantly higher in gnathostomiasis patients than in patients infected with other parasites ($P < 0.001$) (Fig. 1). It has been found that while serum IgG4 antibody is increased with length of infection or chronic disease (Aalberse et al. 1983), the serum IgG1 antibody is increased concomitantly with IgE antibody in helminth infections (Zakroff et al. 1989).

In previous reports, the sensitivity of ELISA for anti-*G. spinigerum* L3 IgG antibody ranged from 59% to 87%, while the specificity ranged from 79% to 96% (Suntharasamai et al. 1985; Maleewong et al. 1988; Anantaphruti 1989). Our ELISA results for anti-*G. spinigerum* L3 total IgG antibody showed the higher sensitivity (95%) but a markedly lower specificity (8%) than these previous reports. This was most likely to be explained by the low cut-off value used in our study (data not shown), a number which was derived from healthy control subjects in whom parasitic infections were rigorously excluded by CBC, blood, and stool examinations.

Similar to previous reports (Suntharasamai et al. 1985; Maleewong et al. 1988), our ELISA results for anti-*G. spinigerum* L3 total IgG antibody showed cross-reactivity with all patients with nematode infections, especially *P. cantonensis*. The cross-reactivity is probably caused by common antigens, such as cuticle proteins, which are shared by several nematodes (Stites et al. 1982; Grieve, 1990; Sakolvaree et al. 1997; Lewis et al. 1999). *P. cantonensis*, like *G. spinigerum*, is common in Southeast Asia and can cause eosinophilic meningitis or meningoencephalitis. Since *P. cantonensis* can cross-react with *G. spinigerum* IgG in the ELISA, parastrongyliasis can be differentiated from gnathostomiasis only by a combination of diet history and a specific ELISA. A history of eating raw terrestrial snails or slugs, or poorly cooked molluscan *Achatina fulica,* is most helpful in most cases (90.5%) of parastrongyliasis. However, some of these patients also eat raw fishes. When the disease is suspected, a highly specific anti-parastrongyliasis total IgG ELISA can be used to confirm the diagnosis (91–96% sensitivity and 73–76% specificity) (Jaroonvesama et al. 1985; Dekumyoy et al. 2000; Geiger et al. 2001).

Interestingly, sera from all patients with *O. viverrini* (3) and with *B. hominis* (2) showed cross-reactions when tested with the IgG ELISA for gnathostomiasis. Using Western blot analysis, it has been shown that *O. viverrini* contains many proteins that can cross-react with several parasites, including *G. spinigerum* (Sakolvaree et al. 1997). *B. hominis* is one of the most common intestinal parasites in Thailand (0.8–44% prevalence) (Wilairatana et al. 1996; Taamasri et al. 2002; Waikagul et al. 2002). Previous studies have also demonstrated that sera from patients with protozoa, including *B. hominis*, may cross-react with helminths (Sakolvaree et al. 1997). Further study to determine the specific protein(s) for diagnosis of each parasite would be useful.

We found that the use of IgG subclass antibodies improved the overall sensitivity and specificity of the

anti-*G. spinigerum* L3 ELISA. In particular, we found that the highest sensitivity (98%) and NPV (94%) was achieved by anti-*G. spinigerum* L3 IgG1 antibody, while the highest specificity (88%) and PPV (93%) was found with the IgG2 antibody (Table 2). The detection of IgG2 antibody against *G. spinigerum* L3 reduced cross-reactivity to most parasites, particularly *P. cantonensis*. The IgG antibody is involved in antibody-dependent cell-mediated cytotoxicity (Boot et al. 1989), which is an effective mechanism for parasitic elimination (Davidson 1985). Therefore, we suggest that combining the results of ELISAs for anti-*G. spinigerum* L3 IgG1 and IgG2 antibodies could increase the sensitivity and specificity (Table 2) for diagnosis of gnathostomiasis. Anti-*G. spinigerum* L3 IgG1 antibody could be used to screen presumptive gnathostomiasis patients, and anti-*G. spinigerum* L3 IgG2 antibody to confirm the diagnosis.

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