IgG4: The Best Immunoglobulin to Diagnosis of Sparganosis

IgG4: อิมมูโนโกลบูลินที่ดีที่สุดในการวินิจฉัยโรคพยาธิสปาร์กาโนซิส

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ABSTRACT

Human sparganosis is caused by infection with larvae (spargana) and demonstrate clinical organ and tissue symptoms. This pilot study aimed to develop serodiagnosis for sparganosis using crude sparganum antigen, molecularly proven as *Spirometra erinaceieuropaei* (*erinacei*). The antigen reacted with antibodies of sparganosis (5 cases), twenty seven parasitic infections (188), and healthy controls (30) to analyze total IgG, IgG_{1-4} and IgE by ELISA. Detection of IgG4 showed the best result with 80% sensitivity and 96.3% specificity. Eight false-positives were found from trichuriasis, hookworm infection, trichostrongyliasis, trichinellosis, and four of paragonimiasis. In addition, immunoblot could confirm unsatisfied reactions of IgG_{1-3} and IgE-ELISA. However, a further improvement of analysis should evaluate the test by using more sparganosis cases and purification technique of crude antigen.

บทคัดย่อ

โรคสปาร์กาโนซิสในคนเกิดจากการกินตัวอ่อนสปาร์กานัมทำให้เกิดอาการแสดงทางคลินิกที่อวัยวะและเนื้อเยื่อ การศึกษานำร่องนี้มุ่งที่จะตรวจโรคนี้ด้วยแอนติเจนชนิดหยาบเตรียมจากสปาร์กานัมของ *Spirometra erinacei-europaei* (*erinacei*) นำมาทำปฏิกิริยากับแอนติบอดีของคนที่เป็นโรคสปาร์กาโนซิส (5 ราย) โรคปรสิตอื่น ๆ 27 โรค (188 ราย) และคนปกติ (30 ราย) เพื่อตรวจจับ total IgG, IgG1-4 และ IgE โดยวิธีอีไลซ่า พบว่า IgG4 ให้ผลดีที่สุดที่ความไว 80% และความจำเพาะ 96.3% เกิดผลบวกปลอมจาก โรคพยาธิแส้ม้า โรคพยาธิปากขอ โรคพยาธิทริโคสตรอง-จิริเอซิส โรคพยาธิทริชิเนลโลซิส โรคละ 1 รายและโรคพยาธิใบไม้ปอด 4 ราย นอกจากนี้วิธีอิมมูโนบล็อทสามารถยืนยันผลที่ไม่ดีของ IgG1-3 และ IgE จากวิธีอีไลซ่าได้ อย่างไรก็ตามงานวิจัยนี้จะมีการศึกษาต่อด้วยจำนวนซีรัมโรค สปาร์กาโนซิสมากขึ้นและเตรียมแอนติเจนให้บริสุทธิ์เพื่อผลการวิเคราะห์ที่ดีขึ้นของการทดสอบ

Key Words: Sparganosis, IgG4, Indirect ELISA

คำสำคัญ: โรคพยาธิสปาร์กาโนซิส IgG4 วิธีอีไลซ่า

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Introduction

Human sparganosis is caused by infection with plerocercoid or procercoid larvae (sparganum) of pseudophyllidean tapeworms belonging to Genus *Spirometra* spp. One of those is *Spirometra erinaceieuropaei* (*erinacei*) which is common in Asia particularly, eastern and southeastern Asia and Europe. The sporadic cases are reported in United States, Africa, South America and Australia (Miyazaki, 1991; Liu *et al*, 2013). In Thailand, 52 cases of sparganosis have been sporadically reported in all parts of country, from 1943-2010 (Anantaphruti *et al*, 2011). Sparganosis, humans can be infected by at least one of the three ways, 1) eating raw or undercooked meat of the second intermediate/paratenic hosts containing plerocercoid, 2) drinking water containing copepods infected with procercoid and 3) the application of traditional treatment with raw, fresh infected flesh of frogs or snakes for ulcers or painful eyes. The parasites can invade several parts of human body, most common-subcutaneous tissues and eyes and others-brain, viscera, lymph ganglia, etc. The surgical removal of worms is the best treatment but it is not always applicable depending on the location of the worm migrations. Besides spargana-tissue parasite, *Gnathostoma* and other tissue parasites cause similar symptoms to sparganosis, especially erythematous swelling and migratory tracks in the cutaneous or subcutaneous tissues. Serodiagnosis for sparganosis has been developed and adapted for mostly detection of IgG antibody. In 1984, Kim and colleagues developed ELISA using crude spargana antigen, which evaluation showed 85.7% of sensitivity and 95.7% of specificity. Cross reaction determined from normal controls and only four diseases and occurred with some of saginata taeniasis sera. Crude antigens and partially purified antigens from spargana still demonstrate cross-reaction with other different diseases when mostly, IgG antibody detected (Choi *et al*, 1988; Kong *et al,* 1994; Park *et al,* 2001; Cui *et al* 2011). However, those studies analyse the antigens with few diseases for cross reactivity. Therefore, this study on diagnosis of sparganosis attempted to develop ELISA using crude sparganum antigen in detection of IgG, IgG1-4, including IgE to this tissue parasite. Also, cross-reaction was determined by several different parasitic infections and healthy controls.

Materials and Methods

Parasite collection

Several naturally infected frogs were purchased from local markets in LAO PDR. Spargana (plerocercoid larvae) were collected and washed with normal saline solution (NSS), 3 times and finally washed with distilled water (DW). Larvae were kept at -70 °C at the Department of Helminthology, Faculty of Tropical Medicine, Mahidol University. The spargana samples were molecularly identified as *Spirometra erinaceieuropaei* (*erinacei*) by Asst.Prof. Megumi Sato.

Preparation of antigen

Crude somatic antigens were prepared from frozen larvae following the procedure by Chung *et al,* (2000) with some modifications. Briefly, larvae were ground in DW using a pestle and a motar in cold condition with ice. The homogenate of parasite was sonicated by ultrasonicator (Ultrasonic Processor XL, CT, USA) at 1-min intervals for 5 min. The suspension was centrifuged at 15,000*g* for 1 hr (Avanti^{TM}30 centrifuge, BECKMAN) and the supernatant was collected and used as crude somatic antigen.

Serum sample

Serum samples were obtained from the Department of Helminthology, Faculty of Tropical Medicine, Mahidol University. Serum samples comprised with 5 spargana-confirmed sera, 30 from healthy individuals and 188 with other parasitic infections, identified by detection of parasites agents; strongyloidiasis, hookworm infection, capillariasis, ascariasis, trichuriasis, bancroftian filariasis, brugian filariasis, dirofilariasis, trichostrongyliasis, taeniasis, hymenolepiasis nana, opisthorchiasis, paragonimiasis heterotremus, haplorchiasis, entamoebiasis, giardiasis, *Blastocystis hominis* infection, and malaria; by detection of parasites agents and serodiagnosis; gnathostomiasis, trichinellosis, angiostrongyliasis, cysticercosis, echinococcosis, schistosomiasis, fascioliasis, and clinical features of creeping eruption. Only 2 diseases were identified by antibody detection; toxocariasis and toxoplasmosis. Each serum sample was stored at -70ºC until used.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA was performed in microtiter plates as described by Dekumyoy *et al* (1998). All ELISA conditions were provided by the checkerboard titration and only detections of IgG and IgG4 were further analyzed because IgG1-3 and IgE could not present a good checkerboard titration conditions (see also detection of these immunoglobulins by immunoblot). The ELISA procedure, 50 µl of antigen dilutions (0.4 µg/ml for IgG , 0.25 µg/ml for IgG4, in carbonate-bicarbonate buffer) were incubated in a microtiter plate (Nunc, Denmark) at 37ºC, and overnight at 4ºC. The unbound sites of the wells were coated with 3% skim milk (PBS, pH 7.4 - sodium azide, 0.02%). The diluted serum samples (1:800 for both IgG and IgG4) were prepared by diluents (washing solution-T-PBS containing 0.02% NaN_3-0.2% bromphenol blue solution), and then the antigen-antibody complexes were treated with diluted conjugate (1:2,000, horseradish peroxidase-labeled anti-human IgG and IgG_4, Southern Biotech, USA). The reaction was visualized with substrate [(2, 2-azino-dis-(3-ethyl-benzothiazoline-6-sulfonate)], and stopped reaction with 1% SDS. The OD values were determined at 405 nm using a spectrophotometer (Titertek Multiskan® PLUS, Labsystems, Findland).

In this study, checkerboard titrations of those IgG1-3 and IgE did not show good conditions between positive and negative controls. An immunoblot analysis was done by following the procedures; (Chung *et al,* 2000; Dekumyoy *et al*, 1998(b)) separated 30 µg antigen/well (5 mm-well) of SDS-PAGE, then, the fractionated antigens in an electrophoresed gel were electrotransferred onto nitrocellulose sheet (0.45µm, PALL, Pall Corporation, USA) by Semi-dry transfer cell (ATTO, Japan). The blot was treated with 3% skim milk-0.02% sodium azide-PBS pH 7.4 and six small strips were treated with positively diluted serum (1:100, by 0.02% NaN_3-Tween20-PBS). The strips were individually exposed by diluted peroxidase conjugates with T-PBS (1:1,000, total IgG, IgG1-4 and IgE). Finally, substrate solution (2, 6-dichlorophenol indophenol) was poured on the strips for 2-3 min. The reaction was stopped by washing the strips with distilled water.

Ethics statement

This study was protocol was approved by the Scientific Ethics Committee of Mahidol University, MUTM 2012-040-01.

Results

Due to IgG1-3 and IgE were not further analyzed in full scale ELISA because of unsatisfied results of checkerboard titration. Therefore, an observation of immunoblot was done by reaction of the antigen and pooled positive serum (1:100) and encountered with individual conjugates (1:1,000). It was found that IgG4 resulted many reactively strong bands mean while IgG showed weaker reaction than IgG4 and also mostly different MWs. It was not surprised in results of checkerboard titration of IgG1, IgG2, IgG3 and IgE because reactions did not detect any bands or probably indistinct bands (Fig. 1). This encouraged the further reactions of ELISA with IgG and IgG4.

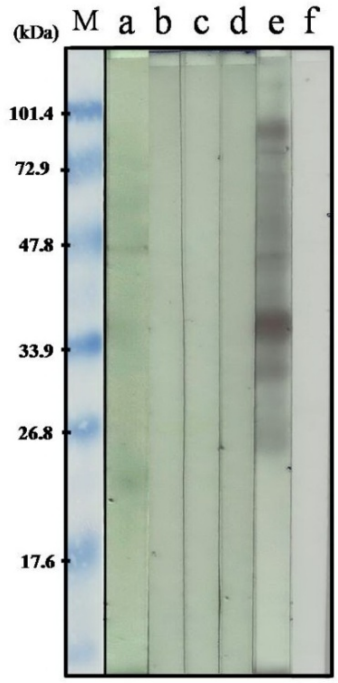

Figure 1 Demonstration of immunoblot on observation of immunoglobulin antibodies, IgG, IgG1-4 and IgE to immune complexes of sparganum antigen and pooled sparganosis serum. M = Low molecular weight of protein standard markers, Conjugates; a = IgG, b-e = IgG1-4 and f = IgE

The IgG-ELISA determined all serum samples above and showed 80% of sensitivity and 95.4% of specificity at the cut-off value, 0.502 (mean +2 SD). Ten false-positives were from cysticercosis (4/10), angiostrongyliasis (1/10), toxocariasis (1/10), gnathostomiasis (1/10), paragonimiasis (1/10), creeping eruption (1/3), dirofilariasis (1/2) and 1 false-negative. Seven diseases produced cross-reaction in detection of IgG using crude sparganum antigen. This crude antigen was not immunogenic to IgG antibodies of all protozoan infections and fifteen helminthic infections including normal controls when IgG detected (Fig. 2 and Table 1).

Detection of IgG4 yielded 80% of sensitivity and 96.3% of specificity at the cut-off value, 0.096 (mean+1SD). This immunoglobulin on cross-reactivity gave lower numbers of cases and diseases than those of IgG antibody. Eight false-positives from five diseases were found as follows; trichuriasis (1/9), trichostrongyliasis (1/10), hookworm infection (1/10), paragonimiasis (4/10), trichinellosis (1/10) and 1 false-negative case occurred. This false negative gave quite low ELISA-OD value of IgG4 antibody, which is the same case of total IgG analysis. Cross-reaction in this test demonstrated different helminthic infections from those of IgG analysis except paragonimiasis. In contrast, cysticercosis gave 4 false-positives to detect IgG but did not show false-positive to IgG4. Controversial, the antigen was immunogenic to IgG antibodies to *Paragonimus* worms due to numbers of cross-reaction increased from one to four cases. Most of false positive cases gave OD-values far from those of their groups. Crude somatic antigen reacted with some of nematodes and trematode infections, not with cestode infections (Fig.3).

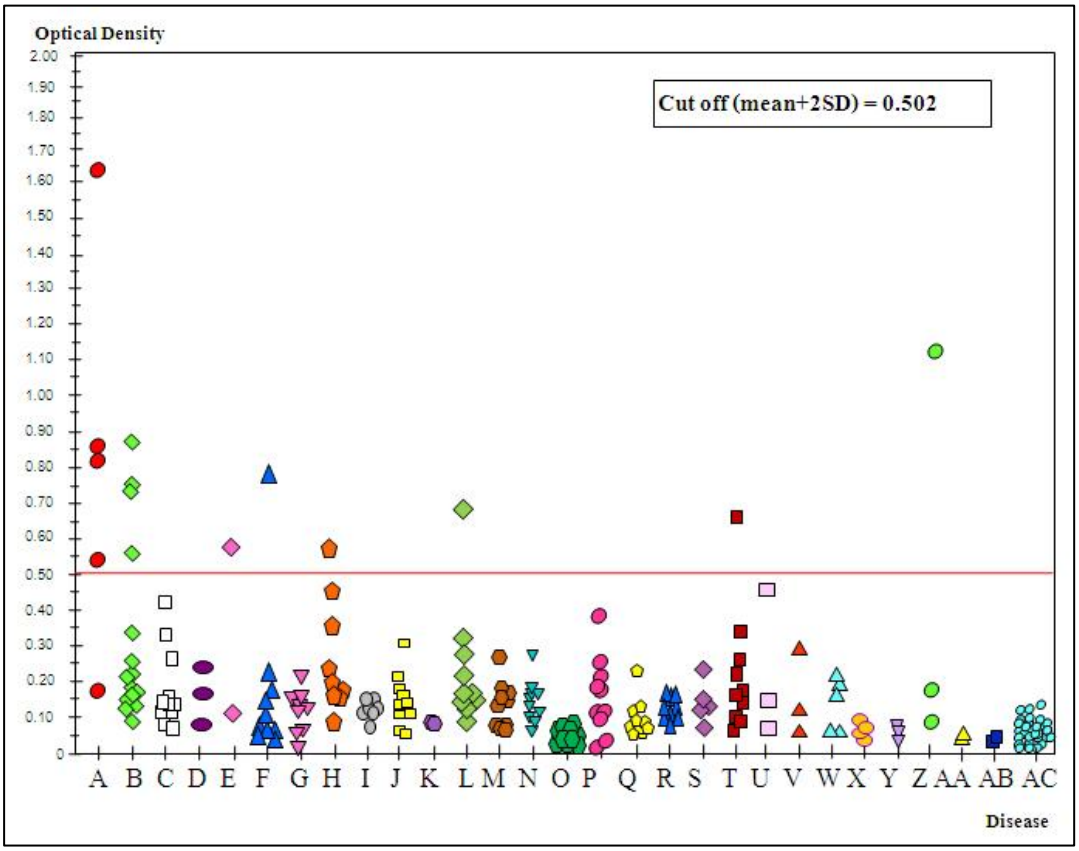

Figure 2 Scatter patterns of ELISA-OD values of sparganosis cases, other parasitic infections and negative serum controls, reacted with IgG antibody. Sparganosis (A), Cysticercosis (B), Taeniasis saginata (C), Echinococcosis (D), Dirofilariasis (E), Angiostrongyliasis (F), Trichuriasis (G), Toxocariasis (H), Ascariasis (I), Trichostrongyliasis (J), Capillariasis (K), Gnathostomiasis (L), Hookworm infection (M), Brugian filariasis (N), Bancroftian filariasis (O), Strongyloidiasis (P), Trichinellosis (Q), Opisthorchiasis (R), Haplorchiasis (S), Paragonimiasis heterotremus (T), Fascioliasis hepatica (U), *Blastocystis hominis* infection (V), Hymenolepiasis nana (W), Amoebiasis (X), Giardiasis (Y), Creeping eruption (Z), Malaria (AA), Toxoplasmosis (AB) and Normal controls (AC). Cut-off value was 0.502

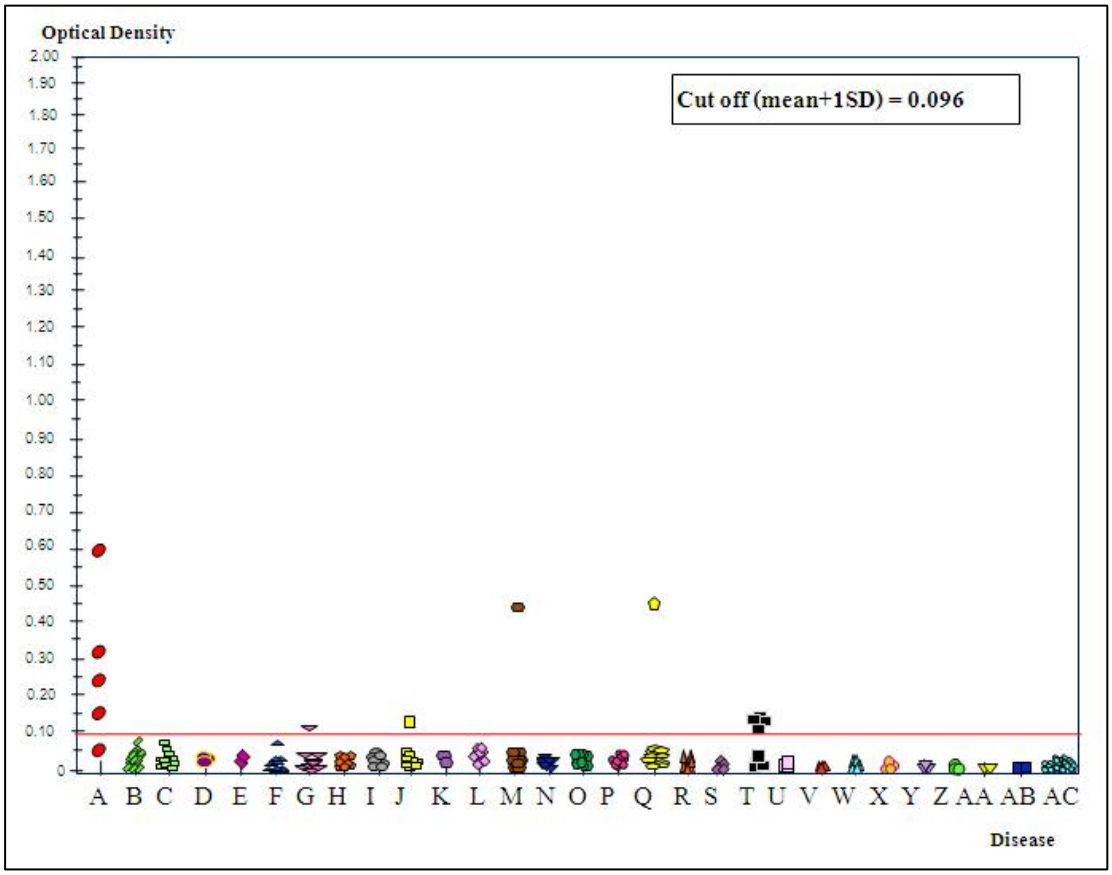

Figure 3 Scatter patterns of ELISA-OD values of sparganosis cases, other parasitic infections and negative serum controls, reacted with IgG4 antibody. Sparganosis (A), Cysticercosis (B), Taeniasis saginata (C), Echinococcosis (D), Dirofilariasis (E), Angiostrongyliasis (F), Trichuriasis (G), Toxocariasis (H), Ascariasis (I), Trichostrongyliasis (J), Capillariasis (K), Gnathostomiasis (L), Hookworm infection (M), Brugian filariasis (N), Bancroftian filariasis (O), Strongyloidiasis (P), Trichinellosis (Q), Opisthorchiasis (R), Haplorchiasis (S), Paragonimiasis heterotremus (T), Fascioliasis hepatica (U), *Blastocystis hominis* infection (V), Hymenolepiasis nana (W), Amoebiasis (X), Giardiasis (Y), Creeping eruption (Z), Malaria (AA), Toxoplasmosis (AB) and Normal controls (AC). Cut-off value was 0.096

Table 1 Comparison of ELISA positivity derived from reactions between sparganum antigen and all serum samples in detection of IgG and IgG4 antibodies.

Disease/sample	No.	IgG			IgG4		
		Pos.	Neg.	% pos.	Pos.	Neg.	% pos.
Sparganosis	5	4	1	80	4	1	80
Angiostrongyliasis	10	1	9	10	-	10	0
Ascariasis	7	-	7	0	-	7	0
Bancroftian filariasis	10	-	10	0	-	10	0
Brugian filariasis	10	-	10	0	-	10	0
Capillariasis	3	-	3	0	-	3	0
Dirofilariasis	2	1	1	50	-	2	0
Gnathostomiasis	10	1	9	10	-	10	0
Hookworm infection	10	-	10	0	1	9	10
Strongyloidiasis	10	-	10	0	-	10	0
Toxocariasis	10	1	9	10	-	10	0
Trichinellosis	10	-	10	0	1	9	10
Trichostrongyliasis	10	-	10	0	1	9	10
Trichuriasis	9	-	9	0	1	8	11.1
Echinococcosis	3	-	3	0	-	3	0
Hymenolepiasis nana	5	-	5	0	-	5	0
Cysticercosis	10	4	6	40	-	10	0
Taeniasis saginata	10	-	10	0	-	10	0
Haplorchiasis	10	-	10	0	-	10	0
Fascioliasis hepatica	3	-	3	0	-	3	0
Opisthorchiasis	9	-	9	0	-	9	0
Paragonimiasis heterotremus	10	1	9	10	4	6	40
Creeping eruption	3	1	2	33.3	-	3	0
Amoebiasis	4	-	4	0	-	4	0
Blastocystis hominis infection	3	-	3	0	-	3	0
Giardiasis	3	-	3	0	-	3	0
Malaria	2	-	2	0	-	2	0
Toxoplasmosis	2	-	2	0	-	2	0
Normal controls	30	-	30	0	-	30	0

Discussion and Conclusions

Total IgG is the common immunoglobul in to be studied in finding a based line data and also development of serodiagnosis. In this study, IgG performs in a good result of indirect ELISA with 80% of sensitivity and 95.4% of specificity. Unfortunately, our study could obtain only 5 spargana-confirmed cases.

However, several other parasitic diseases and detection of IgG1-4 were included in this sero-analysis using crude somatic antigen. Specificity of this IgG-ELISA, ten false-positives are found from seven diseases; cysticercosis (4/10) gives 40% cross-reactivity to detection of IgG. Moreover, creeping eruption and dirofilariasis were available in each three and two cases only. If more cases are analyzed by crude antigen, more false positive cases may be high due to helminthes have complex structures therefore, crude somatic antigen always contain various cross-reactive molecules. Four cases of cysticercosis cross-react with sparganum crude antigen, which this disease is caused by cestode worm as sparganosis. This may be co-infection with sparganum because most of false positives produce high ELISA-OD values far searated from those of their groups. In addition, high antibody levels of these cases cross-react with the antigen.

It differs from one false positive from toxocariasis, which may be happened by high antibodies to *Toxocara* parasite because ELISA-OD values of toxocariasis cases continuously run in a line. However, it cannot omit mixed infection with sparganum. Analysis of the antigen by IgG4, 80% of sensitivity and 96.3% of specificity were determined. Eight false-positives were found and alternatively, four of paragonimiasis cases produced 40% of cross-reactivity increasing from one case. In this study, antibodies from each one of nematode infections and four from paragonimiasis cross-react with the antigen but cestode infections do not react with the antigen. For immunoglobulin subclasses, IgG4 gives the best immunoglobulin among 4 subclasses to sparganosis analysis. In the study, the false negative case ocurrs in the detection of IgG and IgG4. This serum sample belongs to the same patient who was a small female child at the time of collection. Therefore, this case may have low antibody by herself because of antibody response based on age dependence.

Other researches on sparganosis in detection of IgG, crude antigen and purified antigen by gelatin-affinity chromatography show some cross-reactions with other parasitic infections by indicating with specificities, 89% and 95.6%, respectively. However, 92.6% of sensitivity produced in both antigens with a total of 27 sparganosis cases (Kong *et al*, 1991). Cho *et al* (1990) produced three kinds of *Spirometra mansoni* sparganum antigen by simple extraction, gel filtration using SephacrylS-300 column chromatography and monoclonal antibody affinity chromatography. Sensitivities of three kinds of antigens were 96.4%. Specificity of ELISA using mAb-purified antigen showed a highest value at 97%, produced from 7 different diseases. Cui *et al* (2011) demonstrated cross-reactivity between crude antigen from *Spirometra mansoni* spargana and IgG antibodies of cysticercosis (3/11), echinococcosis (5/10) and paragonimiasis (9/20) by indirect ELISA. All above indicates that cross-reaction is mainly found from antibodies with crude sparganum antigens and also partially purified and purified antigens.

The pilot study suggested that IgG4-ELISA can be used in the serodiagnosis of human sparganosis;

wever, further studies should evaluate the test more exhaustively and conclusively.

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