ISO-ELECTRICFOCUSING OF *BITHYNIA* SNAIL ANTIGENS FOR IgG-AND IgG$_{1-4}$ -ELISA DETECTION OF HUMAN OPISTHORCHIASIS

Wallop Pakdee[1], Jitra Waikagul[1], Thareerat Kalambaheti[2], Akira Ito[3] and Paron Dekumyoy[1]

[1]Department of Helminthology, [2]Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; [3]Department of Parasitology, Asahikawa Medical College, Hokkaido, Japan

Abstract. Diagnosis of opisthorchiasis is confirmed by the presence of characteristic eggs and worms. However, misdiagnosis may occur in light infections, and also due to the morphological similarity of opisthorchid eggs to other species. A finding of specific immune mediators can help confirm infection. This study used indirect ELISA to detect total IgG and IgG$_{1-4}$ with selected antigens of *Bithynia siamensis goniomphalos* extract, which were derived by liquid-phase iso-electricfocusing (IFE). Antigens (Iso-AgF) from 20 IEF fractionated fractions were selected based on a high ELISA-OD ratio between pooled-positive and pooled-negative sera. Iso-AgF 7, 7, 6, 2, and 10 resulted in high OD-ratio to total IgG, IgG1, 2, 3, and 4, respectively. A full-scale ELISA was conducted with sera from 50 opisthorchiasis cases, 196 from other parasitic-disease cases, and 35 healthy controls. Iso-AgF7 to IgG$_1$ showed the best result, with sensitivity, specificity, positive and negative predictive value of 100, 96, 86, and 100%, respectively, at a cut-off 0.221. Low cross-reactivity to IgG$_1$ was found in one case each of gnathostomiasis, trichinellosis, toxocariasis, angiostrongyliasis, bancroftian filariasis, enterobiasis, neurocysticercosis, and taeniasis. Thus, Iso-AgF7 to IgG$_1$ was a good candidate antigen to be developed for detection of antibodies against *Opisthorchis viverrini*.

Key words: *Bithynia* antigen; liquid-phase iso-electricfocusing, opisthorchiasis, IgG$_1$-ELISA

INTRODUCTION

Millions of cases of fish-borne trematodiasis caused by *Opisthorchis viverrini* and/or *Clonorchis sinensis* have been reported in the Greater Mekong Sub-region of Thailand, Lao PDR, Vietnam, and Cambodia (WHO, 1995), with about 7 million opisthorchiasis cases estimated in northern Thailand (Jongsuksuntigul and Imsomboon, 2003). The characteristic eggs of small liver flukes are the standard indicator of infection, and in addition, DNA detection of those flukes has been applied in clinical specimens (Parvathi *et al*, 2007; Thaenkham *et al*, 2007). However, antibody detection remains useful for such diagnosis, using several antigens produced by simple to complicated tech-

Correspondence: Paron Dekumyoy, Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, 420/6 Ratchawithi Road, Bangkok, 10400, Thailand.
Tel: 66 (0) 2354 9100 to 4 ext 9180-1; Fax: 66 (0) 2643 5600
E-mail: tmpdk@mahidol.ac.th

niques. These antigens are derived from adult worms and larval stages as crude, excretory-secretory (ES-antigen), and partially purified antigens. In addition, a shared or mimic antigen from snail intermediate hosts has been studied as an alternative antigen for detecting opisthorchiasis antibody (Srivatanakul *et al*, 1985; Wongratanacheewin *et al,* 1988; Amornpunt *et al*, 1991; Akai *et al*, 1995; Watthanagulpanich *et al*, 1997; Sripa and Kaewkes, 2000; Wongsarojt *et al*, 2001; Waikagul *et al*, 2001; 2002).

In helminth infections, the detection of immunoglobulin G is the focal point of the antigen-antibody reaction. A more useful technique is to detect subclasses of IgG namely, IgG_1, IgG_2, IgG_3, and IgG_4, which can differentiate and specifically detect infections, such as gnathostomiasis (Nuchprayoon *et al*, 2003; Anantaphruti *et al*, 2005; Laummaunwai *et al*, 2007). The aim of the current study was to analyze 20 antigens fractionated using liquid-phase iso-electric focusing (IEF) with total IgG and IgG subclasses, and to improve the antigen preparation for detecting opisthorchiasis in routine analysis.

MATERIALS AND METHODS

Serum samples

Serum samples were obtained from the Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, comprising 50 opisthorchiasis sera by worm detection, 196 with other parasitic infections, and 35 from healthy individuals. Several heterologous serum samples were identified by detection of parasitic agents, viz. strongyloidiasis, hookworm infection, capillariasis, ascariasis, trichuriasis, bancroftian filariasis, brugian filariasis, dirofilariasis, sparganosis, taeniasis, hymenolepiasis nana, paragonimiasis heterotremus, haplorchiasis, entamoebiasis, giardiasis, *Blastocystis hominis* infection, and malaria; and by either parasitic agents or serodiagnosis, viz. gnathostomiasis, trichinellosis, angiostrongyliasis, neurocysticercosis, echinococcosis, schistosomiasis, fascioliasis, and creeping eruption. Only 2 diseases were identified by antibody detection, toxocariasis and toxoplasmosis. Each serum sample was stored at -70ºC until used. The study protocol was approved by the Scientific Ethics Committee of Mahidol University, MUTM 2008-052-01.

Preparation of crude *B. s. goniomphalos* snail antigen

Bithynia siamensis goniomphalos snails were bred and cultured in the laboratory of Applied Malacology Center, Department of Social and Environmental Medicine, Faculty of Tropical Medicine, Mahidol University. The snail bodies were prepared by crushing, finding parasites under stereomicroscope, and extraction by a glass pestle and mortar in distilled water (DW) and alumina on ice. The homogenate was sonicated in a sonicator (Ultrasonic Processor XL, CT) 20 times for 1 minute each, using probe No. 419 and power No. 4, and then centrifuged at 18,000*g* for 60 minutes at 4ºC. The supernatant was filtered through glass wool to eliminate snail mucus. The filtrate (crude somatic antigen) was determined using Pierce Coomassie for its protein content Plus Protein Assay reagent kit (Pierce chemical, Rockford, IL).

Iso-electricfocusing of antigens

Crude antigen (110 mg) was fractionated by preparative IEF using Rotofor cell (Bio-Rad), according to the manufacturer's protocol. The antigen was desalted by dialysis and then mixed with 40 ml of Tris buffer and 2 ml of ampholyte [40% (w/v),

pH 3-10; Bio-Rad] to make 2% ampholyte solution. The electrolyte for the anode and cathode was 0.1M H_3PO_4 and 0.1M NaOH, respectively. Samples were subjected to IEF at a constant power of 12 W at 4°C for 4 hours. Twenty IEF antigen fractions (Iso-AgF1-20) were simultaneously aspirated from the cell and delivered to the fraction tubes. All fractionates were measured for pH and then dialyzed against DW. The protein content of each fraction was determined.

Selection of antigens for ELISA

Due to the production of several snail IEF antigen fractions, a system of antigen selection was determined using the ELISA application of Voller *et al* (1979) and Dekumyoy *et al* (1998) with minor modification, to compare the ELISA-OD ratios between those of positive pooled sera and negative controls. Each antigenic fraction was diluted to 2 µg/coating buffer and analyzed by using fixed dilutions of pooled serum controls at 1:200, and conjugates (total IgG) at 1:2,000, and for IgG_{1-4} at 1:1,000. Antigen showing a high ELISA-OD ratio was used for further full-scale ELISA.

Indirect ELISA was used to evaluate the selected antigen, using the method described by Dekumyoy *et al* (1998). Checkerboard titration of a selected antigen against pooled positive and negative sera was performed to establish the optimal conditions for the tests. In brief, 50 µl aliquots of antigen dilutions were incubated in a microtiter plate (Nunc, Denmark) at 37°C for 1 hour, and overnight at 4°C. The unbound sites of the wells were coated with 1% BSA in PBS, pH 7.4 containing 0.02% sodium azide. Antigens were reacted with diluted serum samples, and the wells were washed with 0.02% NaN_3 - 0.2% bromphenol blue solution, and then treated with each conjugate (horseradish peroxidase-labeled anti-human IgG and IgG_{1-4}, Southern Biotech). The reaction was visualized with substrate [(2, 2-azino-dis-(3-ethyl-benzothiazoline-6- sulfonate)], and terminated with 1% SDS. The OD values were determined at 405 nm using a spectrophotometer (Titertek Multiskan® PLUS, Labsystems, Findland).

RESULTS

Twenty snail IEF fractionated antigens were pre-analyzed to obtain a sensitive antigenic fractionated antigen to IgG and IgG_{1-4} in pooled opisthorchiasis serum. Antigenic analysis was determined by reaction between pooled opisthorchiasis and healthy control sera. When using the same selection conditions, only dilutions of anti-human IgG and IgG_{1-4} were different, at 1:2,000 and 1:1,000, respectively. Pooled antibodies to *O. viverrini* showed OD values to total IgG detection in the range 0.365-0.955, whereas pooled healthy control sera gave a lower range, 0.106-0.274. In comparing ELISA-OD ratios, Iso-AgF7, at pH 7, was selected due to its highest ratio value of 3.6 to total IgG. In the detection of IgG subclass, opisthorchiasis OD values, and those of healthy control sera, were not greatly different from each other: IgG_1, opisthorchiasis range 0.251-0.095 and healthy control, 0.218-0.077; IgG_2 0.222-0.071 and 0.226-0.071; IgG_3 0.205-0.065 and 0.189-0.053, and IgG_4 0.271-0.136 and 0.191-0.130. Finally, Iso-AgF7 (pH 7), Iso-AgF6 (pH 6.5), Iso-AgF2 (pH 4.1) and Iso-AgF10 (pH 7.3) were selected based on their highest ratio values, at 1.49, 1.09, 1.34, and 1.42 to IgG_{1-4}, respectively. Then, a full-scale ELISA was used to analyze these antigens using serum antibodies from opisthorchiasis, other parasitic infections, and negative controls.

By checkerboard titration, the condi-

tions for total-IgG ELISA were 2 µg/ml of Iso-AgF7, and dilutions of serum and anti-human IgG of 1:200 and 1:2,000, respectively. Fifty opisthorchiasis sera gave OD values ranging from 0.399 to 0.935, with mean OD value of 0.655 ± 0.113 SD, compared with 0.400 ± 0.098 OD of healthy control sera. A threshold value was considered at 0.498 (mean + SD) to evaluate the test. The results showed that 48 of 50 (96%) opisthorchiasis sera were positive, 50% (98/196 cases) for other parasitic infections, and 37% of healthy control sera (13/35) showed false positive. Sensitivity, specificity, positive and negative predictive value of the test was 96, 52, 30, and 98%, respectively (Table 1). The mean OD of the opisthorchiasis group is significantly higher than that of the heterologous group ($p < 0.05$). Iso-AgF7 to total IgG was reactive to serum antibodies of helminthic infections, except for creeping eruption cases and protozoan infections.

When detecting IgG subclasses, the optimal concentration of the fractionated antigen Iso-AgF7 was determined to be 4 µg/ml, which reacted with 1: 100 diluted serum and 1:2,000 diluted anti-human IgG_1. Opisthorchiasis sera produced a range of OD values from 0.222 to 0.72 over the cut-off value. Tests using this antigen showed excellent sensitivity (100%), at cut-off point of 0.221, and low antigenicity to heterologous infections and healthy controls, with a specificity of 96% and positive and negative predictive value of 86 and 100%, respectively. It revealed significant differences ($p < 0.001$) in IgG_1 absorbance values for opisthorchiasis compared with other parasitic infections and negative controls. Iso-AgF7 showed low cross-reactivity to other helminthiases: gnathostomiasis (1/12), trichinellosis (1/10), toxocariasis (1/10), angiostrongyliasis (1/10), bancroftian filariasis (1/10), enterobia-

Table 1

Accuracy, sensitivity, specificity, positive and negative predictive values of indirect ELISA by detection of total IgG and IgG_{1-4} antibodies to IEF fractionated *Bithynia* snail antigens for serodiagnosis of human opisthorchiasis.

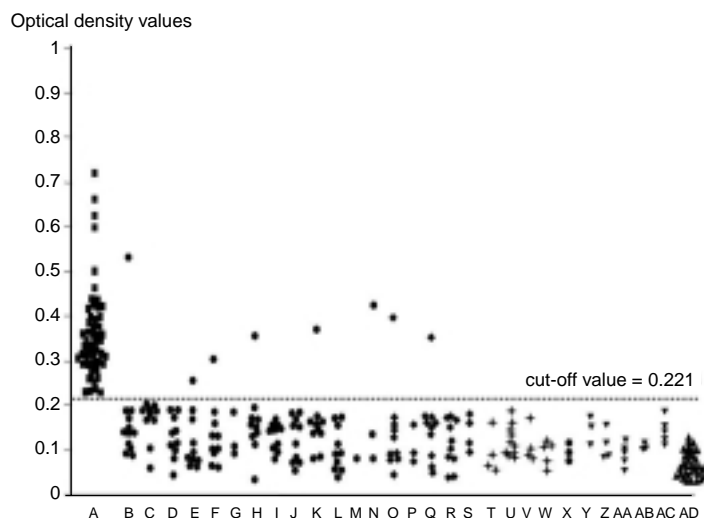| Evaluation of ELISA	Total IgG+Iso-AgF7	IgG_1+Iso-AgF7	IgG_2+Iso-AgF6	IgG_3+Iso-AgF2	IgG_4+Iso-AgF10
Accuracy	81	97	80	78	86
Sensitivity	96	100	90	74	88
Specificity	52	96	66	81	84
Positive predictive value	30	86	36	37	54
Negative predictive value	98	100	97	99	97

Optical density values

Fig 1–Scatter patterns of ELISA-OD values of *Bithynia* antigen Iso-AgF7 (4 µg/ml) reacting with IgG$_1$ (1:2,000 dilution) of serum samples (1:100 dilution). Opisthorchiasis (A), gnathostomiasis (B), strongyloidiasis (C), hookworm infection (D), trichinellosis (E), toxocariasis (F), capillariasis (G), angiostrongyliasis (H), ascariasis (I), trichuriasis (J), bancroftian filariasis (K), brugian filariasis (L), dirofilariasis (M), enterobiasis (N), neurocysticercosis (O), sparganosis (P), taeniasis (Q), echinococcosis (R), hymenolepiasis nana (S), schistosomiasis (T), paragonimiasis heterotremus (U), fascioliasis (V), haplorchiasis (W), creeping eruption (X), entamoebiasis (Y), giardiasis (Z), *Blastocystis hominis* infection (AA), malaria (AC), toxoplasmosis (AB), and healthy controls (AD). Cut-off value was 0.221.

sis (1/3), neurocysticercosis (1/10), and taeniasis (1/10). Serum antibodies of trematodiases and protozoan infections did not present false positives with this antigen (Fig 1). This antigen was highly reactive to serum antibodies to *O. viverrini* but low antigenicity was found for other diseases and healthy controls.

For IgG$_2$, ELISA conditions were 4 µg/ml of Iso-AgF6, with dilution of serum and anti-human IgG$_2$ of 1:100 and 1:1,000, respectively. The OD values for the opisthor-

chiasis sera were in the range of 0.105 to 0.421. Sensitivity, specificity, positive and negative predictive value was 90, 66, 36 and 97%, respectively (Table 1), at cut-off of 0.192. For IgG$_3$- and IgG$_4$-ELISA the conditions were 2 µg/ml of Iso-AgF2 and Iso-AgF10, and dilutions of serum at 1:100 and anti-human IgG$_3$ and IgG$_4$ at 1:500. Using Iso-AgF2 to IgG$_3$, the OD values for opisthorchiasis were in the range 0.198 to 0.515, and evaluation of the test yielded a sensitivity of 74%, specificity 81%, positive predictive value 37%, and negative predictive value 99%. For Iso-AgF10 to IgG$_4$, the range of OD-values was 0.118 to 0.683 for opisthorchiasis sera with sensitivity, specificity, positive and negative predictive value of 88, 84, 54 and 97%, respectively (Table 1), at cut-off 0.221. It was noted that these antigens from opisthorchiasis sera to IgG$_{2-4}$ produced sensitivity of 88-90%, but cross-reactions were still high with antibodies from several diseases and healthy controls.

DISCUSSION

Antigens from different snail intermediate hosts of *Opisthorchis viverrini* have been studied previously (Chanawong *et al*, 1990; Wathanakulpanich *et al*, 1997; Waikagul *et al*, 2001; 2002). *Bithynia siamensis goniomphalos* snail antigen has been analyzed by total IgG antibody in sera of opisthorchiasis and other diseases (Wathanakulpanich *et al*, 1997; Waikagul *et al*, 2001, 2002). Two kinds of *B .s. goniomphalos* antigens have been produced

by two different techniques, electroelution and Sephacryl S-200 HR gel filtration chromatography (Waikagul *et al*, 2002). A 53 kDa was obtained by electroelution technique and cross-reaction only occurred with hymenolepiasis (1/4) and strongyloidiasis (1/10). The sensitivity and specificity of the total IgG-ELISA was 98.4 and 98%, respectively. However, 5/61 opisthorchiasis cases gave false negative results. When using a cocktail of antigens obtained from Sephacryl S-200 HR chromatography, sensitivity and specificity was 88.5 and 88%, respectively (Waikagul *et al*, 2002).

In this study, crude *Bithynia* snail extract was separated by liquid phase IEF technique. The iso-electric separation of crude antigen might limit cross-reaction with antibodies of other diseases. Also, it is possible that some reactive molecules to opisthorchiasis antibodies may not be separated in the pH gradient of 3-10. Although only 28% of the IEF antigen was recovered from 110 mg of crude antigen, it was possible to obtain good antigenic materials to antibodies against *O. viverrini* worms and poor reactivity to antibodies from other infections. This can be observed from the unsatisfactory discrimination between pooled opisthorchiasis sera and pooled healthy control sera, but a full-scale ELISA of Iso-AgF7 to IgG_1 gave excellent sensitivity (100%) and specificity (96%). It seems that separation by gradient pH can provide a number of antigenic molecules to opisthorchiasis and eliminate some cross-reactive molecules to antibodies from other diseases and healthy controls.

Cross-reactivity of only one serum was observed in gnathostomiasis, toxocariasis, trichinellosis, angiostrongyliasis, bancroftian filariasis, enterobiasis, neurocysticercosis, and taeniasis. This reaction may be due to a prior infection with *O. viverrini*, because most OD values for these sera were separated from these groups, and millions of Thai people have probably been infected with this parasite (Jongsuksuntigul and Imsomboon, 2003). In this study, the antibodies from trematodiases and protozoan infections were less responsive to the antigenic epitopes of Iso-AgF7. In addition, the simple structures of protozoans may cause low antibody response, or they may carry different antigenic epitopes from Iso-AgF7. Although trematode structures are more complicated than protozoa, antibodies against trematode parasites did not react strongly with Iso-AgF7. In contrast with total IgG to Iso-AgF7, cross-reaction was very strong in all helminthiases, with low specificity, at 5%.

Several studies have reported on the usefulness of liquid phase IEF technique. Ko and Ng (1998) reported that fractioned antigens from the cystic fluid of *Taenia solium* metacestodes, using Rotofor cell, drastically reduced cross-reactions in several heterologous pig sera. These antigenic fractions were negative by double-antibody ELISA to other antisera against common parasites. IEF-fractionated antigens (10-26 kDa), pH 9.2-9.6, from the cystic fluid of *T. solium* metacestodes were found to be specific and sensitive for differential serodiagnosis of neurocysticercosis, especially from alveolar or cystic echinococcosis by ELISA and enzyme-linked immunoelectrotransfer blot (Ito *et al*, 1998). In addition, highly purified antigens were derived by single-step iso-electrofocusing using Rotofor cell, and resulted in an antigen-B-rich fraction (8 kDa) from *Echinococcus granulosus* cyst fluid, and an Em-18-rich fraction from *E. multilocularis* protoscolex extract (Ito *et al*, 1999). Neither purified antigen cross-reacted with serum antibodies from 7 diseases, including hepatoma and normal controls, especially

cysticercosis (Ito *et al,* 1999), while other publications showed cross-reactions with those antigens (Leggett *et al,* 1992; Shambesh *et al,* 1995; Nirmalan, 1997).

In summary, IEF antigen Iso-AgF7 yielded excellent sensitivity (100%) and specificity (96%) for IgG$_1$ detection. Detection of IgG$_1$ to opisthorchiasis was best compared with other subclasses and total IgG. Further study should examine electro-elution of selected molecules of Iso-AgF7 to improve subsequent detection of opisthorchiasis.

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