EVALUATION OF *BITHYNIA FUNICULATA* SNAIL ANTIGENS BY ELISA-SERODIAGNOSIS OF HUMAN OPISTHORCHIASIS

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Abstract. Four batches of crude somatic antigens from: (1) *Opisthorchis viverrini* adult worms, (2) *Bithynia funiculata*-whole body, (3) *B. funiculata*-head-foot, and (4) *B. funiculata*-visceral mass were assayed against sera from 81 opisthorchiasis patients, 30 parasite-free healthy individuals, and 50 individuals infected with other helminthic infections, and their antibody levels determined. By IgG-ELISA, the antigenic reactive proteins were found in both the head-foot and the visceral mass of *B. funiculata* snails, but the whole snail antigens gave the best results. Furthermore, it was as good as when *O. viverini* antigens were used. Antibody levels of sera from patients with opisthorchiasis assayed against antigens from whole *B. funiculata* snails were significantly higher than those of the other two groups. The cut-off value for positivity at 0.228 gave 80.2% sensitivity and 81.2% specificity. Cross reactions were observed with sera from patients with paragonimiasis and strongyloidiasis. No cross reactions were found to occur with sera from healthy individuals.

INTRODUCTION

Opisthorchis viverrini is a trematode that spends part of its developmental cycle in snails of the genus *Bithynia*. A previous study concerning the relationships between *O. viverrini* adult worms and its snail intermediate host, *Bithynia*, has confirmed that antigen sharing occurs as previously reported (Chanawong *et al*, 1990). Some shared antigens were demonstrated to react with sera from opisthorchiasis patients and their immunodiagnostic potential was evaluated by Western blot technic (Watthanakulpanich *et al*, submitted for publication). This finding leads to the interesting possibility of using *Bithynia* snails as an alternative diagnostic antigen for opisthorchiasis. The advantages of using snails as starting antigens are that large amounts of material can be obtained rapidly and at comparatively lower cost than parasite antigens which require maintenance of a complex life cycle (Rivera-Marrero and Hillyer, 1985). In this study, crude somatic antigens of whole *B. funiculata* snails, as well as isolated head-foot and visceral mass, were used for diagnosis of opisthorchiasis by the enzyme-linked immunosorbent assay (ELISA). The diagnostic parameters of the test: sensitivity, specificity and predictive values were determined (Galen, 1980).

MATERIALS AND METHODS

Snails

Bithynia (Digoniostoma) funiculata snails were collected from Ban Hua Phai, Mae Mo District, Lampang Province, northern Thailand where opisthorchiasis has not been reported. The snails were identified individually based on morphological criteria : shell with a strong carina and funnel-shaped umbilicus; the animal's body scattered with yellow pigments on dorsal aspect as well as on the ventral foot, and with black melanin on the verge (The Tropmed Technical Group, 1986; Chitramvong, 1992).

Parasites

Metacercariae of *O. viverrini* were obtained from small fresh water cyprinoid fish from the northeastern provinces of Thailand: *Cyclocheilicthys siaja* (Pla Mae Sadeng) were collected from a pond at Pakkha Subdistrict, Watthana Nakhon District, Sa Kaeo Province; and *Cyclocheilicthys apogon* (Pla Khao Na) were collected from Nong Bua Lam Phu District, Udon Thani Province. Every fish sample that was presumed to harbor metacercariae in the fins was chopped in one whole batch, and then transferred into a beaker containing 1% acid-pep-

sin. The fish pulp was incubated for 2 to 3 hours at 37°C and frequently shaken. The digested suspension was poured through a wire-meshed funnel with pore size of 180 microns and allowed to settle in a sedimentation flask which was filled with normal saline solution (NSS). Several washes with NSS were done by pouring off about two-thirds of the supernatant at 15 minute intervals until the supernatant was clear. The sediment was examined under a stereomicroscope for metacercariae of *O. viverrini*, using criteria described by Vajrasthira *et al* (1961). Adult golden Syrian hamsters, *Mesocricetus auratus*, were infected orally with 40 to 60 active metacercariae. Four weeks after the infection, the metacercariae had already developed into sexually mature adult flukes judging from the appearance of eggs in fecal examinations by simple smear. The hamsters were sacrificed, and *O. viverrini* adult worms were collected from the common bile ducts, gall bladders and main hepatic ducts. They were then washed 3 to 4 times with NSS, and the final wash was done with distilled water and the worms were kept at -20°C until used.

Preparation of antigens

Each *Bithynia funiculata* snail was placed in a 3.5 cm diameter plastic cup containing 10 ml of dechlorinated tap water and kept under fluorescent light for 8 to 10 hours. The presence of cercariae in water was observed through a stereomicroscope. The snails positive for cercariae were discarded. The uninfected snails were then gently crushed and the shell removed under a stereomicroscope. Each snail was washed with NSS several times and then examined for parasitic infections under the microscope. Only parasite-free snails were lyophilized and manually homogenized together with alumina using a glass pestle and mortar on ice, the homogenate was then sonicated (Sonicator Ultrasonic Processor; Model XL2020-010, Heat System, Inc, USA; Standard probe No. 419) for 15 times at 2 minutes each and then centrifuged at 30,000 rpm at 4°C for 1 hour. The supernatant was collected and kept frozen at -20°C until used. Another two sets of antigens from *Bithynia* snails; from the head-foot and the visceral mass, were prepared in the same manner as above.

The antigen extracted from *O. viverrini* adult worms was prepared by homogenization using a glass tissue grinder with a small amount of distilled water at 4°C. Further procedures were mostly carried out as previously described above, the only variation being the speed of centrifugation which was performed at 15,000 rpm at 4°C for 1 hour. Protein concentrations of all extracts were determined by Lowry's method (1951).

Sera

Serum samples were categorized into 3 groups: opisthorchiasis, normal healthy controls and other helminthic infections. Group A consisted of 81 sera specimens of individuals exclusively infected with opisthorchiasis from Prachin Buri Province, confirmed by finding corresponding eggs in feces using cellophane-covered thick smear method (Suzuki, 1981). Group B consisted of 30 sera samples from apparently healthy individuals visiting the Out-Patient Clinic at the Children's Hospital for their annual medical check up and were chosen from persons residing in non-endermic areas and who gave negative fecal examination results for any parasitic infection. Group C consisted of 50 sera specimens from infected individuals with other helminthic infections and who are also negative for *O. viverrini* eggs. Their distribution according to helminthic infection is as follows: paragonimiasis 23, taeniasis 6, sparganosis 1, strongyloidiasis 7, hookworm infection 5, gnathostomiasis 4, capillariasis 2 and toxocariasis 2. All sera specimens were frozen at -20°C until used.

Indirect enzyme-linked immunosorbent assay (Indirect ELISA)

An ELISA was performed in a microtiter plate as described elsewhere (Voller *et al*, 1976; Dharmkrong-at *et al*, 1986). The optimal concentrations of four prepared antigens (*O. viverrini* adult worms, whole *B. funiculata* snails, head-foot and visceral mass parts of snails), serum and conjugate dilutions were predetermined by checkerboard titration. Wells of microtiter plate (Nunc, Denmark) were filled with 100 µl of antigens in carbonate-bicarbonate buffer pH 9.6 each containing a protein concentration of 20 µg/ml. The plate was incubated in a humidified chamber at 37°C for 1 hour and transferred to 4°C for further incubation overnight. The unbound proteins were eliminated by washing for 5 times with PBS-T (Tween-20 in PBS, pH 7.4). Wells were filled with 150 µl of 1% BSA in PBS-T

and incubated at 37°C for 1 hour. After washing, 100 µl of diluted (1 : 400) of opisthorchiasis sera, normal healthy controls and other helminthiasis were allowed to react with the antigens (whole *B. funiculata* snails, head-foot and visceral mass) and diluted sera (1 : 800) to react with *O. viverrini* antigen. An hour of incubation at 37°C was allowed and then washed as above. Then 100 µl of peroxidase conjugated anti-human IgG (Dakopatts, Denmark) diluted 1 : 1,000 in PBS-T was added into each well and incubated at 37°C for another 1 hour. After the final wash, 100 µl of freshly prepared substrate solution containing paraphenylenediamine dihydrochloride (Fluka Chemicals) was added to each well. The reaction was allowed to proceed for 30 minutes in the dark and then stopped by adding 50 µl of 1N NaOH. The absorbance of the reactions were determined at 492 nm with an ELISA reader (Titertex Multiskan Plus, MK II) against blanks.

RESULTS

Optimal conditions

Checkerboard titration determined the optimal concentration to be at 20 µg/ml for all antigens extracted from *O. viverrini* adult worms, whole *B. funiculata* snails and the two isolated parts: head-foot and visceral mass. The optimal dilution of sera was 1:800 for *O. viverrini* antigen and 1:400 dilution for all *B. funiculata* antigens. The optimal dilution of the conjugate, 1:1,000, was used in the system throughout the study.

Humoral immune response in patients with proven opisthorchiasis

The mean ($\bar{X}$), standard deviation (SD) and range of absorbance values of sera from opisthorchiasis patients upon ELISA evaluation against crude somatic antigens of *O. viverrini* adult worms were determined to be 0.228, 0.173, 0.038-0.792, respectively. These figures were significantly higher than those of normal healthy controls ($p < 0.001$). It was found that the cut off value at 0.099 gave the best sensitivity and specificity (Fig 1). The sensitivity, specificity, and positive and negative predictive values were 80.2%, 80.0%, 80.2% and 80.0%, respectively.

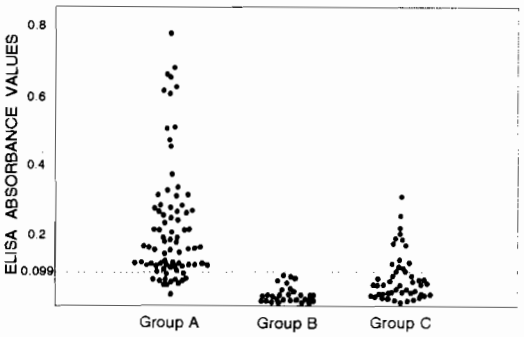

Fig 1–ELISA absorbance values of sera from opisthorchiasis patients (Group A, N = 81), normal healthy individuals (Group B, N = 30) and other patients with helminthic infections (Group C, N = 50) assayed against crude somatic antigens of *O. viverrini* adult worms.

When crude somatic antigen extracted from whole *B. funiculata* snails was used, the $\bar{X}$, SD and range of the absorbance values of sera from opisthorchiasis patients and those from normal healthy controls were also statistically different ($p < 0.001$). The sensitivity, specificity, positive and negative predictive values were calculated using various cut off levels of the normal healthy controls, after which 0.228 was determined to be the cut off value (Fig 2). The values were 80.2%, 81.2%, 81.2% and 80.2%, respectively.

Employing the crude somatic antigens extracted from the head-foot and visceral mass of *B. funiculata* snails against sera from opisthorchiasis patients, normal healthy controls and those with other

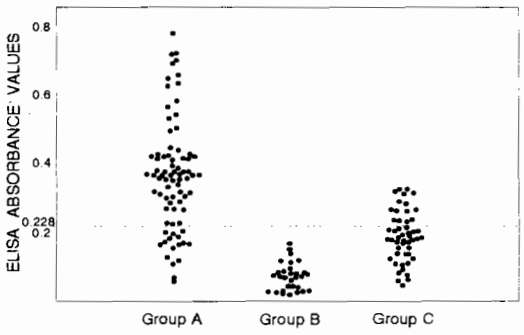

Fig 2–ELISA absorbance values of sera from opisthorchiasis patients (Group A, N = 81), normal healthy individuals (Group B, N = 30) and other patients with helminthic infections (Group C, N = 50) assayed against crude somatic antigens extracted from whole *B. funiculata* snails.

helminthic infections which were used for selecting optimal cut off values produced absorbance values at 0.213 and 0.359 respectively (Figs 3, 4). The sensitivity, specificity, positive and negative predictive values were 75.3%, 70.0%, 71.8% and 73.7%, respectively. And the performance characteristics of the test at absorbance 0.359 were 74.1%, 72.5%, 73.2% and 73.4%, respectively.

DISCUSSION

The crude somatic antigens of *O. viverrini* adult worms and those of the whole *B. funiculata* snails gave equally satisfactory results by IgG-ELISA. It

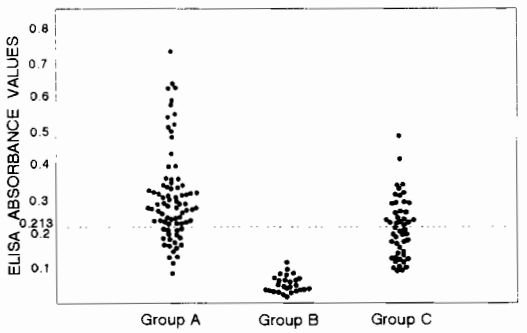

Fig 3–ELISA absorbance values of sera from opisthorchiasis patients (Group A, N = 81), normal healthy individuals (Group B, N = 30) and other patients with helminthic infections (Group C, N = 50) assayed against crude somatic antigens extracted from the head-foot of *B. funiculata* snails.

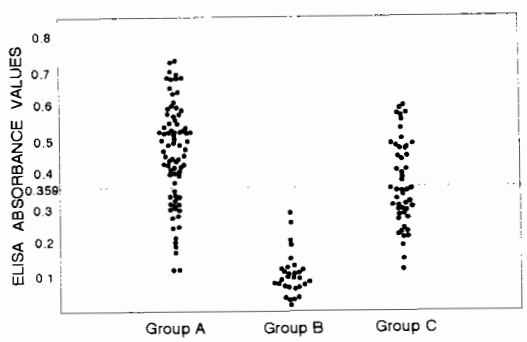

Fig 4–ELISA absorbance values of sera from opisthorchiasis patients (Group A, N = 81), normal healthy individuals (Group B, N = 30) and other patients with helminthic infections (Group C, N = 50) assayed against crude somatic antigens extracted from the visceral mass of *B. funiculata* snails.

is owing to the whole snail body extract containing shared antigenic molecules in both the head-foot and the visceral mass where the components are just as antigenic as those contained in crude somatic extracts of *O. viverrini* adult worms specific for all infected sera (Watthanakulpanich *et al*, unpublished). Sensitivity of the test using both antigens-adult worms and whole snails, came out equally at 80.2%. While the other two sources of antigens-the head-foot and the visceral mass, both gave lower sensitivity at 74.1%. Sixteen out of 81 cases gave false negative reactions when the crude somatic antigen of *O. viverrini* adult worms was employed. The same 16 cases also gave false negative reactions when that of the whole *B. funiculata* snails was used. 68.8% of these cases had eggs counts of less than 1,000 epg. Since fecal egg output is correlated with worm burden (Pungpak *et al*, 1990; Sithithaworn *et al*, 1991), basing on the classification by Upatham *et al*, (1982), these cases, therefore, had light infection. Moreover, there was also a significant correlation observed between log transformed serum IgG levels and fecal egg counts when the crude somatic antigen of *O. viverrini* adult worms was used ($r = 0.61$, $p < 0.0001$) (Elkins *et al*, 1991). Comparing the sensitivity between the crude somatic antigens of *O. viverrini* adult worms and those of whole *B. funiculata* snails, the former should be more sensitive in detecting *O. viverrini* infected sera than the latter since the antigen was made from *O. viverrini* itself. Although in this study, both antigens presented equal degree of sensitivity (80.2%), the former was assayed against a more diluted sera (1:800) than the latter (1:400) as determined by checkerboard titration. Theoretically, stimulated antibodies of an infection produced by correlated antigens can be more easily detected than the other antigens. It was also found that tests performed using the crude somatic antigens of *O. viverrini* adult worms appeared to be more reproducible than that using antigen prepared from *B. funiculata* snails.

With regard to cross reactions, the crude somatic antigens from adult worms and those from whole snails gave their respective specificity at 80.0% and 81.2%. The other two antigenic extracts - the head-foot and the visceral mass, gave lower specificity readings at 70.6% and 73.2%, respectively. The false positive reactions could be observed from all crude somatic extracts. Sera from capillariasis, sparganosis and hookworm infection gave no cross reactions with crude somatic antigens

from adult worms and whole snails. A relatively high degree of cross reactions occurred when the antigens were assayed against sera from paragonimiasis (11 out of 23 cases), strongyloidiasis (3 out of 7 cases) and toxocariasis (1 out of 2 cases) when using antigens from *O. viverrini* adult worms. Cross reactions were likely resulting in the same false positive cases with paragonimiasis (12 out of 23 cases) and strongyloidiasis (2 out of 7 cases) when crude somatic antigens of the whole *B. funiculata* snails were used. Whether or not it is a true cross reaction cannot be ascertained though those individuals had no *O. viverrini* eggs detectable at the time of blood collection, previous infection could not be ruled out. Another possibility is that the *O. viverrini* infection is still in the early stages where in the worms have not yet reached the adult stage, and a third possibility that these were light infections which were not diagnosed by fecal examination. Previous studies on humoral immune responses in experimental opisthorchiasis had shown that low levels of antibodies could be detectable as early as the second week of infection when the parasites are still in the juvenile stage (Sirisinha *et al*, 1983; Chawengkirttikul *et al*, 1988). The crude somatic antigens of *B. funiculata* snails used in this study, though giving minimal cross reactions with a few kinds of helminthic infections tested, may react with many others that were not available for testing.

The significant differences ($p < 0.001$) between the seropositivity of sera with and without *O. viverrini* infection and between those with *O. viverrini* infection and those with other helminthic infections when both of antigens, *O. viverrini* adult worms and whole *B. funiculata* snails were used suggest that this approach could indeed be used as a screening method for opisthorchiasis. However, if used alone it probably is still unreliable at this point of time in determining between present and past infections. From this preliminary attempt, we can conclude that the snail intermediate host's antigens have high potential in becoming a useful diagnostic tool for opisthorchiasis. Purification of this antigen may further increase both the sensitivity and the specificity of ELISA; and subsequent application of this *Bithynia* antigen for diagnosis of opisthorchiasis can be more acceptable.

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