Antigenic Community between *Opisthorchis viverrini* Adult Worms and Their Intermediate Hosts, *Bithynia* Snails as a Support for Concomitant Antigens

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Abstract

The concept that parasites share antigens with their snail intermediate hosts has received considerable experimental support. In this study, comparison between three species of *Bithynia* snails: *B. funiculata, B. siamensis siamensis*, *B. siamensis goniomphalos* and crude somatic antigens of *Opisthorchis viverrini* adult worms were immunologically characterized with antibodies (IgG) in sera of opisthorchiasis patients by SDS-PAGE and Western blot analysis. All of these snail antigens reacted with *O. viverrini* infected human sera, which revealed only 13-15 protein bands by Western blot analysis when compared with sera from healthy controls. *B. funiculata* was proven to have more shared antigens with *O. viverrini* than the other two species, particularly three pairs with molecular weights (MW) of 29 and 30, 47 and 50, and 86 and 90 kDa, which reacted consistently with all infected sera. Extensive cross reactions of the *B. funiculata* snail antigen with sera from patients with other parasitic infections still occurred. However, the three pairs of proteins mentioned above mostly failed to give any cross reaction with the sera of paragonimiasis, schistosomiasis, fascioliasis, sparganosis, larval toxocariasis, trichuriasis and capillariasis, or at least just one or two protein bands out of the six appeared to cross react with sera of taeniasis, cysticercosis, echinococcosis, gnathostomiasis, angiostrongyliasis, trichinosis, ascariasis, filariasis, strongyloidiasis, hookworm infections and HIV infections. This study suggests that the antigen prepared from *B. funiculata* snails, one of the snail intermediate hosts of *O. viverrini*, could possibly be used as an alternative diagnostic antigen for opisthorchiasis in the future.

Keywords: shared antigens, *Bithynia funiculata*, *B. siamensis siamensis*, *B. s. goniomphalos*, *Opisthorchis viverrini,* Western blot analysis

Introduction

Liver fluke infection due to *Opisthorchis viverrini* is distributed mainly in Southeast Asian regions, including Thailand, Laos PDR, Cambodia and South Vietnam. It was estimated that about 8.6 million people were infected with opisthorchiasis in northeastern Thailand [1]. Despite effective chemotherapy and a decrease in prevalence, opisthorchiasis is still consistently a public health problem in Thailand.

Several investigators have studied the phenomenon of antigen sharing between a parasite and its snail intermediate host by developing a characterization technique for specific detection. It was initiated by Fairley [2], who utilized an extract of *Biomphalaria boissyi* snails to diagnose schistosomiasis in humans and monkeys by a complement fixation test. Other parasite-snail immune relationships were assessed as follows: *S. mansoni* - *B. glabrata* [3-7];

Fasciola hepatica - *Galba truncatula* [8]; *S. haematobium* - *Bulinus* (*Physopsis*) *africanus* [9]; *Trichobilhazia ocellata* - *Lymnaea stagnalis* [10]; *S. japonicum* - *Oncomelania* snails [11-12]; *S. mansoni* and *Fasciola hepatica* - *B. glabrata* [13]; *Parastrongylus cantonensis* - *B. glabrata* [14]. In 1990, Chanawong and colleagues demonstrated two common antigens that are shared among *O. viverrini* adult worms and three species of non-infected snail intermediate hosts (*Bithynia*) by immunoelectrophoresis [15]. This finding leads to the interesting possibility of using *Bithynia* snails as antigens by ELISA [16] and gelatin particle indirect agglutination test [17] in the diagnosis of human opisthorchiasis.

The objectives of the present study were to further characterize shared antigens between *O. viverrini* and *B. funiculata* using SDS-PAGE and Western blot technique for the immunodiagnosis of human opisthorchiasis.

Materials and methods

Snail intermediate hosts

Three species of *Bithynia* snails were collected from different sources: *B. funiculata* from various rice fields at Ban Hua Phai, Mae-Moh District, Lampang Province, northern Thailand; *B. s. goniomphalos* from Ban Sa-ard, Khon Kaen Province, northeastern Thailand; and *B. s. siamensis* from Kasetsart University, Bang Khen, Bangkok, central Thailand. The snail species were identified individually based on morphological criteria summarized by both the Tropmed Technical Group [18] and Chitramvong [19].

Parasites

O. viverrini adult worms were collected from the common bile ducts, gall bladders and main hepatic ducts of infected adult golden Syrian hamsters, *Mesocricetus auratus,* after four-week maintenance. All worms were washed 3 times with normal saline solution and repeatedly washed with distilled water. They were kept at -20˚C until used.

Sera

Three groups of serum samples were provided: Group A consisted of sera of individuals residing in Prachinburi Province with parasitologically confirmed opisthorchiasis by the recovering of eggs in feces; Group B consisted of sera from apparently healthy individuals residing in non-endemic areas who attended regular checkups at the out-patient clinic of the Children's Hospital and were found not to have any parasitic infection. Group C consisted of sera from individuals with other parasitic infections proven by parasitological and/or immunological techniques, as follows: paragonimiasis, schistosomiasis, fascioliasis, taeniasis, cysticercosis, echinococcosis, sparganosis, gnathostomiasis, angiostrongyliasis, trichinosis, larval toxocariasis, ascariasis, trichuriasis, capillariasis, filariasis, onchocercosis, strongyloidiasis, hookworm infection and HIV infection. All sera were frozen at -20˚C until used.

Antigens

The shell of each snail species was gently removed under a stereomicroscope and the shellless snails were washed with distilled water. These parasite-free snails were then collected and subjected to lyophilization and manual homogenization by using a glass pestle and mortar with distilled water, after which they were sonicated 15 times, 2 minutes each. The homogenate was centrifuged in an automatic ultracentrifuge (Hitachi Centrifuge, Model SCP 85H2, Hitachi Koki Co, Ltd, Japan) at 30,000 rpm at 4˚C for 1 hour. Protein concentrations of all extracts were determined by Lowry's method [20]. The crude somatic extracts of each *Bithynia* species were collected in small aliquots and kept frozen at -20˚C until used. All *O. viverrini* adult worms were thoroughly ground in a glass tissue grinder and then centrifuged at 15,000 rpm at 4˚C for 1 hour. Further procedures were carried out as previously described above.

Sodium dodecyl sulfate-polyacrylamide gel eletrophoresis (SDS-PAGE)

To compare the pattern of crude proteins of the three snail species and *O. viverrini*, SDS-PAGE was employed, by using a discontinuous gel composed of 4% stacking gel and 13%

separating gel in a vertical slab gel apparatus (AE-6200, ATTO Corporation, Japan). A volume of 7.5 μl each of crude protein (45 μg) was treated with an equal volume of sample buffer (0.5 M Tris-HCl pH 6.8, 7.5% 2-mercaptoethanol, 3.5% SDS) at 100°C for 3 minutes, then loaded into each well. A well with low molecular weight protein markers (Pharmacia) was set as standard. A constant current at 20mA for the stacking gel and 30 mA for the separating gel was supplied. The patterns of protein fractions were visualized by staining with Coomassie brilliant blue and then subsequently characterized.

Western blot analysis

A strip of single well gel was prepared and loaded with a total volume of 945 μg extract after selecting the crude somatic antigen that gave the highest protein profile. The fractioned proteins in the gel were electrotransferred onto a nitrocellulose sheet (0.45 μm) by semi-dry transfer cell "Horiz Blot" (ATTO Corporation, Japan). The electrophoretic transfer was accomplished with a constant current at 200 mA for 4 hours and the nitrocellulose sheet was blocked with 3% gelatin in 0.04% NaN_3-PBS pH 7.4 for 1 hour on a rocking platform, washed with PBS and cut into several strips. The detection of immunoreactive components was performed as follows: exposure of diluted serum samples (1:50) for 4 hours on a rocking platform and then incubated overnight, washed in PBS-Tween for 3 times (10 minutes each time on a rocking platform) and then exposed to peroxidase conjugated anti-human IgG (1:1,000) for 2 hours on a rocking platform. After the final wash, freshly prepared substrate solution (2,6-dichloro indophenol) was added. The appearance of color on the strips was stopped by washing with distilled water several times.

Results

Characterization of crude somatic extracts of three *Bithynia* species and *O. viverrini* adult worms

The *O. viverrini* extract revealed a complex protein pattern comprising approximately 30 protein bands with MW ranging from 16.5 to 98 kDa. The *Bithynia* snail extracts: *B. funiculata*, *B. s. siamensis* and *B. s. goniomphalos* revealed a very similar pattern comprising approximately 28 protein bands with MW ranging from 14 to 90 kDa. The snail extracts from *B. funiculata* exhibited reactive bands which were of 26, 27, 29, 30, 32, 37, 42, 45, 47, 50, 53, 55, 58, 86 and 90 kDa while those of *B. s. siamensis* and *B. s. goniomphalos* showed the same reactive bands as *B. funiculata*, except that *B. s. siamensis* had no 55 and 58 kDa bands and *B. s. goniomphalos* did not have the 29, 55 and 58 kDa bands. Only the extract of *B. s. goniomphalos* showed a reactive band with a MW of 25 kDa (Fig 1).

Specificity of the serum antibody responses

The serum reactivities of 23 patients with confirmed opisthorchiasis against the crude

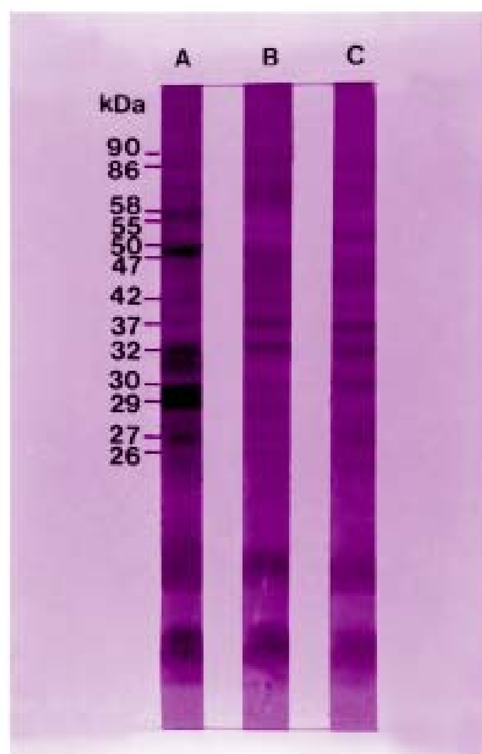

Fig 1 Western blot patterns of the confirmed opisthorchiasis pooled sera against *B. funiculata* (A), *B. s. siamensis* (B) and *B. s. goniomphalos* (C) snail antigens.

somatic extract of *B. funiculata* gave a similar pattern, particularly the three pairs of antigens at MW 29 and 30, 47 and 50 and 86 and 90 kDa (Fig 2). The sera of other patients infected with a variety of parasites also reacted with some of this crude somatic antigen but did not produce a homogeneous pattern. However, the three specific pairs of proteins found to give consistent reactions with all sera of opisthorchiasis were mostly unrecognized when assayed against sera of other helminthiases such as paragonimiasis, schistosomiasis, fascioliasis, sparganosis, larval toxocariasis, trichuriasis and capillariasis; or one to two of each couple might appear to give cross reactions with taeniasis, cysticercosis, echinococcosis, gnathostomiasis, angiostrongyliasis, trichinosis, ascariasis, filariasis, onchocercosis, strongyloidiasis, hookworm infections and HIV infections. Moreover, none of the sera from healthy controls reacted significantly with any of the specific three pairs (Figs 3-6 and Table 1).

Discussion

An attempt to characterize antigens resulted in the evaluation of various antigen preparations from three species of *Bithynia* snails for the detection of specific antibodies in the sera of infected patients. Reducing conditions and Coomassie brilliant blue staining of SDS-polyacrylamide gels showed similar complex protein profiles among crude somatic extracts of three species of *Bithynia* snails and *O. viverrini* worms; this is true especially among the *Bithynia* snails, where the individual profiles can hardly be distinguished from each others. This finding is in agreement with Chung, who showed a similar protein pattern among three species of *Bithynia* snails [21]. This has resulted with some antigenic determinants being so similar or identical to those of *O. viverrini* that the *Bithynia* snails' immune system will find the *O. viverrini* snail stages difficult to detect.

The pooled sera of patients infected with *O. viverrini* that reacted mainly with *O. viverrini* antigen in Western blotting also gave strong reactive bands with the extracts of all three species of *Bithynia* snails, although lower antigenicity was observed. Similarly, the immunological relationship between *B. glabrata* antigens and the sera from patients with parasitologically confirmed parastrongyliasis developed 9 reactive bands with MW of 19.5, 22, 24, 39.5, 48, 60, 70, 110 and 190 kDa. However, only two antigenic components with MW of 24 and 48 kDa appeared to be specific, since they did not give any cross-reactions with other parasitic infections [22]. Thus it would be worthwhile to pursue tests using the crude somatic antigens of *Bithynia* snails which share antigens with liver flukes. The obvious advantages are the large amounts of material that can be obtained rapidly and at a comparatively lower cost than *O. viverrini* antigen, whereby the

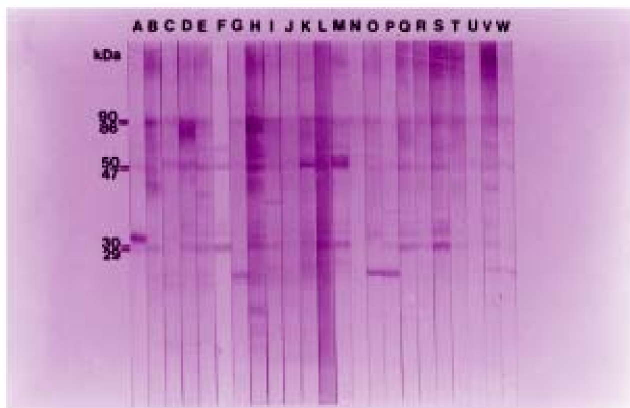

Fig 2 Western blot patterns of the confirmed opisthorchiasis sera (A-W) against *B. funiculata* snail antigen.

Fig 3 Western blot patterns of other helminthic sera (A-X) against *B. funiculata* snail antigen, A: opisthorchiasis, B-D: gnathostomiasis, E-I: strongyloidiasis, J-N: trichinosis, O-P: capillariasis, Q-U: hookworm infection, V-W: ascariasis, X: trichuriasis.

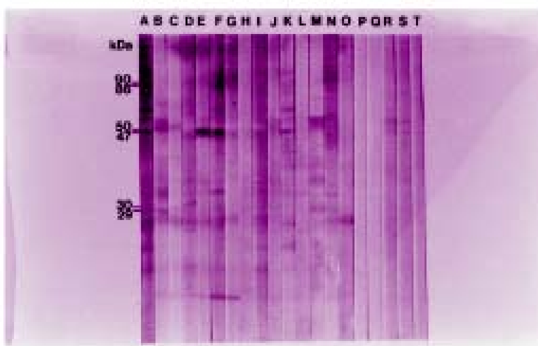

Fig 4 Western blot patterns of other helminthic sera (A-T) against *B. funiculata* snail antigen, A: opisthorchiasis, B-G: angiostrongyliasis, H-I: larval toxocariasis, J-O: taeniasis, P-T: paragonimiasis.

Fig 5 Western blot patterns of other helminthic sera (A-W) against *B. funiculata* snail antigen, A: opisthorchiasis, B-E: schistosomiasis, F-I: fascioliasis, J-M: cysticercosis, N-R: echinococcosis, S-T: sparganosis, U-V: onchocercosis, W: filariasis.

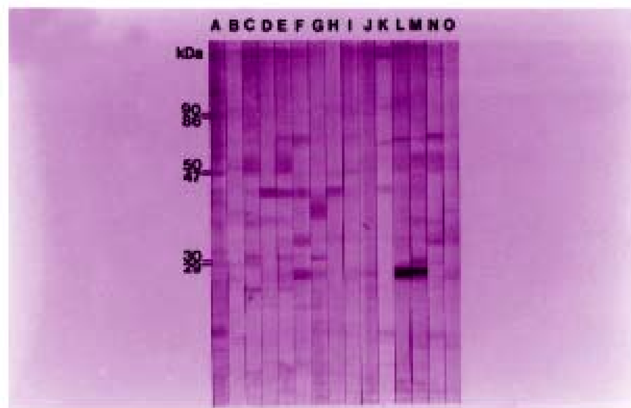

Fig 6 Western blot patterns of other helminthic sera (A-O) against *B. funiculata* snail antigen, A: opisthorchiasis, B-J: healthy controls, K-O: HIV infection.

Table 1 Summary of serum reactivities of patients with opisthorchiasis, other parasitic infections and healthy controls against the three specific pairs of *B. funiculata* antigens.

Sera	Specific bands (kDa)			Patients showing positive reaction
(Total number)	29 & 30	47 & 50	86 & 90	(Total number)
Confirmed cases (23)	23	23	23	23
Paragonimiasis (5)	-	-	-	0
Schistosomiasis (4)	-	-	-	0
Fascioliasis (4)	-	-	-	0
Taeniasis (6)	1	1	-	2
Cysticercosis (4)	-	2	-	2
Echinococcosis (5)	2	3	1 (= 90)	4
Sparganosis (2)	-	-	-	0
Gnathostomiasis (3)	-	1 (= 50)	-	1
Angiostrongyliasis (6)	1	3	-	4
Trichinosis (5)	-	1 (= 47)	-	1
Larval toxocariasis (2)	-	-	-	0
Ascariasis (2)	-	1 (= 50)	1	2
Trichuriasis (1)	-	-	-	0
Capillariasis (2)	-	-	-	0
Filariasis (1)	-	1	-	1
Onchocercosis (2)	-	1	1 (= 86)	2
Strongyloidiasis (5)	-	1 (= 47)	-	1
Hookworm infection (5)	-	1	-	1
HIV infection (5)	1	-	-	1
Healthy controls (9)	-	-	-	0

– : negative reaction
= : cross-reaction only one band from its couple

parasites require the maintenance of a complex life cycle. Furthermore, no significant reaction was observed in normal human sera assayed against antigens produced from the *Bithynia* snails.

The shared antigens in the crude somatic extracts of three species of *Bithynia* snails are nearly identical, as demonstrated by both SDS-PAGE and Western blot analysis. However, the deterioration of some components, especially the 29, 55 and 58 kDa, were observed in the extracts of *B. s. siamensis* and *B. s. goniomphalos* snails when compared with those from extracts of *B. funiculata* snails. Besides, of the three species, *B. funiculata* snails showed the highest infection rate with *O. viverrini* eggs, with both cercaria-shedding snails and non-shedding snails containing rediae and immature cercariae [23]. It seems that the more bands displayed, the higher the infection rate. As Tsuji *et al* [11] reported, the number of precipitin bands of IEP was relative to the degree of susceptibility in the corresponding snails, and the more suitable the snail host, the greater the antigenic similarity to the adult trematode. This observation agreed with the report of Iwanaga *et al* [24] that *Achatina fulica*, which obtained the highest infection rate to *Angiostrongylus cantonensis*, produced the most common antigens against *A. cantonensis* adult worm. Therefore, *B. funiculata* seemed to be a more suitable snail intermediate host than the other two, and was selected as the representative species throughout the study.

In the present study, this snail extract was used to identify the predominant antigen for immunodiagnosis for opisthorchiasis with patients' sera, and simultaneously against those of other helminthic infections, and also with healthy individuals. Sera of all 23 individuals with *O. viverrini* infection had an almost identical pattern of reactivity against the *B. funiculata* snail antigen; however, variations were observed in the presence and intensity of some bands. The prominent antigenic bands with MW of 29 and 30, 47 and 50 and 86 and 90 kDa were found to give consistent reactions with sera from all of the corresponding infections, but not with those from healthy individuals. However, there were some cases of other infections giving positive results against not more than one pair out of the three, except echinococcosis for cases (2 out of 5) which gave cross-reaction against two pairs: 29 and 30, 47 and 50 kDa for the first, and 47 and 50, 86 and 90 kDa for the second. This may have resulted from mixed infection with *O. viverrini*. The results clearly demonstrated that extensive cross-reactions of the crude somatic antigen of *B. funiculata* occurred with not more than one pair, while the reaction against opisthorchiasis infection gave consistent bands with all three specific pairs, 29 and 30, 47 and 50 and 86 and 90 kDa. The use of crude, undefined *B. funiculata* snail antigen is not suitable for the specific immunodiagnosis of human opisthorchiasis. However, this finding suggests that the crude somatic antigen of *B. funiculata* snails is quite immunogenic and can be used instead of the parasites themselves. Purification of the antigen in order to obtain a more refined one is a need for whatever test system aimed at detecting the specific antibodies.

Acknowledgements

This work was supported in part by a grant from the Faculty of Tropical Medicine, Mahidol University, Bangkok. The technical assistance of the Faculty staff is acknowledged. The authors would like to thank Dr Pennapa Subchareon and Miss Napaporn Chaikittisuk for providing sera.

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