EFFICACY OF SODIUM METAPERIODATE (SMP)-ELISA FOR THE SERODIAGNOSIS OF SCHISTOSOMIASIS MEKONGI

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Abstract. Schistosomiasis mekongi is an important public health issue in endemic countries. In this study, we evaluated an indirect immunodiagnostic ELISA method using *Schistosoma mekongi* soluble egg antigen. Sodium metaperiodate (SMP)-ELISA was utilized in order to remove the glycosylated epitopes responsible for false positive reactions and the results using this method were compared with those using conventional ELISA (conv-ELISA). Forty-two serum samples from schistosomiasis mekongi egg-positive patients and 100 serum samples from schistosomiasis-negative Cambodian subjects were tested using both ELISA methods. The ranges of ELISA values for positive and negative sera were distinct on SMP-ELISA, but the ranges of the two groups of sera overlapped on conv-ELISA. Therefore, diagnostic criteria may be established based on the highest ELISA value on negative sera and the lowest ELISA value on positive sera. In the present study, both the sensitivity and specificity of SMP-ELISA reached 100% using the criteria in which an ELISA value ≥ 0.2 was positive.

Keywords: SMP-ELISA, *Schistosoma mekongi*, diagnosis

INTRODUCTION

Schistosomiasis mekongi is a significant public health problem in Lao PDR and Cambodia. A mass drug administration campaign by the National Center for Parasitology, Entomology and Malaria Control (CNM), Ministry of Health, Cambodia, with international cooperation was initiated in 1994 using praziquantel in Kratie Province. At the beginning of the control program, the disease prevalence in primary schools in the Kratie Province, as determined by stool examination using the Kato-Katz method, was 72.9% (Stich *et al*, 1999). As a result of the control program, the prevalence of schistosomiasis decreased dramatically. In 2004, when stool

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surveys were conducted at five sentinel villages, no schistosome eggs were detected and only three cases of schistosomiasis were reported (Sinuon *et al*, 2007).

Despite the success of the control program, the limited sensitivity of the Kato-Katz method is a matter of concern. The detection of eggs from patient stool has become difficult because the intensity and prevalence of the disease have decreased. Therefore, a more sensitive diagnostic technique is required to determine progress of the schistosomiasis control program (Urbani *et al*, 2002; Fenwick *et al*, 2006). Bergquist *et al* (2009) stated that diagnostic methods need to be continually adapted based on the stage of control. Serological diagnosis, such as using an enzyme-linked immunosorbent assay (ELISA), is a potential alternative diagnostic method for the schistosomiasis mekongi control program (Ohmae *et al*, 2004).

We previously conducted a seroepidemiological survey of schistosomiasis japonica using circum oval precipitin (COP) tests (Tanaka *et al*, 1987) and ELISA (Matsumoto *et al*, 1999; Chigusa *et al*, 2006) and have studied *Schistosoma mekongi* control in Cambodia (Ohmae *et al*, 2004).

Immunodiagnosis of schistosomaisis mekongi using antigens of *Schistosoma japonicum* (Zhu *et al*, 2005) and keyhole limpet hemocyanin (Ittiprasert *et al*, 2000) have been carried out, but immunological diagnosis using *S. mekongi* antigen has not previously been reported.

In this study, we performed an ELISA using the soluble egg antigen (SEA) of *S. mekongi* and utilized sodium metaperiodate (SMP)-ELISA. The method of Alarcón de Noya *et al* (2000) was used in order to remove the glycosylated epitopes responsible for false positive reactions occurring with immunodiagnosis of *S. mansoni* infection. In this study, a comparison between SMP-ELISA and conventional ELISA was performed, and we discus suitable criteria for the methods.

MATERIALS AND METHODS

Serum samples

A mass drug administration campaign was conducted in Cambodia: individuals in the target population were diagnosed by stool examination and serological testing as part of the National Schistosomiasis Control Program for Cambodia. The 42 positive control sera (Group A) used in this study were collected from schistosome egg-positive residents in 2003 ($n = 34$) and 2009 ($n = 8$) in Kratie Province, Cambodia. The 34 sera collected from *S. mekongi* infected patients in 2003 were pooled and used as the positive reference serum, which was distributed into sample tubes and stored at -40°C until use.

Negative control sera were collected from volunteers without risk for schistosomiasis mekongi infection: 100 Cambodians (Group B) in 2002 and 25 Japanese (Group C) in 2003. Group B was comprised of schoolchildren in Phnom Penh, located more than 120 km away from the endemic area. Furthermore, these subjects had no history of visiting the endemic area. Serum samples were collected by CNM as a part of the parasitic diseases control program.

Individuals in Group C were healthy Japanese volunteers without a history of traveling abroad. The purpose of the study was explained and written informed consent was obtained from all volunteers.

Antigen

Schistosoma mekongi (Laotian strain) was maintained in *Neotricula aperta* snails and mice (ICR strain) at the Laboratory of Tropical Medicine and Parasitology,

Dokkyo Medical University, Japan. SEA was prepared using the method described by Matsuda *et al* (1984). Eggs of *S. mekongi* were isolated from infected mice intestines by digestion method using actinase E (No. 122, Kaken Pharmaceutical, Tokyo, Japan) and collagenase (C6885, Sigma-Aldrich, St. Louis, MO), and the collected eggs were subsequently lyophilized. SEA was extracted from lyophilized eggs with carbonate buffer (0.05 M, pH 9.6). After protein content measurement using Bradford reagent (#500-0006, Bio-Rad Laboratories, Hercules, CA), 1 ml aliquots of SEA solution were placed in small tubes and stored at -80ºC until use.

ELISA

The ELISA was performed as described by Matsuda *et al* (1984) and Hirose *et al* (2005) with some modifications. In this study, the standard ELISA technique used in our laboratory was designated as conventional-ELISA (conv-ELISA). *S. mekongi* SEA was dissolved in carbonate buffer at a concentration of 2 μg protein/ml just before sensitization of the ELISA plate (No.762070, Greiner Bio-One, Frickenhausen, Germany). Each well of the ELISA plate was sensitized overnight with 0.1 ml of *S. mekongi* SEA diluent. After washing the wells with T-PBS (0.15 M phosphate buffered-saline containing 0.05% Tween 20), the inner surfaces of the wells were blocked with 0.12 ml of BSA/T-PBS solution (T-PBS containing 1% bovine serum albumin) for 10 minutes.

We used horseradish peroxidase (HRP)-conjugated anti-human IgG goat serum (55252, MP Biochemicals, LLC-Cappel Products, Soton, OH) for the enzyme-conjugated antibody and 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) di-ammonium salt (ABTS)(A-1888, Sigma-Aldrich, St. Louis, MO) as a substrate for HRP. The optimal concentrations of antigen, test sera, and enzyme conjugate were determined using checkerboard titration. The test serum was diluted to 1:200 with BSA/T-PBS, and 0.1 ml of the dilution was put in each well. After incubation of the plate at 37ºC for 45 minutes, the wells were washed three times with T-PBS. Zero point one milliliter of HRP conjugate diluted to 1:1,200 was then added, and the plate was incubated at 37ºC for 60 minutes. After washing, 0.2 ml of ABTS solution (0.03% ABTS, 0.25 M citric acid, 0.25 M sodium dihydrogen phosphate, 0.003% H_2O_2) was put in each well and the plate was kept at room temperature for 1 hour to allow enzyme reactions. The optical density (OD) of each well was read by a microplate reader (MTP-120, CORONA ELECTRIC, Ibaraki, Japan) at 415 nm.

For SMP-ELISA, SMP treatment of antigen was performed as described by Alarcón de Noya *et al* (2000) except for the concentration of SMP solution. The *S. mekongi* SEA-sensitized plate was prepared using the same method as conv-ELISA and was washed with 50 mM sodium acetate buffer (pH 4.5). Each well was treated with 0.1 ml of 0.5 mM SMP solution (0.5 mM SMP in sodium acetate buffer) at room temperature for 1 hour in the dark. After briefly washing the treated plate with PBS, 0.12 ml of 50 mM sodium borohydride in PBS was added to the wells and the plate was incubated for 30 minutes at room temperature. After washing three times with T-PBS, the plate was subjected to ELISA in a similar manner as conv-ELISA, except the dilution of HRP conjugate was 1:6,000. Optimal concentrations of SMP solution and enzyme conjugate were determined using checkerboard titration to make the positive reference serum produce an OD value comparable with that of the conv-ELISA.

Evaluation of cross-reaction with other parasites

A parasitological survey was conducted in Kratie Province, Cambodia in May 2005 as part of the National Schistosomiasis Control Program. In this survey, eggs from other helminths were found at positivity rates of 14.6-25.8% by stool examination in a village, Talous, located 5 km south of the city of Kratie. According to a previous survey, Talous was known as an area with low transmission of *S. mekongi*. Therefore, we compared the results of stool examination with those of the two ELISA methods in samples obtained from Talous to evaluate cross-reaction with other parasite infections by ELISA. Blood and stool were collected from 151 residents in Talous. Blood samples were collected on blood sampling filter papers (Advantec Toyo Kaisha, Tokyo, Japan) after finger pricking. The blood samples on filter paper were dried and transported to the laboratory in Japan. Discs (3 mm in diameter) were cut out from the blood sampling filter paper and placed individually into wells of deep-well microplates. Each disc with blood was immersed in 400 µl of BSA/T-PBS containing 0.5% skimmed milk, shaken vigorously, and incubated overnight at 4ºC after vigorous shaking. The resulting extract was estimated to have a 1:200 dilution of the plasma specimen and was tested by the ELISA methods as described above. For reasons described in the Discussion section, we used the criterion that ELISA values ≥ 0.2 were positive. Stool examination was conducted using a formalin-detergent technique (Waikagul *et al*, 1997). In brief, 0.5 ml of each stool sample was suspended in a formalin-detergent solution (10% liquid dish-washing detergent, 2% formalin in water) and incubated for 30 minutes at room temperature. The suspension was filtered through gauze into another tube and then shaken vigorously. The suspension was allowed to settle for 3 hours, after which the supernatant was discarded. The remaining pellet was dissolved in 10% formalin to give a volume of 1 ml. The resulting specimen was mixed well, then 0.04 ml of the specimen was examined under the microscope. Each sample was examined twice.

Statistical analysis

The software program Microsoft Excel (Microsoft Office Excel 2003, Microsoft, Washington, WA) was used to calculate the correlation coefficient (r).

The two-sided probability (P) for the Fisher's exact probability test was calculated by standard statistical software (Dr. SPSS 2 for Windows, Version 11.0.1J, SPSS, Chicago, IL). P-value < 0.05 was considered significant.

RESULTS

The results of the two ELISA methods are shown in Fig 1 and Table 1. The range of ELISA values in Group A was wider with the SMP-ELISA on both the higher and lower sides. The highest ELISA value of Group B with the conv-ELISA (0.578) was markedly lower with the SMP-ELISA (0.198). The range of ELISA values from Group C was very narrow in comparison with the other groups, and there was little difference between the two ELISA methods. With conv-ELISA, the lowest value in Group A (0.330) was lower than the highest value in Group B. Therefore, 10 individuals (23.8%) from Group A and 6 individuals (6%) from Group B had ELISA values ranging from 0.330 to 0.578. In contrast, the distribution of the ELISA values from Group A was different than those from Groups B and C with SMP-ELISA.

The correlation between conv-ELISA and SMP-ELISA, from Groups A and B is

Table 1
A comparison of the ELISA methods for Schistosomiasis mekongi.

	conv-ELISA			SMP-ELISA		
	Group A	Group B	Group C	Group A	Group B	Group C
No. examined	42	100	25	42	100	25
Minimum	0.330	0.006	0.004	0.233	0.015	0.010
Maximum	1.232	0.578	0.054	1.526	0.198	0.058
Mean	0.755	0.089	0.018	0.694	0.058	0.029
SD	0.211	0.107	0.014	0.314	0.041	0.011
Mean + 3SD		0.410	0.059		0.180	0.061

Table 2
Relationship between the ELISA results and other parasitic infections detected by stool examination (n = 151).

			Stool examination [a]					
			A. lumbricoides		Hookworms		*Echinostoma* sp	
			Positive	Negative	Positive	Negative	Positive	Negative
			22	129	35	116	39	112
conv-ELISA [b]	Positive	25	3	22	7	18	9	16
	Negative	126	19	107	28	98	30	96
	Fisher's exact probability test (P)		1.000		0.605		0.217	
SMP-ELISA [b]	Positive	8	2	6	3	5	3	5
	Negative	143	20	123	32	111	36	107
	Fisher's exact probability test (P)		0.329		0.388		0.426	

[a]Stool examination was conducted using the formalin-detergent method.
[b]ELISA values ≥ 0.2 were considered positive.

shown in Fig 2. There were significant correlations between the two ELISA methods in both groups. The correlation coefficient of Group A was higher (r = 0.951, <0.001) than the correlation coefficient of Group B (r = 0.744, <0.001).

Stool examination and ELISA were conducted to determine cross-reactivity with other parasites (Table 2). Of 151 subjects, 25 (16.6%) and 8 (5.3%) had positive ELISA results with the conv-ELISA and the SMP-ELISA, respectively. No *S. mekongi* eggs were detected. Eggs from *Ascaris lumbricoides*, hookworms, and *Echinostoma* sp were detected in samples from 22, 35, and 39 individuals, respectively. Most individuals who had eggs of other parasites had negative ELISA results on both ELISAs, but some individuals had positive ELISA results. The ELISA positivity rates

Table 3
False positive and false negative in each criteria.

	conv-ELISA			SMP-ELISA		
	Group A	Group B	Group C	Group A	Group B	Group C
≥0.2	42 (100%)	9 (9%)	0 (0%)	42 (100%)	0 (0%)	0 (0%)
<0.2	0 (0%)	91 (91%)	25 (100%)	0 (0%)	100 (100%)	25 (100%)
≥X (B)[a]	41 (98%)	2 (2%)	0 (0%)	42 (100%)	2 (2%)	0 (0%)
<X (B)[a]	1 (2%)	98 (98%)	25 (100%)	0	98 (98%)	25 (100%)
≥X (C)[b]	42 (100%)	45 (45%)	0 (0%)	42 (100%)	33 (33%)	0 (0%)
<X (C)[b]	0	55 (55%)	25 (100%)	0 (0%)	67 (67%)	25 (100%)

[a]X (B) Mean + 3SD of Group B in each the method. The values are shown in Table 1.
[b]X (C) Mean + 3SD of Group C in each the method. The values are shown in Tabele 1.

among egg-positive patients were 13.6-23.1% with conv-ELISA and 7.7-9.1% with SMP-ELISA. The Fisher's exact probability test did not show bias with the ELISA for intestinal parasites.

DISCUSSION

SMP treatment of SEA was performed in order to destroy the glycosylated epitopes responsible for false-positive results (Alarcón de Noya *et al*, 2000). Because glycosylated epitopes can be recognized by antibodies in *Schistosoma mekongi* infected patients, the OD values of the patients decreased with the SMP-ELISA when we used enzyme conjugate at the same dilution rate as that used for conv-ELISA (data not shown). In this experiment, we used a higher concentration of enzyme conjugate with the SMP-ELISA. SMP treatment can increase or decrease ELISA values based on serum type and result in expansion of the range of ELISA values for Group A with SMP-ELISA. Differences in the effect of SMP treatment on ELISA values appear to depend on the composition of target epitopes in each serum sample. Most antibodies in sera with high ELISA values in Group B, which consisted of subjects from a non-endemic area in Cambodia, recognized the glycosylated epitopes in SEA.

Diagnostic criteria are established based on the protocol and/or purpose of the study. Many researchers have used mean + 3SD for the OD values of negative control sera as a cut-off limit (Alarcón de Noya *et al*, 2000). Some authors used criteria that depended on the distribution of ELISA values for positive and negative controls (Hirose *et al*, 2005). In this study, Cambodian (Group B) and Japanese (Group C) individuals were examined as negative controls. Using the mean + 3SD for the negative control for Group B as a cut-off value (Table 3), the sensitivity and specificity of conv-ELISA were both 98%, and the sensitivity and specificity with SMP-ELISA were 100% and 98%, respectively. Using the same criteria for Group C (Table 3) results in false positives with conv-ELISA of 45% and with SMP-ELISA of 33%.

The range of ELISA values for positive sera was distinct from that for negative sera with SMP-ELISA. Therefore,

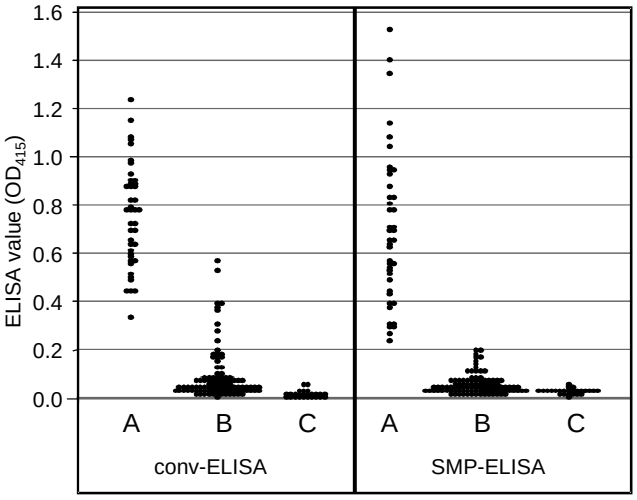

Fig 1–Distribution of ELISA values for sera from *S. mekongi* infected patients and from subjects in non-endemic areas by the two ELISA methods. A: *S. mekongi* infected patients ($n = 42$); B: subjects in a non-endemic area in Cambodia ($n = 100$); C: subjects in Japan ($n = 25$).

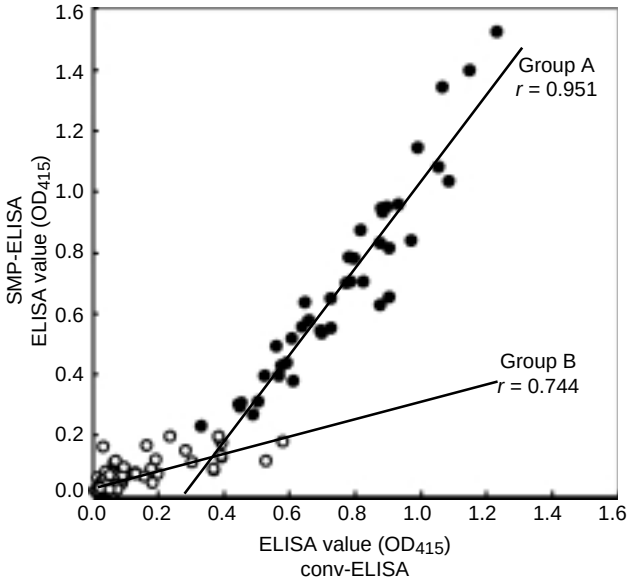

Fig 2–Plot of OD values comparing conv-ELISA to SMP-ELISA to evaluate the correlation between the two ELISA methods. The OD values of sera from *S. mekongi* infected patients (solid circle) and Cambodians living in a non-endemic area (open circle) are shown. Group A: *Schistosoma mekongi* infected patients ($n = 42$); Group B: persons in a non-endemic area in Cambodia ($n = 100$).

diagnostic criteria can be established based on the highest ELISA value for negative sera and the lowest ELISA value for positive sera. Using the criterion in which an ELISA value ≥0.2 was positive, both the sensitivity and specificity of SMP-ELISA reached 100% (Table 3).

Concerning negative control sera, the range of ELISA values for Group B (Cambodian) was higher than for Group C (Japanese). This is possibly due to a difference in antigens to which each group were exposed, both qualitatively and quantitatively. Our results suggest SMP-ELISA can reduce the influence of cross reactive antigens.

A positive ELISA reaction without schistosome eggs indicates one of three scenarios: 1) active infection with *S. mekongi* but no eggs were detected, 2) *S. mekongi* has already been treated but residual antibodies still existed, and (3) non-specific cross-reaction was detected. The lower sensitivity of the stool examination compared to the ELISA may explain why scenario 1) might occur. In low transmission areas, such as in Talous, it is difficult to detect eggs in stool samples because of lower disease intensity (Urbani *et al*, 2002; Fenwick *et al*, 2006). Scenario 2) is a characteristic feature of diagnostic methods detecting specific antibodies. This reaction indicates the subject had a risk for infection. In

general, residual antibodies tend to diminish progressively, and withdrawal periods vary between individuals (Hayashi *et al*, 2000). In scenario 3), a non-specific cross-reaction, might be one of the causalities, at least with the conv-ELISA (Fig 1). Most patients with intestinal parasite eggs had negative ELISA values, although some had positive values. The present data suggest infection with one of these three intestinal parasites should not result in a significant cross-reaction. Given the results of Table 3, it can be seen that the positive reactions on SMP-ELISA are most likely caused by scenarios 1) or 2). Assuming that all positive ELISA tests were due to cross-reaction with other parasites, the maximum rate of false positives with SMP-ELISA would be 9.1% (2/22). In another survey in a *Schistosoma mekongi* endemic area in Champasack Province, Lao PDR in 2006, we detected *Opisthorchis viverrini* eggs without *S. mekongi* eggs in 13 of 41 individuals who took the survey (Nakamura *et al*, 2006; unpublished data). Three of 13 opisthorchiasis patients had positive ELISA values, and 14 out of 41 had positive ELISA values on SMP-ELISA. The ELISA positivity rate for *O. viverrini* egg-positive persons (23%) was lower than the positivity rate of the targeted 41 persons (34%). These data suggest cross-reaction should not be significant with these helminths, although further studies are required for validation.

We conclude the use of SMP-ELISA improves diagnostic specificity and sensitivity for schistosomiasis mekongi. This method should become a powerful tool for diagnosing infection.

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