DEVELOPMENT OF THE RAPID AND SIMPLE ELISA (WHOLE BLOOD-ELISA) USING COCONUT WATER-TWEEN AS A WASH SOLUTION FOR WHOLE BLOOD SAMPLE FROM *SCHISTOSOMA JAPONICUM*-INFECTED RABBIT AND HUMAN

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Abstract. PBS-Tween as a wash solution, prepared with distilled water, is used in ELISA. In areas where schistosomiasis is endemic, however, distilled water is hard to come by. We have modified a WHOLE BLOOD-ELISA test to use coconut water-Tween as a wash solution, because coconut water is easy to come by and cheap in the tropics. We applied the test to whole blood samples from rabbits and humans infected with *Schistosoma japonicum*. This modified WHOLE BLOOD-ELISA was confirmed to be a rapid, simple, and cost-effective method.

INTRODUCTION

The most widely used method for diagnosing schistosomiasis, a parasitic helminth infection, is enzyme-linked immunosorbent assay (ELISA), first published by Engvall and Perlmann (1971). Modifications include the microplate-based ELISA (Voller *et al,* 1974; Matsuda *et al,* 1984), k-ELISA (a kinetic-dependent modification) (Tsang *et al,* 1980), the Falcon Assay Screening Test system (FAST-ELISA) using microsomal adult worm antigen (Hancock and Tsang, 1986), the dot-ELISA, blotted onto nitrocellulose paper (Boctor *et al,* 1987), RAST-ELISA (Weiss *et al,* 1978; Ismail *et al,* 1989), transferable solid-phase (TSP)-ELISA, based on FAST-ELISA (Moser *et al,* 1990), and Diffusion-In-Gel (DIG)-ELISA (Kamal *et al,* 1994). However, there are few reports of rapid, simple, cost-effective ELISA methods for screening in the mass treatment of schistosomiasis (Hamilton *et al,* 1998).

We introduce a rapid and simple immunodiagnostic method using coconut water-Tween as a wash solution for whole blood samples from *Schistosoma japonicum*-infected rabbit and humans.

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MATERIALS AND METHODS

Parasite antigen

S. japonicum (Yamanashi strain) was maintained in *Oncomelania nosophora* snails and mice (ICR strain). *S. japonicum* eggs were isolated from the intestines of infected mice by digestion with pronase and collagenase (Matsuda *et al,* 1984), freeze-dried, and stored at -80ºC until use.

Whole blood samples of rabbits and humans

Positive whole blood samples (2-3 ml) were collected in each experiment from four rabbits (Japanese white), each infected with 400 cercariae of *S. japonicum*. The samples were treated with 0.001% heparin and immediately used for the experiments. Positive sera were separated by centrifugation for 5 minutes at 1,710*g* at room temperature and then stored at -80ºC until use. Normal whole blood samples were collected from two control rabbits (Japanese white) with no history of schistosomiasis, and treated and stored in as described above.

Human whole blood samples were donated by the residents of Palo, Leyte, the Philippines,

in August 2002, and treated with 0.001% heparin. Samples were either used immediately or treated with 5% sodium azide (1 drop reagent/1 ml sample) and stored in a refrigerator. *S. japonicum* eggs were confirmed by Kato-Katz thick smear technique (Katz *et al,* 1972) and formalin-detergent technique (Moody, 1986). The first stool examination was performed by Kato-Katz thick smear technique. After one month, feces were again collected from the residents who were stool negative from *S. japonicum* eggs and were examined successively for 1 to 5 days by both Kato-Katz thick smear and formalin-detergent techniques. Some of the people were also infected with *Ascaris lumbricoides*, hookworm, or *Trichuris trichiura.* Positive human serum samples were separated by centrifugation at 1,710*g* for 5 minutes at room temperature and then stored at -80ºC until use. Negative human serum samples were donated by the students of Dokkyo University School of Medicine, Japan, with no history of schistosomiasis and treated and stored as described above.

ELISA procedure

WHOLE BLOOD-ELISA, a rapid and simplified ELISA, was modified from a conventional ELISA as follows. ABTS [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt] was used as a substrate for horseradish peroxidase (HRP) conjugate (Matsuda *et al,* 1984). The wells of microtiter plates were treated (sensitized) with 0.1 ml of *S. japonicum* crude egg antigen at a concentration of 10 μg protein/ml in carbonate buffer (0.05 M, pH 9.6) in a humidified plastic box at 37ºC for 2 hours. The plates were washed with PBS-Tween (0.15 M phosphate-buffered saline containing 0.05% Tween 20), air-dried, wrapped in a plastic bag, and kept at -80ºC until use. After binding of antibodies and conjugates, the wells were washed three or five times with PBS-Tween or coconut water-Tween (Coco-Tween). The coconut water obtained from coconuts were filtered, and added with 0.05% Tween 20.

The pH of coconut water is generally 4 to 5. To determine whether pH affected the ELISA results, we compared unadjusted Coco-Tween (pH 5.1), adjusted Coco-Tween (pH 7.2) and PBS-Tween (pH 7.2) in the rabbit blood assay.

The optimal dilutions for whole blood and conjugate were determined by checkerboard titration (data not shown). Whole blood samples or conjugates were diluted to the optimal concentration with PBS-Tween containing 1% bovine serum albumin (Fraction V, Sigma Chemical, USA) or with Coco-Tween containing 1% bovine serum albumin. They were then placed in the sensitized wells, incubated at room temperature for 5 minutes, and then washed with a wash solution. For the rabbit samples, 0.05 ml of goat anti-rabbit IgG conjugated with HRP (Southern Biotechnology Associates, USA) diluted to the optimal concentration were added to the wells, and the plates were incubated at room temperature for 5 minutes. For the human samples, 0.05 ml of anti-human IgG conjugated with HRP (ICN Pharmaceuticals, USA) diluted to the optimal concentration were added to the wells, and incubated as indicated above. After washing, 0.1 ml of ABTS (Sigma Chemical) as a substrate were added to each well, and the plates were kept at room temperature for 10 minutes. ABTS was prepared by the dissolutions: 30 mg of ABTS in a mixture of 50 ml each of 0.1 M citric acid and 0.1 M sodium dihydrogen phosphate with 0.01 ml of 30% H_2O_2. The optical density (OD) of the reaction product was measured with a microplate reader (Corona, Japan) at 415 nm.

WHOLE BLOOD-ELISA using Coco-Tween as a wash solution for samples from *S. japonicum*-infected rabbits and humans

Whole rabbit blood samples (0.05 ml) diluted 1:10 were incubated for 5 minutes, then 0.05 ml of conjugate diluted 1:1,000 were added, and the plates were incubated for 5 minutes. OD values were measured 10 minutes after transfer of 0.1 ml of substrate. Coco-Tween with unadjusted pH (5.1), Coco-Tween adjusted to pH 7.2, and PBS-Tween (pH 7.2) were compared as wash solutions. The plates were washed five times with each wash solution. Positive and negative samples were tested.

Whole human blood samples (0.05 ml) diluted 1:10 that were positive by stool examinations of *S. japonicum* eggs were incubated for 5 minutes, then 0.05 ml of conjugate diluted 1:1,000 were added, and the plates were incubated for 5 minutes. OD values were measured

10 or 20 minutes after transfer of 0.1 ml of substrate. Because the pH of the Coco-Tween was shown to have no effect on the results, Coco-Tween with unadjusted pH and PBS-Tween (pH 7.2) were compared as wash solutions. The plates were washed three times with each wash solution.

Data analysis

Microsoft Excel was used to calculate the geometric mean, SD, and correlation coefficient (*r*). The Wilcoxon-Mann-Whitney test and Student's *t*-test were used to assess the significance of difference of OD values between WHOLE BLOOD-ELISA using Coco-Tween (unadjusted pH), Coco-Tween (pH 7.2), and PBS-Tween for rabbit samples, and *r* was used to investigate the correlation of OD values between WHOLE BLOOD-ELISA using Coco-Tween (unadjusted pH) and PBS-Tween for human samples.

RESULTS

In blood from infected rabbits, the OD value in PBS-Tween was significantly higher ($p < 0.001$) than the values in Coco-Tween (Table 1). There was no significant difference between pH-adjusted and unadjusted Coco-Tween ($p = 0.1174$). In blood from uninfected rabbits, the OD value in PBS-Tween was significantly lower ($p < 0.001$) than the values in Coco-Tween (Table 1). There was no significant difference between pH- adjusted and unadjusted Coco-Tween ($p = 0.1826$). Thus, the pH of Coco-Tween did not affect the results of WHOLE BLOOD-ELISA.

At 10 minutes after transfer of substrate, the OD values of whole blood samples from most subjects were higher in PBS-Tween than in Coco-Tween (Table 2, "A/C"). The OD values of most subjects were still higher in PBS-Tween at 10 minutes than in Coco-Tween at 20 minutes (Table 2, "B/C"), though slightly less so.

There was a high correlation ($r = 0.926$, $p < 0.001$) between the OD values of PBS-Tween and Coco-Tween at 10 minutes. In blood from uninfected humans, the OD values from pH-unadjusted Coco-Tween at 10 and 20 minutes were very low, and were therefore satisfactory as reference values. Although some of the subjects were infected with *Ascaris lumbricoides*, hookworm, and *Trichuris trichiura*, cross-reactivity was not observed. Therefore, Coco-Tween with unadjusted pH is a suitable wash solution for WHOLE BLOOD-ELISA in mass screening tests.

DISCUSSION

Rapidity, simplicity, and cost-effectiveness are important factors in the use of ELISA for mass screening in the field. We have developed a rapid and simple ELISA that uses ABTS as a substrate for HRP conjugate (Hirose *et al,* 2005a;b). This WHOLE BLOOD-ELISA has the following advantages: it takes 20-30 minutes; it

Table 1
Mean optical density values of whole blood samples from rabbits in WHOLE BLOOD-ELISA using Coco-Tween or PBS-Tween as wash solution.

Wash solutions	Coco-Tween	Coco-Tween	PBS-Tween
pH	5.1 (unadjusted)	7.2	7.2
OD ± SD (infected)[a]	2.25 ± 0.05 NS	2.30 ± 0.05 NS	2.52 ± 0.07***
OD ± SD (uninfected)[b]	0.41 ± 0.02 NS	0.46 ± 0.08 NS	0.16 ± 0.05***

Five replicates, in OD values in each of Coco-Tween (pH 5.1), Coco-Tween (pH 7.2) and PBS-Tween.
[a] Whole blood samples from rabbits infected with *S. japonicum.*
[b] Whole blood samples from uninfected rabbits.
NS, not significant at the 5% level in OD values between WHOLE BLOOD-ELISA using Coco-Tween (pH 5.1) and Coco-Tween (pH 7.2).
***$p < 0.001$: significant difference in OD values between WHOLE BLOOD-ELISA using PBS-Tween and Coco-Tween (pH 5.1 and 7.2).

Table 2
Optical density values of whole blood samples from humans in WHOLE BLOOD-ELISA using Coco-Tween (unadjusted pH) or PBS-Tween as wash solution in a field screening test of schistosomiasis japonica in Palo, Leyte, the Philippines.

WHOLE BLOOD-ELISA	A (10 min)[a]	B (20 min)[b]	C (10 min)[a]	A/C	B/C
Wash solution	Coco-Tween, pH 5.2	Coco-Tween, pH 5.2	PBS-Tween, pH 7.2		
Pos control ± SD	0.981 ± 0.056	1.158 ± 0.052	1.279 ± 0.088		
Neg control ± SD	0.065 ± 0.068	0.067 ± 0.090	0.097 ± 0.031		
No.	Average	Average	Average		
1	1.026 (1.045, 1.006)	1.165 (1.180, 1.150)	1.355 (1.418, 1.291)	0.757	0.860
2	0.942 (0.932, 0.951)	1.105 (1.105, 1.128)	1.291 (1.291, 1.411)	0.729	0.856
3	0.785 (0.783, 0.787)	0.971 (0.968, 0.974)	1.132 (1.142, 1.122)	0.693	0.858
4	0.433 (0.436, 0.429)	0.516 (0.531, 0.501)	0.767 (0.794, 0.740)	0.564	0.673
5	0.855 (0.905, 0.804)	0.997 (1.055, 0.938)	1.031 (1.035, 1.027)	0.829	0.967
6	0.927 (0.928, 0.926)	1.087 (1.063, 1.111)	1.268 (1.240, 1.295)	0.731	0.858
7	0.963 (0.971, 0.954)	1.089 (1.148, 1.029)	1.367 (1.367, 1.367)	0.704	0.796
8	0.460 (0.454, 0.465)	0.553 (0.538, 0.567)	0.836 (0.832, 0.839)	0.550	0.661
9	0.258 (0.295, 0.221)	0.319 (0.363, 0.275)	0.602 (0.686, 0.518)	0.429	0.530
10	0.918 (0.897, 0.938)	1.044 (0.981, 1.107)	1.356 (1.355, 1.357)	0.677	0.770
11	0.717 (0.726, 0.707)	0.822 (0.829, 0.814)	1.138 (1.125, 1.151)	0.630	0.722
12	0.647 (0.639, 0.654)	0.774 (0.739, 0.808)	1.203 (1.176, 1.229)	0.538	0.643
13	0.737 (0.762, 0.712)	0.904 (0.941, 0.866)	1.055 (1.081, 1.028)	0.699	0.857
14	0.895 (0.891, 0.899)	0.963 (0.990, 0.935)	1.183 (1.128, 1.237)	0.757	0.814
15	0.785 (0.819, 0.751)	0.894 (0.924, 0.863)	1.224 (1.183, 1.264)	0.642	0.730
16	0.689 (0.690, 0.687)	0.812 (0.809, 0.815)	1.125 (1.143, 1.106)	0.612	0.722
17	0.832 (0.855, 0.808)	0.948 (0.992, 0.903)	1.088 (1.110, 1.066)	0.764	0.871
18	0.783 (0.766, 0.799)	0.865 (0.856, 0.873)	1.104 (1.070, 1.137)	0.709	0.783
19	0.892 (0.885, 0.899)	1.010 (0.976, 1.044)	1.303 (1.310, 1.295)	0.685	0.775
20	0.777 (0.769, 0.784)	0.945 (0.951, 0.939)	0.944 (0.900, 0.988)	0.823	1.001
21	0.875 (0.889, 0.861)	0.997 (1.003, 0.990)	1.243 (1.220, 1.265)	0.704	0.802
22	1.038 (1.055, 1.020)	1.188 (1.226, 1.150)	1.382 (1.403, 1.360)	0.751	0.860
23	1.053 (1.074, 1.031)	1.228 (1.250, 1.206)	1.259 (1.167, 1.350)	0.836	0.976
24	0.201 (0.210, 0.192)	0.246 (0.246, 0.245)	0.502 (0.502, 0.501)	0.401	0.490
25	1.041 (1.076, 1.005)	1.209 (1.243, 1.174)	1.346 (1.324, 1.367)	0.773	0.898
26	0.998 (1.000, 0.996)	1.192 (1.216, 1.168)	1.287 (1.300, 1.274)	0.775	0.926
27	0.676 (0.680, 0.671)	0.896 (0.917, 0.875)	0.940 (0.878, 1.002)	0.719	0.953
28	1.015 (1.013, 1.016)	1.221 (1.233, 1.209)	1.474 (1.438, 1.509)	0.688	0.829

[a] OD values were measured at 10 minutes after transfer of substrates.

[b] OD values were measured at 20 minutes after transfer of substrates.

OD values were duplicates shown in parenthesis in screening test samples and were 5 replicates in each of positive control and negative control.

can use whole blood samples; the volumes of blood samples, conjugates, and substrates are half those used in a conventional ELISA; and temperature does not have to be controlled.

Because distilled water is hard to get in large quantities in places where schistosomiasis is endemic, we tested whether coconut water, which is cheap and easy to obtain in the tropics, could be substituted. The OD values of the subjects tested by WHOLE BLOOD-ELISA using Coco-Tween with unadjusted pH were not significantly different from those obtained with PBS-Tween, and the OD values of uninfected blood samples were very low. Therefore, Coco-Tween can be used as a wash solution in WHOLE BLOOD-ELISA for screening in areas where schistosomiasis is endemic.

Since the pH of coconut water is usually 4-5 and the pH of PBS-Tween is 7.2, it was uncertain if coconut water could be used to make a

wash solution. However, we obtained nearly the same results using Coco-Tween and PBS-Tween. The composition of coconut water (RCSTA, 2000) is water (94.3%), potassium (0.23%), sodium (0.011%), calcium (0.011%), phosphorus (0.011%), magnesium (0.006%), and vitamins (<0.001%). This composition makes coconut water suitable as a wash solution.

Some of the subjects tested were simultaneously infected with *Ascaris lumbricoides*, hookworm, or *Trichuris trichiura*; however, there was no cross-reactivity. Therefore, Coco-Tween with unadjusted pH can be used as a cost-effective or emergency wash solution as a replacement for PBS-Tween in mass testing by WHOLE BLOOD-ELISA.

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