# A RAPID AND SIMPLIFIED ELISA USING WHOLE BLOOD SAMPLES OF *SCHISTOSOMA JAPONICUM*-INFECTED RABBITS

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Abstract. A rapid and simplified ELISA using whole blood samples of *Schistosoma japonicum*-infected rabbits was compared with a conventional ELISA. This whole-blood ELISA has advantages. The volume of crude egg antigens, whole blood samples, and conjugates was only 0.05 ml. The incubation time was shortened to 5 minutes. Wells were washed three to five times with PBS-Tween after each procedure. Optical density values were measured in 10 minutes after transfer of 0.1 ml of substrate. Constant temperature was not necessary. The entire procedure took only 20-30 minutes.

## INTRODUCTION

The most widely used method for diagnosing schistosomiasis, a parasitic helminth infection, is enzyme-linked immunosorbent assay (ELISA), first published by Engvall *et al* (1971). Modifications include the microplate-based ELISA (Voller *et al*, 1974), 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), and the dot-ELISA, blotted onto nitrocellulose paper (Boctor *et al*, 1987). More recently, 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) have been introduced. However, there are few reports of rapid, simple, cost-effective ELISA methods for mass screening for schistosomiasis (Hamilton *et al*, 1998).

We introduce a rapid, simplified immunodiagnosis method using whole blood samples of *Schistosoma japonicum*-infected rabbits.

## 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 held at -80ºC until use.

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### Whole blood and serum samples

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 whole blood samples were treated with heparin and immediately used for experiments. The positive sera were separated by centrifugation for 5 minutes at 1,710*g* at room temperature. Normal whole blood samples and sera were collected from two control rabbits (Japanese white) with no history of schistosomiasis, and treated in the same way with positive samples.

### Conventional ELISA procedure

A conventional ELISA using ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] as a substrate for horseradish peroxidase (HPR) conjugate was done according to Matsuda *et al* (1984). The wells of microtiter plates were 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 plastic box with moisture at 37ºC for 2 hours. The plates were washed three times for 5 minutes each 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. The wells were simi-

larly washed after the binding of antibodies and conjugates.

The optimal dilutions for sera and conjugate were determined as described in the next section by checker-board titration. Test samples (serum; 0.1 ml) diluted to the optimal concentration with BSA, PBS-Tween containing 1% bovine serum albumin (Fraction V, Sigma Chemical, USA) were placed in sensitized wells, incubated at 37ºC for 45 minutes, and then washed. Then, 0.1 ml of goat anti-rabbit IgG conjugated with HPR (Southern Biotechnology Associates, USA) diluted to the optimal concentration was added, and the plates were incubated at 37ºC for 1 hour. After washing, 0.2 ml of ABTS (Sigma Chemical, USA) as a substrate was added to each well, and the plates were kept at room temperature for 10 to 60 minutes to allow enzyme reactions. ABTS was prepared by the following procedure: 30 mg of ABTS was dissolved in a mixture of 50 ml each of 0.1 M citric acid and 0.1 M sodium phosphate dibasic, with 0.01 ml of 30% $H_2O_2$. The optical density (OD) of the reacting product was measured by a microplate reader (Corona, Japan) at 415 nm.

Optimal concentrations of serum and conjugate

In the conventional ELISA, 0.1 ml of serum diluted 1:25, 1:50, 1:100, 1:200, or 1:400 was incubated for 45 minutes, then 0.1 ml of conjugate diluted 1:250, 1:500, 1:1,000, or 1:2,000 was added to each serum dilution, and the plates were incubated for 1 hour. OD values were measured at 10 minutes after transfer of 0.2 ml of substrate.

In the simplified ELISA, 0.1 ml of whole blood sample or of serum diluted 1:25, 1:50, 1:100, 1:200, or 1:400 was incubated for 10 minutes, then 0.1 ml of conjugate diluted 1:250, 1:500, 1:1,000, or 1:2,000 was added to each whole blood sample or serum dilution, and the plates were incubated for 10 minutes. OD values were measured at 10 minutes after transfer of 0.2 ml of substrate. Pooled blood samples and pooled sera were tested.

Whole blood as test samples, and the volume of whole blood, conjugate, and substrate

In the simplified ELISA, 0.05 ml of whole blood sample or of serum diluted 1:1, 1:2, 1:4, 1:8, 1:16, or 1:32 was incubated for 5 minutes, then 0.05 ml of conjugate diluted 1:1,000 was added to each whole blood sample or serum dilution, and the plates were incubated for 5 minutes. OD values were measured at 30 and 60 minutes after transfer of 0.1 ml of substrate.

Incubation time of whole blood and of conjugate, and temperature condition

In the simplified ELISA (whole-blood ELISA), 0.05 ml of whole blood samples diluted 1:1, 1:4, and 1:16 were incubated for 5, 10, or 20 minutes, then 0.05 ml of conjugate diluted 1:1,000 was added to each well, and the plates were incubated for 5 minutes. In a variation, 0.05 ml of whole blood samples diluted 1:1, 1:4, or 1:16 were incubated for 5 minutes, then 0.05 ml of conjugate diluted 1:1,000 was added to each well, and the plates were incubated for 5, 10, or 20 minutes. OD values were measured at 60 minutes after transfer of 0.1 ml of substrate. The procedure was done at room temperature.

Incubation time of whole blood

In the whole-blood ELISA, whole blood samples diluted 1:1, 1:10, 1:100, 1:1,000, 1:5,000, 1:25,000, 1:125,000, or 1:625,000 were incubated for 5 or 45 minutes. OD values were measured at 30 minutes after transfer of 0.1 ml of substrate.

Incubation time of substrate and number of washings by PBS-Tween

In whole-blood ELISA, whole blood samples diluted 1:1, 1:2 and 1:10 were incubated at room temperature. OD values were measured at 10 and 30 minutes after transfer of substrates. Plates were washed as described above, three, five or seven times with PBS-Tween.

Statistical methods

For statistical study, differences between 2 groups were examined for significance using Student's *t*-test. A p-value less than 0.05 was considered to be statistically significant.

## RESULTS

Optimal concentration of conjugates

The optimal concentration of conjugate was investigated by a comparison of a conventional ELISA (Matsuda *et al*, 1984) and the simplified

ELISA (Fig 1). The 1:1,000 dilution of conjugate in the conventional ELISA gave the highest correlation (r= -0.92824, p=0.00047) between the dilution of serum and the OD value [Fig 1 (3)]. Therefore, we used the 1:1,000 dilution of conjugate in the other experiments.

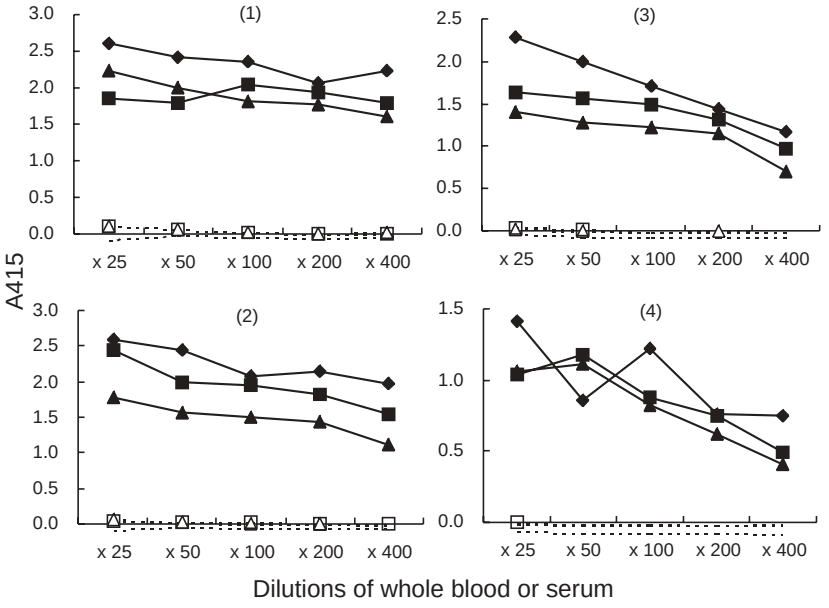


Fig 1–Optimal concentration of conjugate in the simplified ELISA and conventional ELISA. (1) conjugate diluted 1:250, (2) conjugate diluted 1:500, (3) conjugate diluted 1:1,000, (4) conjugate diluted 1:2,000. (▲) Whole blood from infected rabbits in the simplified ELISA, (■) serum from infected rabbits in the simplified ELISA, (◆) serum from infected rabbits in conventional ELISA, (△) whole blood from normal rabbits in the simplified ELISA, (□) serum from normal rabbits in the simplified ELISA, (◇) serum from normal rabbits in conventional ELISA.

Whole blood as test samples, and the volume of whole blood, conjugate, and substrate

Whole blood as test samples and the volume of whole blood, conjugate, and substrate were studied in the simplified ELISA using whole blood samples or sera diluted 1:1, 1:2, 1:4, 1:8, 1:16, or 1:32 (Fig 2). Each OD value of whole blood samples was nearly the same as that of the sera at 30 and 60 minutes after the transfer of substrate. This result shows that whole blood samples can be used as a substitute for sera. The volumes used were 0.05 ml of crude egg antigens, whole blood samples, and conjugates, and 0.1 ml of substrate, half the volume needed in the conventional ELISA. The incubation times were much shorter, too.

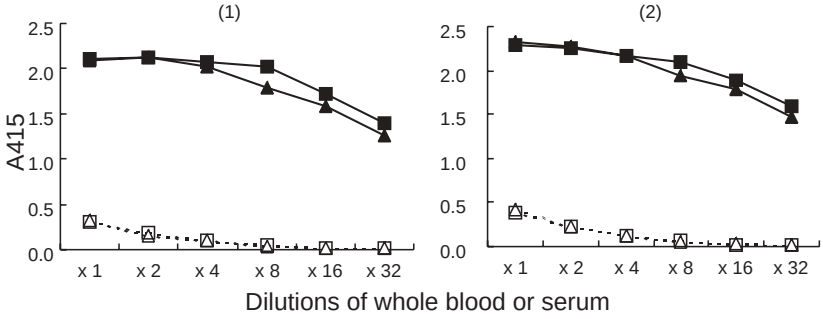


Fig 2–Whole blood as test samples, and the volume of whole blood, conjugate, and substrate in the simplified ELISA. (1) 30 minutes after transfer of substrate, (2) 60 minutes after transfer of substrate. (▲) Whole blood from infected rabbits, (■) serum from infected rabbits, (△) whole blood from normal rabbits, (□) serum from normal rabbits.

The simplified ELISA also tested with whole blood samples or sera from individual rabbit diluted 1:1, 1:2, or 1:4. As shown in Fig 3, each OD value for whole blood samples was nearly the same as that of the sera. The results of this experiment were the same as in Fig 2. This confirms that whole blood samples can be used in place of sera in our rapid and simplified ELISA. The volumes of crude egg antigen, whole blood sample, conjugate, and substrate were half the volume needed in the conventional ELISA. The incubation times were much shorter, too. The simplified ELISA gave satisfactory OD values.

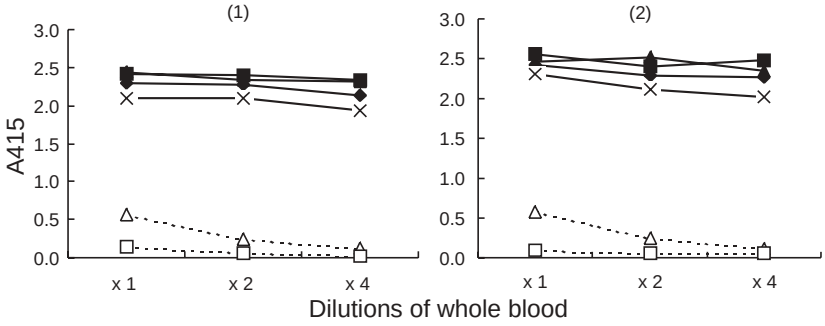


Fig 3–Whole blood as test samples, and the volume of whole blood, conjugate, and substrate in the simplified ELISA. (1) 30 minutes after transfer of substrate, (2) 60 minutes after transfer of substrate. (▲) Infected rabbit A, (■) infected rabbit B, (◆) infected rabbit C, (x) infected rabbit D, (△) normal rabbit E, (□) normal rabbit F.

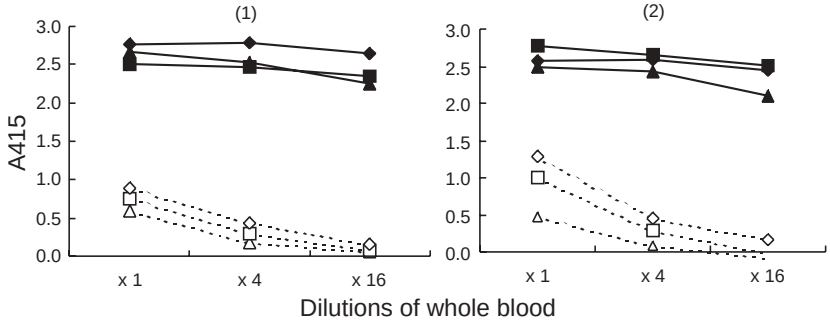


Fig 4–Incubation times of whole blood and of conjugate, and temperature during the experiment. (1) Incubation time of whole blood: (▲,△) 5, (■,□) 10, and (◆,◇) 20 minutes; and incubation time of conjugates: 5 minutes. (2) Incubation time of whole blood: 5 minutes; and incubation time of conjugates: (▲,△) 5, (■,□) 10, and (◆,◇) 20 minutes. (▲,■,◆) Infected rabbits; (△,□,◇) normal rabbits.

### Each incubation time of whole blood and of conjugate, and temperature condition

Incubation times of whole blood samples and of conjugate and temperature conditions were studied in the whole-blood ELISA using whole blood samples diluted 1:1, 1:4, or 1:16 at room temperature. As shown in Fig 4, each OD value at 5, 10, and 20 minutes of incubation time in whole blood samples was almost the same as that at 5, 10, and 20 minutes of incubation time of the conjugates. Thus, the OD values were not influenced by the incubation time. As shown in Fig 4, constant temperature was also not necessary in the whole-blood ELISA.

### Incubation time of whole blood

The incubation time was studied with the whole-blood ELISA using whole blood samples diluted 1:1, 1:10, 1:100, 1:1,000, 1:5,000, 1:25,000, 1:125,000, or 1:625,000 (Fig 5). Although OD values were slightly lower at 5 minutes incubation than at 45 minutes, the curve of OD at 5 minutes was almost the same as at 45 minutes. Values were nearly the same at 5 and 45 minutes in whole blood samples diluted 1:1 to 1:100. Five minutes of incubation time was a satisfactory time for the whole-blood ELISA.

### Incubation time of substrate

The incubation time of the substrate was examined by whole-blood ELISA using whole blood samples diluted 1:1, 1:2, or 1:10 (Tables 1). The 1:10 dilution produced the biggest difference in OD values between infected and normal rabbits at 10 and 30 minutes after transfer of substrate. This difference was slightly larger at 10 minutes (5.11-9.54) than at 30 minutes (4.34-9.37). Since the OD values of normal rabbits increased greatly from 10 minutes (0.13-0.28) of incubation time to 30 minutes (0.19-0.50), the higher OD values at 30 minutes of incubation time would interfere with a screening test for schistosomiasis.

We think that 10 minutes of incubation time of substrates is reasonable, because speed is one of the most important factors in mass-screening tests.

### Number of washings by PBS-Tween

Since OD values of whole blood samples diluted 1:1 and 1:10 from normal rabbits were still higher than the ones of whole blood samples diluted 1:100 to 625,000 as shown in Fig 5, the number of washings by PBS-Tween was investigated in whole-blood ELISA (Table 1). The 1:10 dilution produced the biggest difference between

Table 1
Optical densities of samples after 3, 5, or 7 washes and after 10-minute and 30-minute incubations in whole-blood ELISA. Each experiment was repeated five times and mean OD±SD was shown.

| No. of washings | Dilutions | OD after washes and 10-minute incubation | | | OD after washes and 30-minute incubation | | |
|---|---|---|---|---|---|---|---|
| | | (a) Infected rabbits | (b) Normal rabbits | Ratio (a)/(b) | (a) Infected rabbits | (b) Normal rabbits | Ratio (a)/(b) |
| 3 | x 1 | 1.52 ± 0.03 | 0.88 ± 0.01 | 1.73 [a] | 2.17 ± 0.09 | 1.32 ± 0.04 | 1.64[a] |
| | x 2 | 1.47 ± 0.02 | 0.74 ± 0.03 | 1.99[a] | 2.08 ± 0.09 | 1.13 ± 0.03 | 1.84[a] |
| | x 10 | 1.43 ± 0.04 | 0.28 ± 0.02 | 5.11[a] | 2.17 ± 0.14 | 0.50 ± 0.05 | 4.34[a] |
| 5 | x 1 | 1.51 ± 0.03 | 0.87 ± 0.04 | 1.74 [a] | 2.16 ± 0.12 | 1.26 ± 0.03 | 1.71[a] |
| | x 2 | 1.52 ± 0.08 | 0.61 ± 0.04 | 2.49[a] | 2.11 ± 0.13 | 0.97 ± 0.07 | 2.18[a] |
| | x 10 | 1.43 ± 0.04 | 0.20 ± 0.02 | 7.15[a] | 2.04 ± 0.07 | 0.36 ± 0.02 | 5.67[a] |
| 7 | x 1 | 1.41 ± 0.05 | 0.81 ± 0.03 | 1.74[a] | 1.99 ± 0.15 | 1.15 ± 0.04 | 1.73[a] |
| | x 2 | 1.40 ± 0.04 | 0.55 ± 0.02 | 2.55[a] | 1.95 ± 0.09 | 0.80 ± 0.01 | 2.44[a] |
| | x 10 | 1.24 ± 0.02 | 0.13 ± 0.01 | 9.54[a] | 1.78 ± 0.04 | 0.19 ± 0.02 | 9.37[a] |

Values are mean OD values±SD (n=5)
[a]p=0.001 indicates a significance between the OD values of infected and normal rabbits.

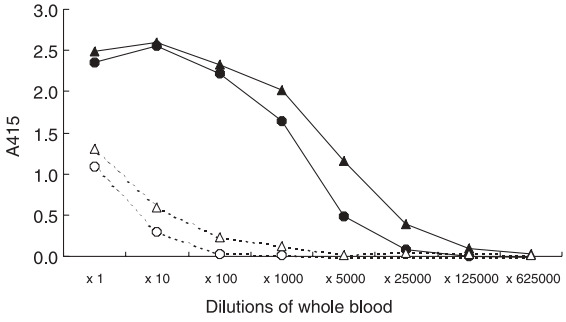


Fig 5–Incubation time of whole blood in whole-blood ELISA. (●) Infected rabbits at 5 minutes of incubation time, (○) normal rabbits at 5 minutes of incubation time, (▲) infected rabbits at 45 minutes of incubation time, (△) normal rabbits at 45 minutes of incubation time.

OD values of infected rabbits (a) and OD values of normal rabbits (b) at 10 and 30 minutes after transfer of substrate. The ratio (a/b) of OD values at 10 minutes of incubation time was 5.11 after 3 washings, 7.15 after 5, and 9.54 after 7. The ratio at 30 minutes was 4.34 after 3 washings, 5.67 after 5, and 9.37 after 7. Seven washings produced the biggest ratios. The OD values of infected rabbits were lower after 7 washings (1.24-1.78) than after 3 (1.43-2.17) and 5 (1.43-2.04).

We think that 3 or 5 washings are reasonable, because rapidity and simplicity are the most important factors in mass-screening tests.

## DISCUSSION

Enzyme-linked immunosorbent assay (ELISA) has been widely used for mass screening of schistosomiasis. Various methods have been introduced, as listed in the Introduction. k-ELISA uses single disposable polystyrene cuvettes instead of micro-titration plates (Tsang *et al*, 1980,1982), FAST-ELISA uses polystyrene beads on sticks molded to the lid of micro-titration plates (Hancock *et al*, 1986), and dot-ELISA uses nitrocellulose-paper-blotted antigens (Boctor *et al*, 1987). RAST-ELISA determines serum IgE levels by paper radioimmunosorbent assay (Weiss *et al*, 1978; Ismail *et al*, 1989), and TSP-ELISA uses transferable solid phases that consist of polystyrene pins molded onto lids (Moser *et al*, 1990). DIG-ELISA uses diffusion of capillary blood spotted onto filter papers (Kamal *et al*, 1994).

In contrast to almost all conventional ELISAs, which use serum, our whole-blood ELISA, using whole blood, is more rapid and simple. On the other hand, unlike the whole-blood ELISA, DIG-ELISA collects whole blood samples on filter papers by finger prick for transportation of samples collected in the field and for storage of blood samples for later examination. Many methods of ELISA generally take 1 to 3 hours, although FAST-ELISA is much faster. Since k-ELISA requires a steady 25ºC and RAST-ELISA needs special fa-

cilities for radioimmunoassay, these methods are not simple or cost-effective.

Our whole-blood ELISA has the following advantages. (1) It can use whole blood samples instead of sera. (2) It requires only 0.05 ml of whole blood samples and of conjugates, instead of the 0.1 ml used in the conventional ELISA. (3) The incubation time of whole blood and conjugate was shortened to 5 minutes owing to the high concentrations of whole blood and conjugate, instead of 45 minutes for serum and 60 minutes for conjugates in the conventional ELISA. (4) OD could be measured at 10 minutes after transfer of substrate, instead of up to 30 minutes in the conventional ELISA. (5) A constant temperature was not required, unlike in the conventional ELISA. (6) The entire procedure took 20 to 30 minutes, instead of over 3 hours in the conventional ELISA.

FAST-ELISA also uses an incubation time for sera and of conjugates of 5 minutes, and takes 30 minutes at room temperature. However, FAST-ELISA requires separation of serum. This gives whole-blood ELISA the advantage. Thus, whole-blood ELISA should be more suitable for mass-screening of children.

We expect whole-blood ELISA to be adopted owing to its simplicity (no need of centrifuges) and rapidity (20-30 minutes in operation time). Future sensitive and specific immunodiagnostic research will be necessary to expand the use of whole-blood ELISA to various areas of endemic human diseases. A check for cross-reactivity will be important.

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