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# An Enzyme-Linked Immunosorbent Assay Method for Diagnosis of Parasitic Infections in Field Laboratories

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**Key words :** rapid diagnosis, enzyme-linked immunosorbent assay, parasitic infections, field testing, antigen potency

## Introduction

Diagnosis of parastic diseases in developing countries mainly depends on clinical manifestations. The unpopularized use of immunodiagnosis is fundamentally attributed to the difficulty in access to electricity, and in taking a sufficient amount of blood occasionally because of people's instinctive repulsion toward the bleeding (N. Iwamura, personal communication). The following disadvantages claimed for existing methods also may constitute an obstacle to their performance : (i) indispensability of the skilled technique and the objective judgment of the results, based on rich experience of trained technicians, as in the indirect or passive hemagglutination test and the indirect or direct fluorescent antibody test, (ii) use of costly and massive equipments in a restricted laboratory area, as in the radio immunoassay, and (iii) lack of sensitivity or rapidity as in precipitin tests or the complement fixation test (Houba, 1980 ; Braude, 1981).

To perform immunodiagnosis under such unfavorable conditions, therefore, we must devise a new assay system, which is simple, rapid and sensitive, as well as economical and reproducible, and which runs without electric apparatus and technical expertise. In the present paper, a rapid enzyme-linked immunosorbent assay (ELISA) system using whole blood as the source of antibodies was developed to meet above requirements, and its practicability for medical care and health services in the tropics was evaluated.

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## Materials and Methods

*Antigen*

The antigen of *Toxoplasma gondii*, the RH strain, was prepared as previously described (Konishi and Takahashi, 1983 ; Takahashi *et al.*, 1985). The soluble antigens prepared from *Ascaris lumbricoides*, *Dirofilaria immitis*, *Fasciola hepatica*, *Metagonimus yokogawai*, *Taenia saginata* and *Dipylidium caninum* were also used. In examination for maintenance of antigen potency, the antigen was fixed by an incubation with 0.37 % formalin at 37°C for 1 hr. After being dialyzed against phosphate-buffered saline (PBS), the antigens adjusted to 15 μg/ml (*T. gondii*), 25 μg/ml (*A. lumbricoides*, *T. saginata*, *F. hepatica*), 30-40 μg/ml (*D. caninum*, *M. yokogawai*) or 100 μg/ml (*D. immitis*), were used for sensitization of iron beads.

*Serum and whole blood samples*

Human serum samples including those positive for *D. immitis*, *F. hepatica*, *M. yokogawai* and *T. gondii* were provided from the Central Laboratory of Kobe University. A total of 299 samples obtained from patients 20-49 years old were used for evaluation of the ELISA system with shorter reaction periods ; 56 samples for reproducibility, 200 for comparison with the ordinary system and 43 for comparison with other serological tests. A total of 696 human serum samples used for evaluation of visual readings were provided

from the Miki Health Center of Hyogo Prefecture, which were collected from healthy individuals 20–77 years old at medical examinations. Dog serum samples were supplied by Dr. Uga of this laboratory with data for helminthic infections at necropsy. These sera were stored at $-20°C$ until use for the test.

Human whole blood samples, 2 positive and 2 negative for *Toxoplasma* antibodies, were taken from a basilic vein of 22- to 37-year-old individuals. A total of 39 dog whole blood samples were collected from stray dogs captured by the Animal Administration Office of Hyogo Prefecture. Three whole blood samples positive for *Toxoplasma* antibodies were obtained from a white rabbit which had been infected intraperitoneally first with about 100 cysts (the S-273 strain, harvested from mouse brain) and after 4 weeks with $2\times10^3$ tachyzoites of the RH strain. Four whole blood samples were taken from three ICR mice infected intraperitoneally with about 40 cysts as above and one unifected mouse as a negative control.

Part of each whole blood sample (2 $\mu$l) was taken with a micro-pipette ("Microcaps", Drummond Scientific Co.,USA) and promptly diluted in 100 $\mu$l of ELISA diluent (PBS containing 1 %bovine serum albumin, 0.05 % Tween 20, and 0.02 % $NaN_3$) which had been previously distributed in microplate wells for the first reaction of ELISA test. The 1 : 51 dilution was determined corresponding to the standard serum dilution of 1 : 100, because the use of undiluted whole blood led to a considerable amount of nonspecific reactions in the preliminary experiment. Prompt dilution in the Tween 20-containing PBS did not cause visible coagulation. Another part of the specimen (0.1 ml) was sampled with a filter paper (Strip type, Toyo Roshi Co., Ltd., Japan). After being dried, the blood-soaked filter paper was immersed in the ELISA diluent to elute the blood samples. The eluate was adjusted to 1 : 100 dilution. In cases of human, dog and rabbit whole blood, the rest of the specimen was used for usual serum separation.

*ELISA procedure*

The magnetic processing system was based on the technique proposed by Smith and Gehle (1980) with some modifications described in our previous paper (Konishi and Takahashi, 1983). Polycarbonate-coated iron beads used as the solid phase were simply and simultaneously processed using magnetic transfer devices without any electrical apparatus.

The standard procedure of the ELISA test followed our previous report mentioned above. In brief, the solid phase iron beads sensitized with the antigen were each reacted with 100 $\mu$l of the 100-fold diluted test serum samples at 28°C for 1 hr (the first reaction) and then with 100 $\mu$l of the 100-fold diluted alkaline phosphatase-conjugated anti-human immunoglobulin G at 28°C for 1 hr (the second reaction), followed by the third reaction with 100 $\mu$l of 0.1 % *p*-nitrophenyl phosphate at 28°C for 30 min. The enzyme reaction was halted by adding 50 $\mu$l of 1 N NaOH and the absorbance values were read at a wavelength of 410 nm.

In the improved procedure, the reaction period was shortened and the results were read by naked eyes instead of spectrophotometry. The process of adding the stopping reagent was saved, because the removal of enzyme-labeled beads almost terminated the color development. For evaluation of this system, *T. gondii* was mainly used as an infectious agent and all processes of the ELISA test was performed at 28°C unless otherwise stated. To test rabbit, mouse or dog serum samples, conjugates were prepared from alkaline phosphatase (Sigma Chemical Co., USA) and goat anti-rabbit, -mouse or -dog IgG (Cappel Laboratories, Inc., USA) as described by Engvall and Perlmann (1972). The methylene blue dye test and the latex agglutination (LA) test were performed as previously described (Konishi and Takahashi, 1983).

## Results

*Applicability of whole blood*

Experiments were designed to study the reliability of every step of the assay procedures. At first, the usefulness of whole blood in the first reaction of ELISA test was examined.

Table 1 Comparison of ELISA absorbance values for *Toxoplasma* antibodies on whole blood, serum and blood collected on filter paper

| Blood* source | Sample no. | Sample† | | | Ratio of | |
|---|---|---|---|---|---|---|
| | | Whole blood | Serum | Filter eluate | Serum to blood | Eluate to blood |
| Human | 1 | 0.95 | 0.92 | 0.95 | 0.97 | 1.00 |
| | 2 | 0.59 | 0.55 | 0.61 | 0.93 | 1.03 |
| | 3 | 0.02 | 0.02 | 0.14 | 1.00 | 7.00 |
| | 4 | 0.01 | 0.01 | 0.12 | 1.00 | 12.00 |
| Rabbit | 1 | 2.39 | 2.48 | 2.61 | 1.04 | 1.09 |
| | 2 | 2.24 | 2.52 | 2.44 | 1.13 | 1.09 |
| | 3 | 1.68 | 1.80 | 1.90 | 1.07 | 1.13 |
| Mouse | 1 | 1.39 | ND‡ | 2.32 | — | 1.67 |
| | 2 | 1.13 | ND | 1.80 | — | 1.59 |
| | 3 | 0.51 | ND | 0.79 | — | 1.55 |
| | 4 | 0.10 | ND | 0.15 | — | 1.50 |

* Human whole blood samples obtained from 22- to 37-year-old individuals were positive in nos. 1 and 2 and negative in nos. 3 and 4. Rabbit samples were collected three times from a white rabbit infected previously. Mouse samples of nos. 1 to 3 were obtained from three infected mice and a sample of no. 4 from an uninfected mouse as a negative control.

† Whole blood and serum samples were diluted 1 : 51 and 1 : 100 in ELISA diluent, respectively. Filter eluate samples were adjusted to 1 : 100 dilution. These samples were tested for *Toxoplasma* antibodies in ELISA with the antigen-coated beads sensitized at a protein concentration of 15 μg/ml.

‡ Not determined.

The substitution of whole blood for serum saves the time-consuming and laborious process of serum separation.

The 51-fold diluted whole blood was compared in ELISA value with the 100-fold diluted serum and/or the eluate from filter paper which had been adjusted to 100-fold diluted serum. In addition to human samples, those of rabbit, mouse and dog were used for the comparison. In several cases of human, rabbit and mouse (Table 1), results from the whole blood samples were proportional to all the other ones, except human negative samples collected on filter paper. Although ELISA values were higher in these filter eluate samples, results from whole blood and serum samples were almost equal and such a non-specific reaction was not observed in positive samples. The examinations with 39 dogs also revealed proportional relationships among those three samplings: correlation coefficients were more than 0.80. These data indicate successful application of the whole blood.

*Evaluation of ELISA system with shorter reaction periods*

The reducibility of reaction periods in the ELISA was examined to incorporate more rapidity into this procedure. The ELISA system with 15 min for the first and the third reactions and 30 min for the second reaction (the rapid ELISA system) was constructed, because time courses presented in the previous report (Konishi and Takahashi, 1983) indicate that about 80% of the first reaction was completed in 15 min and about 70 % of the second reaction, in 30 min. An attempt was made to hasten the reaction by continuous shaking of the microplate, but little change was observed in time courses.

The reproducibility of the rapid ELISA system was examined by using 56 human serum samples. The absorbance values obtained in duplicate experiments were highly correlated, providing a correlation coefficient of 0.991.

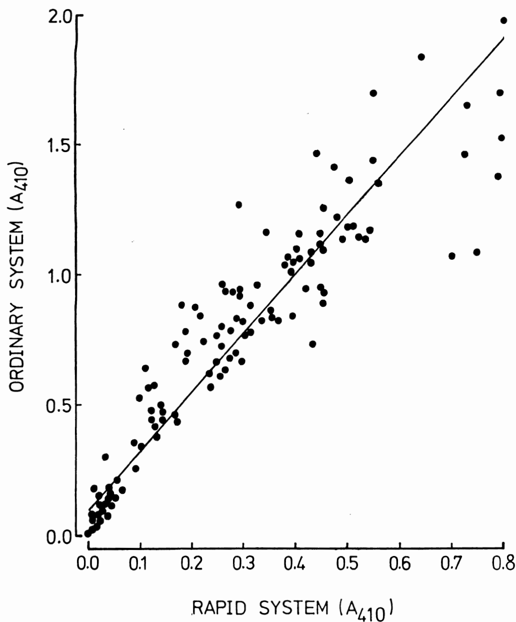


Fig. 1 Comparison between the rapid and ordinary ELISA systems for *Toxoplasma* antibody quantitation. A total of 200 human serum samples collected routinely from patients at our Hospital were examined. Abscissa and ordinate indicate absorbance values at 410nm ($A_{410}$) obtained by the rapid and ordinary systems, respectively. The correlation coefficient was 0.960 with a regression line of Y=2.271X+0.0959.

In other replicate test with positive samples, was seen almost no detectable inter- or intra-plate variation : coefficients of variation were smaller than 5%.

The rapid ELISA system was directly compared with the ordinary ELISA system. As shown in Fig. 1, a significant correlation was observed in 200 human sera ($r$=0.960). Quantitative and qualitative comparisons with other serological tests using 43 human serum samples, also resulted in high correlation coefficients with the dye test ($r$=0.932) and with the LA test ($r$=0.894), as well as good agreements with the dye test (93.0%) and the LA test (83.7%).

To know whether or not the ELISA can be performed at atmospheric temperatures in the tropics, several temperatures for running the test, besides 28°C, were examined. The results indicated that the ELISA values were almost the same at 25°C, 28°C and 32°C, as well as 37°C generally used for laboratory testing, although data were not shown in this paper. Therefore, high correlations with the dye and LA tests were again observed under these conditions.

*Reliability of visual readings*

The reliability of judgment by eyes, which is useful in field laboratories without electric power, was examined. The assay was per-

Table 2 Comparison between judgments by visual and spectrophotometric readings*

| Spectrophotometric† reading | Visual reading‡ | | | | | Total |
|---|---|---|---|---|---|---|
| | +++ | ++ | + | ± | − | |
| +++ | 2 | 0 | 0 | 0 | 0 | 2 |
| ++ | 6 | 59 | 7 | 0 | 0 | 72 |
| + | 0 | 3 | 52 | 4 | 0 | 59 |
| ± | 0 | 0 | 5 | 4 | 1 | 10 |
| − | 0 | 0 | 0 | 6 | 547 | 553 |
| Total | 8 | 62 | 64 | 14 | 548 | 696 |

* A total of 696 human serum samples collected at medical examinations from healthy individuals 22–77 years old, were examined for *Toxoplasma* antibodies in ELISA testing system.

† Reactions were classified as strongly positive (+++ ; >0.767), medially positive (++ ; 0.290–0.767), weakly positive (+ ; 0.115–0.290), doubtful (± ; 0.075–0.115), and negative (− ; <0.115).

‡ Reactions were classified into 5 grades by visually comparing the color intensity of the test sample with those of four control sera showing ELISA values of 0.767, 0.290, 0.115 and 0.075.

Table 3 Maintenance of antigen potency of six helminths and one protozoan sensitized on beads at different temperatures

| Antigen | | Relative ELISA values* of sensitized beads kept | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | in antigen solution | | | | under the dryness | | | |
| | | 4°C | 25°C | 28°C | 32°C | 4°C | 25°C | 28°C | 32°C |
| *Acaris lumbricoides* | Fixed† | 99.8 | 128.6 | 95.9 | 81.2 | 96.7 | 29.4 | 31.2 | 27.2 |
| | Non-fixed | 151.9 | 100.4 | 122.8 | 57.7 | 94.9 | 51.5 | 34.8 | 43.6 |
| *Dirofilaria immitis* | Fixed | 148.3 | 132.6 | 137.1 | 129.1 | 101.8 | 102.8 | 90.6 | 94.1 |
| | Non-fixed | 142.3 | 81.6 | 64.2 | 30.5 | 106.0 | 101.6 | 84.9 | 108.3 |
| *Fasciola hepatica* | Fixed | 109.4 | 94.1 | 93.2 | 72.2 | 92.2 | 52.2 | 48.4 | 56.1 |
| | Non-fixed | 92.7 | 99.0 | 89.7 | 70.8 | 106.9 | 50.3 | 35.3 | 51.6 |
| *Metagonimus yokogawai* | Fixed | ND‡ | ND | 143.3 | ND | ND | ND | 93.0 | ND |
| | Non-fixed | 95.3 | ND | ND | ND | ND | ND | 85.6 | ND |
| *Taenia saginata* | Fixed | 72.9 | 102.8[a] | 90.7[a] | 75.3[a] | 93.6 | 29.9 | 21.8 | 35.6 |
| | Non-fixed | 83.0 | 109.8[a] | 101.9[a] | 63.3[a] | 82.7 | 42.4 | 27.4 | 34.3 |
| *Dipylidium caninum* | Fixed | 117.4 | 115.7 | 99.0[a] | 77.0[a] | 40.2 | 14.3 | 16.2 | 23.6 |
| | Non-fixed | 189.1 | 173.1 | 116.0[a] | 78.0[a] | 62.2 | 23.6 | 20.3 | 25.7 |
| *Toxoplama gondii* | Fixed | ND | ND | 102.0[b] | ND | 88.2[b] | ND | 63.5 | ND |
| | Non-fixed | 97.1[c] | ND | 98.3[b] | ND | 69.5 | ND | ND | ND |

* Relative ratio to the antigen potency of control beads newly sensitized at 37°C for 1 hr. Data described were obtained after incubation of the antigen-sensitized beads for 1 week ([a]), 2 weeks (with no mark), 4 weeks ([b]), and 5 months ([c]).

† Fixed with formalin.

‡ Not determined.

formed not only with two control sera producing the absorbance values of 0.075 and 0.115, border lines between negative and boubtful sera and between doubtful and positive sera, but also with two control sera producing 0.290 and 0.767 which correspond to dye test antibody titers of 1 : 16 and 1 : 1024 respectively. At the end of the third reaction, test samples were judged strongly positive, medially positive, weakly positive, doubtful or negative, by comparing the color intensity of the sample visually with those of the four control sera. In 696 human serum samples collected at medical examinations, the judgment by naked eyes resulted in 95.4 % agreement with the readings by spectrophotometry (Table 2). The consistency in samples classified spectrophotometrically as positive was 85.0 %.

*Maintenance of antigen potency on sensitized beads*

The maintenance of antigen potency on sensitized beads at tropical atmospheric temperatures is also required when antigens are not stored under a refrigerated condition. Beads were sensitized with the formalin-fixed or non-fixed antigen at 37°C for 1 hr. A half of the beads were kept at 32°C, 28°C, 25°C or 4°C in the same antigen solution, and the rest were dried on blotting paper and incubated at the above temperatures under the dryness. These beads were periodically examined for their antigen potency by ELISA using a standard positive control serum. Positive human samples were examined by using *D. immitis*, *F. hepatica*, *M. yokogawai* and *T. gondii* and positive dog samples, by *A. lumbricoides*, *T. saginata* and *D. caninum*. The results are summarized in Table 3, where the potency was represented as a relative ratio to that of control beads newly sensitized at 37°C for 1 hr. When beads were sensitized with and kept in non-fixed antigen solution, higher than 70 % of the antigen potency, enough for the ELISA, was maintained at least for 2

weeks in all the parasite antigens used here at 4°C, in *A. lumbricoides*, *F. hepatica* and *T. gondii* at 28°C, and in *F. hepatica* at 32°C. With two nematodes, the potency was maintained for 2 weeks even at 32°C by using the fixed antigen solution. With two cestodes, more than 50 % decrease in potency was observed on beads that were incubated in fixed or non-fixed antigen solution at 28°C or 32°C for 2 weeks, despite the fact that the potency did remain on these beads for 1 week. The dry condition did not bring about such a long-term maintenance at high temperatures in all antigens, except *D. immitis* and *M. yokogawai* antigens which were stable for 2 weeks at 32°C and 28°C, respectively. In some conditions, the ratios were far greater than 100 % (e.g., 151.9 in non-fixed *A. lumbricoides* antigen solution at 4°C), probably due to higher efficiency in sensitization at 4°C for 2 weeks than at 37°C for 1 hr.

*Test protocol*

A test protocol constructed based on the above results is schematized as follows: (i) sensitized beads are incubated with 51-fold diluted whole blood for 15 min, (ii) the beads are then placed in the conjugate for 30 min, (iii) reaction with the substrate for 15 min and (iv) the color intensity is judged visually.

## Discussion

In immunological diagnosis of parasitic diseases, various methods have been adopted in laboratories (Houba, 1980; Braude, 1981). Few of these conventional methods, however, are appropriate for medical care and health services in field laboratories, especially in the area where electric power is not easily available. The precipitin test in gels and the intradermal test are practicable without electricity, but they lack rapidity and/or specificity. The circumoval precipitin test for schistosomiasis is suitable (Oliver-Gonzales, 1954) but the principle does not hold in other diseases. Usefulness of the magnetic processing ELISA for epidemiological surveys under field conditions was suggested in a detection system of antibodies to Japanese encephalitis virus (Konishi and Yamaoka, 1983). In the present study, the ELISA system was further developed for the purpose of immunodiagnosis of parasitic diseases at rural areas.

The whole blood was successfully applied to the ELISA system as the source of antibody. Use of as small as 2 $\mu$l amount of whole blood permits us to take blood from ear lobe- or finger-pricks. Thus, the ELISA system takes about 1 hr from bleeding to reading the results. There still remains a possibility of reducing the reaction period as in a 5min −5min −10min system (Saunders and Clinard, 1976). In our ELISA system, however, such reduction did not result in higher reproducibility as revealed in coefficients of variation of less than 5 %.

In comparison with other ELISA system for *Toxoplasma* antibody detection (Voller *et al.*, 1976; Walls *et al.*, 1977; Camargo *et al.*, 1978; Denmark and Chessum, 1978; Balsari *et al.* 1980; Carlier *et al.*, 1980; Lin *et al.*, 1980; van Loon and van der Veen, 1980), the present ELISA system requiring minimum equipments was simple in procedure, rapid in performance and economical in running. Significant correlations and high qualitative agreements with the dye and LA tests at temperatures of 25°C, 28°C, 32°C and 37°C indicate the reliability of this ELISA system for field testing in the tropics.

Antigen potency of two nematodes, two trematodes and one protozoan was maintained on the solid phase at least for 2 weeks at some tropical atmospheric temperatures, when beads were kept in fixed antigen solution. Smith and Gehle (1980) have reported the long-term maintenance of herpes simplex virus antigen on the plastic-bead solid phase at 5°C, consistent with our result with *Toxoplasma* antigen stored at a similar temperature. No change in antigen potency was also reported on microplate wells coated with the soluble egg antigen from *Schistosoma japonicum* after storage under the dryness at 37°C for 1 month (H. Matsuda, personal communication). Although clude antigens of two cestodes were maintained only for 1 week, antigen fractionation and improvement of storage conditions may contribute to a longer period of maintenance.

On the basis of above results, this system

has proved to satisfy most of the requirements for immunodiagnosis of parasitic diseases in field laboratories.

## Summary

An enzyme-linked immunosorbent assay method was improved so that immunodiagnosis of parasitic diseases can be performed under field conditions where electric power is not easily available. To save all procedures of serum separation, whole blood was substituted for serum. The 51-fold diluted whole blood samples showed absorbance values in proportion to those obtained with 100-fold diluted serum samples. The ELISA system with reaction periods shortened to a total of 1 hr, was reproducible and was significantly correlated with the dye test and the latex agglutination test when antibody detecting system to *Toxoplasma gondii* was adopted as a model. Visual readings were reliable with a consistency of 95.4 % with diagnosis by spectrophotometry. Antigen potency of six helminths (*Ascaris lumbricoides*, *Dirofilaria immitis*, *Fasciola hepatica*, *Metagonimus yokogawai*, *Taenia saginata*, *Dipylidium caninum*) and one protozoan (*T. gondii*) was maintained on solid phase iron beads at least for 1 week at such high atmospheric temperatures as 25°C, 28°C and 32°C by incubating the beads in formalin-fixed antigen solution.

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## 野外条件下の寄生虫症診断に適した酵素抗体法の検討


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熱帯地方において寄生虫症の免疫診断を野外で実施するに適した酵素抗体法の各条件を検討改良した．まず血清分離の過程を省略するために血清のかわりに全血を用いたところ，トキソプラズマ抗体検出系で51倍希釈の全血は，100倍希釈の血清とほぼ同等の抗体値を示した．また反応時間の短縮を検討した結果，全反応時間が1時間であってもこの系はなお再現性が高く，色素試験やラテックス凝集反応との間に有意の相関係数が認められた．さらに肉眼での判定は分光光度計を用いた場合と95.4%の一致率であった．回虫，犬糸状虫，肝蛭，横川吸虫，無鉤条虫，犬条虫及トキソプラズマの抗原力価は，ホルマリン固定抗原中に感作ビーズを保存することにより，25°C，28°C，32°Cの条件下で少なくとも1週間維持された．