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Comparative Evaluation of Field Applicability of a Malaria ABC-ELISA with Other Serological Methods

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Abstract

Evaluation of field applicability of an avidin-biotin peroxidase complex (ABC)-enzyme linked immunosorbent assay (ELISA) for malaria, was conducted using 52 sera from Brazilian patients, compared with an indirect fluorescent antibody test (IFAT), ELISA and fluorescence-ELISA. All assays were sensitive and reproducible, and gave reasonable results. An excellent correlation between the IFAT and ABC-ELISA titers was evident with a correlation coefficient of 0.999 in the patients. Compatibilities with the IFAT in other serological techniques also gave excellent coincidental results, however, the ABC-ELISA was considered to be most suitable method for field mass-surveys, because it could be read clearly with the naked eye without the need for special equipment. The lyophilized malaria antigen gave excellent results in any of the ELISA system studied so far.

Key words: Malaria serodiagnosis, IFAT, ABC-ELISA, ELISA, Fluorescence-ELISA

Introduction

Serological surveys of malaria have several advantages over microscopic monitoring. Latent infection with low parasitemia is likely to be overlooked by microscopic observation. However, latent infections should be highlighted in crusades against malaria, particularly at the advanced stage of control. Serological surveys can identify latent infection as well as past infection to read period prevalence by cross sectional studies (Draper, *et al.*, 1980, Voller, *et al.*, 1982). To date, the indirect fluorescent antibody test (IFAT) (Voller, *et al.*, 1971) has been used as the standard reference serological technique. The IFAT results have correlated with microscopic diagnosis in Japanese imported malaria cases (Kano *et al.*, 1990). However, a more simple and rapid method than the IFAT will be needed in field mass surveys. Several methods such as enzyme linked immunosorbent assay (ELISA) (Voller *et al.*, 1977), radioimmunoassay (Voller *et al.*, 1977, Avidor *et al.*, 1987), fluorescence-ELISA (f-ELISA) (Sato *et al.*, 1986) have been reported. However, a particular method suitable for large scale surveys is necessary in rural endemic areas which can afford least laboratory facilities.

Recently, we developed an ABC-ELISA for malaria surveys (Sato *et al.*, 1990). The method was more sensitive than the ordinary ELISA as the final enzyme reaction was amplified by the use of the avidin biotin peroxidase complex system. It does not require any special equipment and can be performed easily in laboratories in tropical areas.

In the present paper, advantages and disadvantages of each serological technique were comparatively studied and discussed.

Materials and Methods

Antigen preparation: *P. falciparum* parasites were propagated by culture *in vitro* (Trager and Jensen, 1976; Miyagami and Waki, 1985). The

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culture was stopped when parasitemia reached at 25 to 30%. The infected red blood cells were washed 3 times with phosphate buffered iso-osmotic saline, pH 7.2 (PBS), then hemolysed with 0.45% NaCl. Free parasites were collected by centrifugation at 11,000 g for 30 min, washed twice with PBS, resuspended in 0.05 M carbonate buffer, pH 9.6, and sonicated for 3 min with a Cell Disruptor 200 (Branson USA). The resulted antigen material was stored after lyophilization. All above operations were carried out at 2°C. Protein content of the antigen was measured using the "BIO-RAD" Dye-Binding Protein Assay. Bovine serum albumin was used as the standard protein.

Test sera: Test sera from malarial patients were collected from 52 donors who were the Japanese immigrants living in an Amazonian settlement, named Tomé-Açu. Sera with acute malaria were collected from 25 imported *P. falciparum* cases in Japan; most of the donor patients were non-immune and the sera were withdrawn at the acute stage of infection. Fifty normal control sera were obtained from healthy Japanese volunteers who had never traveled abroad.

Procedures of the various serological methods (Table 1): The malarial antigen was diluted to an appropriate concentration for each methods. The IFAT was performed according to the method of Voller and O'Neil (1971). The ABC-ELISA was performed by the method described by Sato *et al.* (1990) using a Vectastain^{TM} ABC kit. In this system, the blue color was finally generated in the bottom of the well with positive serum. The final dilution of a specimen which gave this coloring was recorded as the antibody titer. The ELISA was performed by the method of Voller *et al.* (1977) using horseradish peroxidase-conjugated goat anti-human IgG (Hp-IgG) and O-phenylenediamine (OPD) as the substrate. The f-ELISA was performed according to the modified method described by Sato and Suzuki (1986) using the same procedures as the ELISA except for replacing HP-IgG with β-D-galactosidase conjugated anti-human IgG (Gal-IgG). In a final step, 50 μg of 50 μM of 4-methyl umbelliferyl-β-D-galactoside dissolved in 0.05 M phosphate buffer, pH 7.2, was added to each well and incubated at 37°C for 1 hr. The reaction was terminated by adding 10 μl of 1 M NaOH. The hydrolyzed probe, 4-methyl umbelliferone released from the substrate, was measured with a MicroFLUOR reader (Dynatech Product, USA). Results were expressed as relative fluorescence unit (RFU) values. RFU values increased linearly in proportion to the amount of 4-methyl umbelliferone present. In the ELISA and f-ELISA, the measured values were read as positive reaction when they were over twice the mean value of normal sera at each dilution.

Materials: Polystyrene microplates for the ABC-ELISA and ELISA were purchased from Greiner Co. LTD. (Germany). Polystyrene black plates for the f-ELISA were obtained from Dynatech Products (USA). Fluorescein-conjugated anti-human IgG antibody was obtained from Hoechst Co. LTD. (Germany). The Vectastain^{TM} ABC kit was obtained from MBL KK. (Nagoya, Japan). OPD and 4-methyl umbelliferyl-ß-D-galactoside was obtained from Sigma Co. LTD. All other chemicals were refined products from Wako Pure Chemicals Co. (Osaka, Japan).

Results

Fifty-two serum samples from Brazil were titrated by the IFAT and the breakdown of the results were as follows: Titer of 15 samples were read as 1:16, 11 samples as being at 1:64, 19 samples at 1:256, and 7 samples at 1:1024. A portion of each tested sample were also subjected to the antibody titration of ABC-ELISA, ELISA and f-ELISA. As shown in Fig. 1, the tested sera were positive at dilutions equal to or higher than 1:64 by the ABC-ELISA, although 2 samples were titrated at 1:16 by the IFAT and were read positive at 1:32 by the ABC-ELISA. By the ELISA, all sera were positive at a serum dilution of 1:64. All the sera were also positive at a serum dilution of 1:64 by the f-ELISA. All samples from 50 healthy volunteers were negative by the IFAT, ABC-ELISA, ELISA and f-ELISA. In addition, 25 sera from acute imported *P. falciparum* cases were also positive at serum dilutions equal to or higher than 1:4 by the IFAT and 1:64 by the ABC-ELISA, ELISA and f-ELISA. In general, sera with high positive titers by the IFAT tended to have high titers in the other serological techniques.

A statistical analysis of the data of 52 patients

Table 1 Comparison of the procedures of the studied serological tests

Method	IFAT	ABC-ELISA	ELISA	f-ELISA
Antigen	Infected red blood cells	SMA (2.5μg/20μl/well)	SMA (0.5μg/50μl/well)	SMA (0.5μg/50μl/well)
Coating material	Glass slide	U-type plate	U-type plate	Black plate
Antigen	Dry, room temp.	Dry, 37°C. 5 hrs	Incubation, 37°C. 3 hrs	
Pre-treatment I	Fixation with acetone	0.5% HIO_4 treatment, 50–100μl, 15 min		
Pre-treatment II	–	Buffer II treatment, 37°C, 30 min		
Reaction I	4-fold dilution serum, 25μl, 37°C, 30 min	2-fold dilution serum, 20μl, 37°C, 90 min	4-fold dilution serum, 50μl 37°C, 90 min	
Washing	PBS	Buffer I		
Reaction II	FITC-IgG, 25μl 37°C, 30 min	Bio-IgG, 20μl 37°C, 90 min	Hp-IgG, 50μl 37°C, 90 min	ß-D-Gal-IgG 50μl, 37°C, 90 min
Washing	PBS	Buffer I		
Reaction III	–	Avi-Bio-HP-complex 20μl, 37°C, 60 min	–	
Washing	–	Buffer I	–	
enzyme reaction	–	4-chloro-1-naphthol. 100μl 37°C, 15 min	OPD, 50μl 37°C, 150 min	4-methyl-umbelliferyl-β-D-galactoside, 50μl 37°C, 1 hr
Reaction stop	washing	washing	lN HCl, 50 μl	lN NaOH, 10μl
Measurement	Fluorescent microscope	Naked eye	ELISA reader at 410 nm	Micro FLUOR reader

SMA; Sonicated malaria antigen, Buffer I; PBS containing 0.1% Tween 20, Buffer II; PBS containing 0.3% BSA, FITC-IgG; fluorescein-labelled IgG, Bio-IgG; biotin-labelled IgG, Avi-Bio-Hp Complex; Avidin biotin horseradish peroxidase complex, OPD; o-phenylenediamine, β-Gal-IgG; β-galactosidase conjugated IgG. The sample was diluted with Buffer II (Buffer I containing 0.3% BSA and 0.05% NaN_3).

obtained from the 4 kind of methods was conducted. Figure 2 shows the relationship between the IFAT and the ABC-ELISA titers. Almost complete correlation was recognized between the weighed mean values of the IFAT and the ABC-ELISA titers with a correlation coefficient of 0.999. The correlation coefficients between the data from the IFAT and ELISA, and from the IFAT and f-ELISA were 0.998 and 0.971, respectively.

The effects of various conditions of preservation on the antigen activity were studied. The wells were plated with antigens which had been preserved under three different conditions: 4°C for 3 months, –30°C for 3 months and –30°C for 3 months after lyophilization for the study by the ABC-ELISA plates as given in Table 2. Sera from malarial pa-

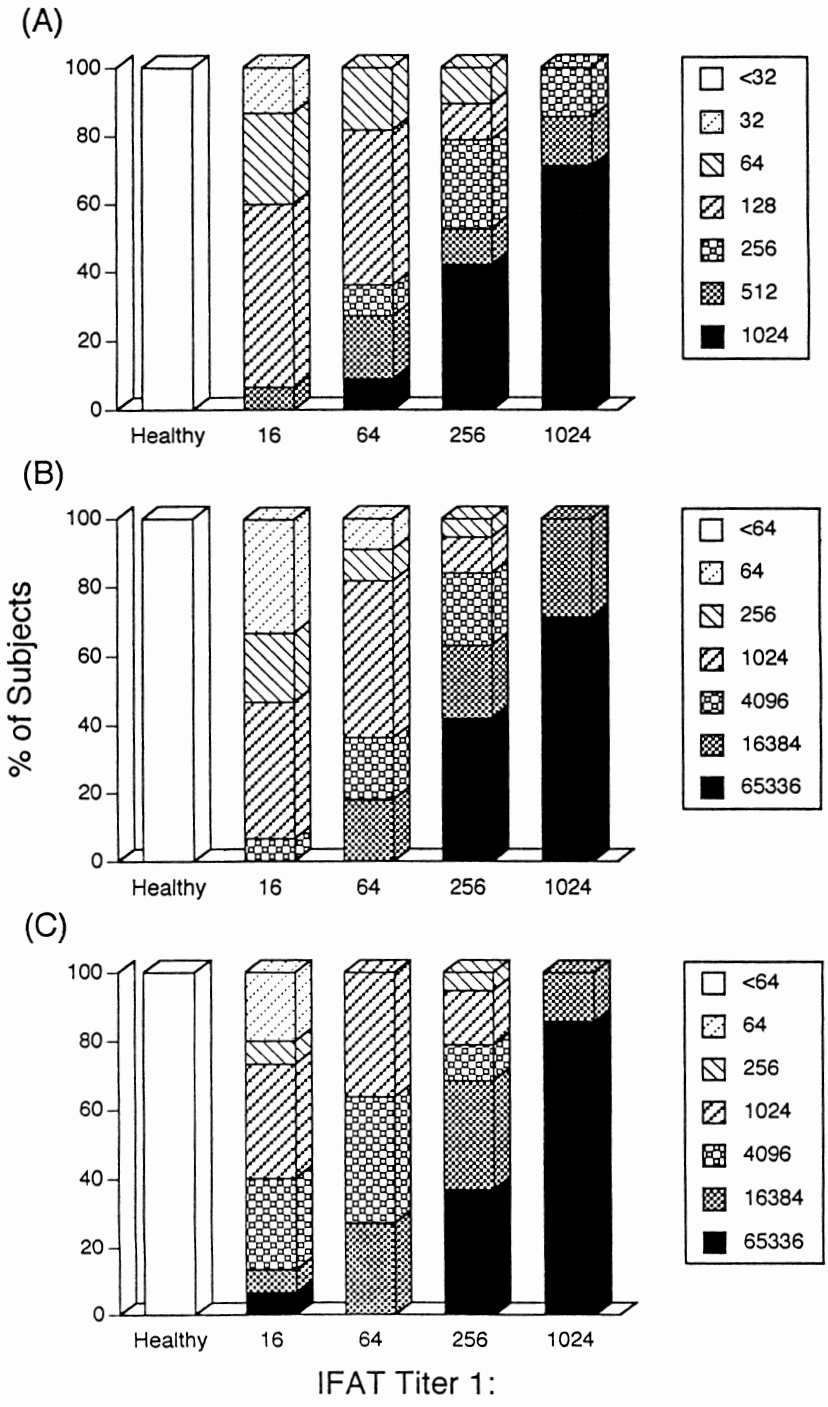

Fig. 1 The results of the serological tests using sera from 52 patients with *P. falciparum* malaria and 50 healthy individuals compared with the titer read by IFAT.
(A); ABC-ELISA, (B); ELISA, and (C); fluorescence-ELISA.

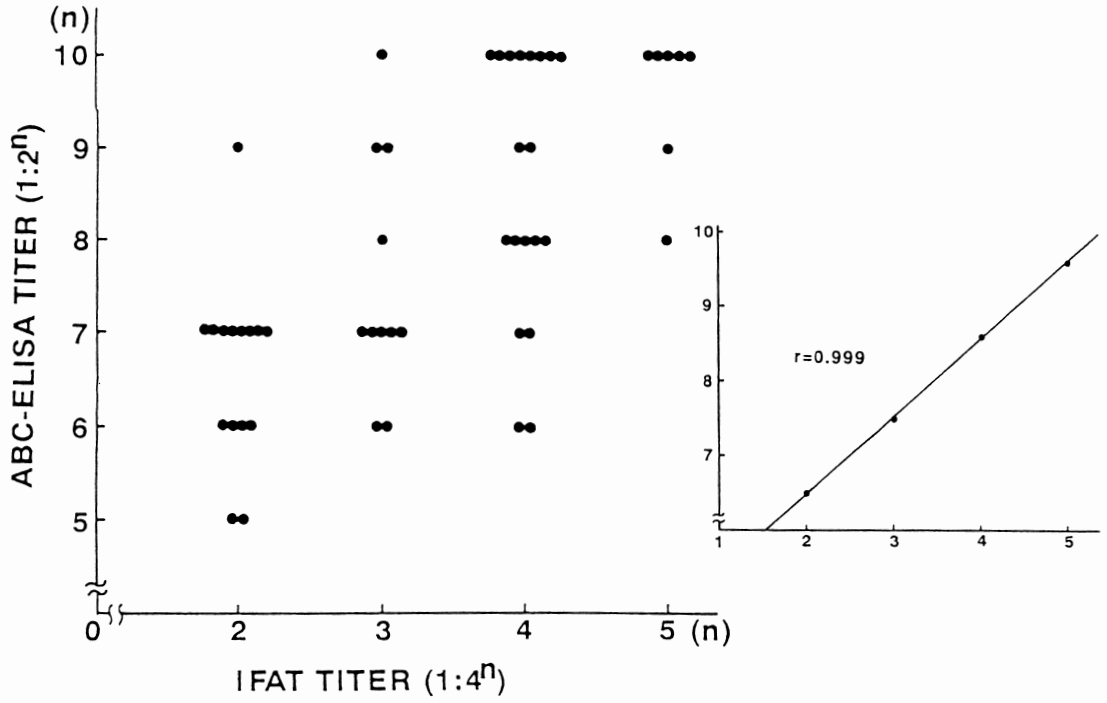

Fig. 2 Correlation between the IFAT and ABC-ELISA results of the sera from 52 Brazilian Japanese patients with *P. falciparum* malaria. The inset in the right corner is a correlation plot of weighed mean values of the IFAT and ABC-ELISA titers of the sera.

tients were positive at all serum dilutions. Non-specific reactions were observed in the sera diluted to 1:64 and 1:128 of healthy subjects when the antigen preserved at 4°C was used. No nonspecific reactions were observed with the antigen preserved after lyophilization at any dilution. One serum (No. 109) out of 15 healthy subjects non-specifically reacted with the antigen that had been stored at –30°C. Thus, the antigen preserved at –30°C after lyophilization retained antigenic activity without causing nonspecific reactions. In addition, activity of the antigen plated on the polystyrene was well preserved at 4°C for 6 months.

Discussion

Epidemiological surveys of malaria by serological methods have been well rationalized. The serological surveys in malaria programme is useful in defining malaria foci in an endemic area, for the assessment of past epidemics, in the differentiation of epidemiological features of a moving population and also in the potential risk assessment of a given area (Suzuki, 1991). We had confirmed that the IFAT is the most reliable serological method particularly in the retrospective malaria diagnosis of individuals (Kano *et al.*, 1991). However, the technique requires a time-consuming manipulation by a highly trained technician. In the present paper, several methods, ABC-ELISA, ELISA, and f-ELISA suitable for a large scale assessment were comparatively studied. Excellent correlation between the IFAT titers and those given by the ABC-ELISA, ELISA and f-ELISA was obtained in the sera equal to or higher than 1:16 measured by IFAT. Our previous report showed that 207 sera taken from healthy Japanese domestic residents did not give positive IFAT titer at 1:4 dilution, but in the treated malaria patients gave titer still persisted at 1:4 around the time of 6–12 months since the onset of the first symptom. Whereas, the sera from Japanese immigrants in endemic Brazilian settlement occasionally manifested positive IFAT

Table 2 Effects of preservation on the antigen activity. The malarial antigen was preserved under three different sets of condition: 4°C for three months, –30°C for 3 months and –30°C for three months after lyophilization. The sera from No. 101 to 115 were obtained from healthy subjects, and PS1 to PS3 were obtained from patients with *P. falciparum* (IFAT, 1:1024 positive)

Antigen preservation	Preserved at 4°C			Preserved at –30°C			Lyophilized		
Serum dilution 1:	32	64	128	32	64	128	32	64	128
Serum No.									
101	+	±	–	+	–	–	±	–	–
102	+	–	–	–	–	–	±	–	–
103	±	±	–	+	–	–	–	–	–
104	+	±	–	–	–	–	±	–	–
105	+	±	–	±	–	–	±	–	–
106	+	±	–	±	–	–	±	–	–
107	+	±	±	±	–	–	–	–	–
108	±	–	–	±	–	–	–	–	–
109	+	+	±	+	±	–	±	–	–
110	+	+	–	±	–	–	–	–	–
111	+	–	–	±	–	–	±	–	–
112	±	–	–	–	–	–	–	–	–
113	+	–	–	+	–	–	–	–	–
114	+	±	–	±	–	–	–	–	–
115	±	–	–	–	–	–	–	–	–
PS1	+	+	+	+	+	+	+	+	+
PS2	+	+	+	+	+	+	+	+	+
PS3	+	+	+	+	+	+	+	+	+

(+); Positive. (±); False positive, (–); Negative

reaction at 1:4 without declared previous infection history. The finding may indicate that the donors titrated at 1:4 consisted of heterogeneous infection histories regardless of apparent clinical manifestations. The sera from the heterogeneous population may be reflected by the different positive titers measured by respective ELISA system in the present study. This point must await for further investigations.

The methods studied in the present work showed respective merits and demerits in the field running as illustrated in Table 3. The ELISA and f-ELISA results agreed with those reported by Voller *et al.* (1977, 1982) in the point that ELISA was simple, sensitive and reliable of assessing malarial antibody in endemic areas. However, ELISA and f-ELISA require expensive apparatus, such as an ELISA reader or a fluoro-densitometer. Occasionally, it is difficult to transport the equipment to endemic areas. Even if these facilities are available, local electricity supplies are often insufficient. The ABC-ELISA will be the most suitable serological test under such conditions. First, the method can be carried out in the area without electric supply. Second, the reacted color in this system can be kept unchanged for one year or even longer, which makes supervision of the results feasible. Disagreements of the assessed values found in low titered sera were minimum and any of the methods studied seems to be acceptable for practical mass surveys according to the field laboratory conditions.

Antigen preparation makes one of the crucial steps in carrying a serological test. The usefulness of the lyophilized antigen in all tested ELISA systems will add a favorable advantage in carrying out the tests in the field. Results in table 2, show that the lyophilized parasite material retained sufficient antigenicity for the tests studied so far without causing unfavorable false reactions, and this finding gives an appreciable advantage of the ELISA sys-

Table 3 Comparative evaluation of the studied serological methods

Comparative points	IFAT	ABC-ELISA	ELISA	f-ELISA
Ag* preservation	Ag spotted glass slide at –80°C	Ag lyophilized	Ag lyophilized	Ag lyophilized
Technique	Highly skilled staff required	Simple	Simple	Simple
No. of test†	50	100	100	100
Special equipment	Fluorescent microscope with a stable electric source	None	ELISA reader with a stable electric source	Fluorescence reader with a stable electric source
Preservation of reactant	2 days	over 1 year	1 day	1 day
Sensitivity	Better	Good	Best	Best
Specificity	Best	Better	Better	Better
Correlation coefficient with IFAT	–	0.999	0.998	0.971
Cost (yen)				
1 case diagnosis	3,000	500	400	650
Mass survey	250	150	90	100
Field applicability	Poor	Best	fair	fair

*Ag; antigen
†No. of test; Number of sample which can be tested by a skilled staff per day.

tems over the IFAT.

Recently, we tried the ABC-ELISA using a V bottom type (V-type) of polystyrene microplate instead of the U-type plate. The results of individuals obtained from the V-type plate were consistent with those from the U-type. The cost of the ABC-ELISA was reduced by using the V-type plate, since all reagent volumes could be halved.

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