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SIMULTANEOUS USE OF *PLASMODIUM FALCIPARUM* CRUDE ANTIGEN AND RED BLOOD CELL CONTROL ANTIGEN IN THE ENZYME-LINKED IMMUNOSORBENT ASSAY FOR MALARIA

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Abstract. An enzyme-linked immunosorbent assay for malaria, based upon duplicate testing of serum samples on both a crude *Plasmodium falciparum* antigen and a red blood cell control antigen, is evaluated. Results were analyzed using the Student's *t*-test for identification of positive serum samples ($t \geq 2.92$, $P \leq 0.05$) and for calculation of the mean difference in absorbance values (ΔABS) obtained between the *P. falciparum* wells and the control wells. Cross-evaluation with the IFA test for *P. falciparum* antibodies gave 89.6% concording positive or negative results. Among discrepant sera 8.35% were ELISA+/IFA− and 2.05% ELISA−/IFA+. In addition, ΔABS values in ELISA were highly correlated to titers obtained in immunofluorescence ($r = 0.80$, $P < 0.001$). The results confirm the high degree of species-specificity of the ELISA using *P. falciparum* crude antigen.

The necessity of the simultaneous use of red blood cell control antigen with a crude plasmodial antigen is demonstrated by comparing the presented results with those obtained on the *P. falciparum* antigen only.

The indirect immunofluorescence test is presently the most widely used technique for the serological diagnosis of malaria. However, the time-consuming reading of fluorescence and the subjective interpretation of borderline fluorescence are two important drawbacks of this test. The enzyme-linked immunosorbent assay (ELISA) offers an attractive alternative since reading of the results can be fully automated.

ELISAs using crude soluble antigens have been applied to serodiagnosis of malaria with various degrees of success; correlation with immunofluorescence was not always satisfactory.[1-3] The risk of false positive reactions due to heterophiles, antiglobulines, or autoimmune antibodies has already been established,[4] but most subsequent reports have not investigated further the problem of nonspecific reactions. Recently, however, an ELISA involving noninfected red blood cell (RBC) antigen was performed in parallel to a titration of antimalarial antibodies, and revealed that many sera reacting with the *Plasmodium falciparum* antigen also had significant levels of antibodies specific to RBC antigens, especially of the IgM isotype.[5]

Criteria that have been applied so far for interpretation of ELISA results were almost uniform: either the negative-positive cut-off value was set at an absorbance value equal to mean + 2 SD or mean + 3 SD of the results obtained with a group of negative control samples,[6-11] or an arbitrarily chosen absorbance value was used as cut-off point.[1-3, 12-14]

This study evaluates an ELISA in which a systematic control for the reactivity of each serum to the RBC contaminants of the crude malarial antigen is built-in and examines more objective criteria for interpretation of ELISA results independent of negative control samples.

MATERIALS AND METHODS

Enzyme-linked immunosorbent assay

Antigen. Antigen for the ELISA was obtained from in vitro cultures of clone B-11 of the Ugandan Palo Alto strain of *P. falciparum.* The material was collected on the sixth day of a nonsynchronized culture presenting a parasitemia of about 9.5% with a majority of mature trophozoites. After centrifugation, the supernatant was discarded and the volume of packed cells was accurately measured. Lysis of the red cells was obtained by addition of a tenfold volume of saponin 0.2% in PBS and followed by centrifu-

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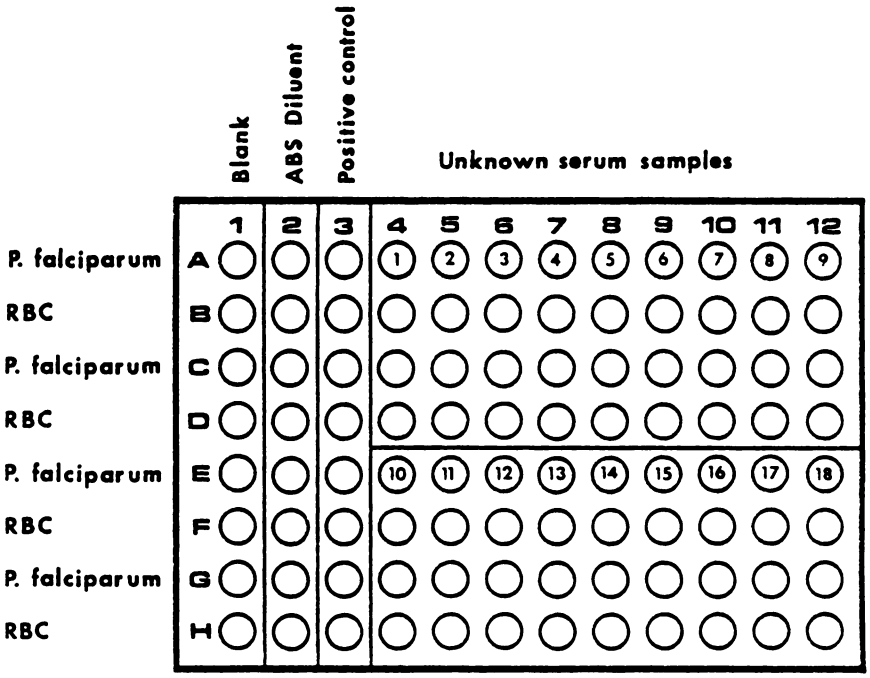

FIGURE 1. Schematic representation of the positions of antigens and serum samples on the ELISA plates.

gation at 15,000 rpm for 30 min. The lysis procedure was repeated twice for complete removal of hemoglobin. The pellet of free parasites was resuspended in 10 ml of carbonate-bicarbonate buffer, pH 9.6, and disrupted by sonication for 30 sec at 60 W using a needle probe (Labsonic® 1510 ultra-sonic homogenizer, B-Braun Melsungen AG, West Germany). The material was then centrifuged at 18,000 rpm for 15 min and 100 μl aliquots of the supernatant were stored at −20°C until used.

Control antigen was prepared from an identical volume of noninfected RBC that had undergone a similar 6-day period of in vitro survival in RPMI medium at 37°C.

The protein contents of the *P. falciparum* and the control antigen were estimated using the BioRad protein assay. The optimal antigen dilution of 1/1,000, corresponding to a protein content of, respectively, 2.3 and 1.9 μg/ml in the *P. falciparum* and the control antigen, was determined by checkerboard titration.

Test procedure. Alternate rows of Dynatech microtiter plates (Greiner) were sensitized by incubation with 200 μl of *P. falciparum* antigen and control antigen, diluted 1/1,000 in carbonate-bicarbonate buffer. The wells of the first column on each plate served as blanks, receiving 200 μl of the antigen diluent. After 3 hr incubation at 37°C, the plates were rinsed with PBS pH 7.2 0.01 M containing 0.05% Tween 20 (PBS-Tween), sealed, and stored at −20°C until used.

Before testing, the stored plates were washed twice more with PBS-Tween. Subsequently they were incubated with 200 μl of the serum samples, diluted 1/200 in PBS-Tween. Only the serum diluent (PBS-Tween) was added to the wells of the first and the second column. Each serum sample was tested in duplicate on both the *P. falciparum* and the control antigen. After 30 min of incubation at 37°C, the plates were washed three times and 200 μl of horseradish peroxidase-labeled anti-human IgG conjugate, diluted 1/10,000 in PBS-Tween was added to all wells, except those of the first column, to which only PBS-Tween was added. Again after 30 min of incubation at 37°C and three rinses with PBS-Tween for removal of free antiglobulin, 200 μl of enzyme substrate were added to all wells and allowed to react for 30 min in the dark at room temperature. The substrate solution consisted of 10 mg o-phenylenediamine in 40 ml of citrate buffer pH 5.5 to which 20 μl of hydrogen per-

oxide was added. The reaction was stopped by addition of 50 μl of sulfuric acid 8N to all wells and absorbance at 492 nm was measured with a Titertek Multiskan ELISA reader, blanking the plate against the wells of column 1.

On each plate the third column was fully assigned to the analysis of a reference positive serum; the four results obtained provide a control of intertest variability.

The positions of antigens and serum samples on the microtiter plates are illustrated in Figure 1.

Calculations

Absorbance values in the wells coated with *P. falciparum* antigen or control antigen which did not react with serum samples (column 2) were called "ABS diluent antigen" and "ABS diluent control." Mean values of "ABS diluent antigen" and "ABS diluent control" are calculated for each plate and subtracted from the absorbance values obtained with the serum dilutions on the *P. falciparum* or control antigen, respectively. This results in "real ABS antigen" and "real ABS control" values, on which SD and coefficients of variation (CV) are calculated. Based upon the information on the reproducibility on duplicate determinations, the Student's *t*-test is applied and allows an objective appreciation of the difference in reactivity of a given serum sample against the two types of antigen. Finally, the mean ΔABS value is calculated (real ABS antigen − real ABS control, mean of two determinations). This ΔABS value illustrates to what extent the serum is reactive to the parasite-specific material of the *P. falciparum* antigen.

In a preliminary assay on different plates, the reference positive serum pool had given a mean ΔABS value of 1.460 (SD = 0.104). In subsequent experiments, all calculated ΔABS values of a given plate were corrected using a coefficient based on the mean ΔABS value of the positive control on that plate.

A computer program was developed for processing of the ELISA results as described above. The program also enabled serious discrepancies among replicates from the same sample to be detected. Warning messages about poor precision appear on the printout whenever the SD on ABS values <0.150 exceeds 0.050 or if CV on ABS values ≥ 0.150 exceeds 20%. Such results were rejected and corresponding samples were retested.

Indirect immunofluorescence

The IFAT procedure used as a reference in the present work has been described in detail earlier.[15] Briefly, serum samples were screened at 1/20 and 1/80 on three human malarial antigens: *P. falciparum, P. ovale,* and *P. vivax.* The *P. falciparum* antigen was prepared from in vitro cultures of an isolate of unknown geographic origin. The *P. ovale* and *P. vivax* antigens were prepared from blood of patients who became infected during a stay in Zaire and India, respectively. Any serum sample positive at 1/80 with one of the antigens was further diluted up to 1/640 for determination of the endpoint titer. The conjugate was used as an FITC-labeled goat anti-human Ig G.A.M. antibody preparation (Pasteur). Cross-examination ELISA/IFAT was performed double blind.

Serum samples

Serum samples were as follows: 635 specimens from immigrants from malaria-endemic areas or from Europeans who had been temporarily exposed to malaria; 126 samples from patients with parasitic diseases other than malaria, including confirmed cases of amebiasis (15), leishmaniasis (13 cases of kala-azar and 2 of cutaneous leishmaniasis), toxoplasmosis (15), African sleeping sickness (10), tuberculosis (10), leprosy (10), ascariasis (11), schistosomiasis (10), filariasis (9), visceral larva migrans (10), and hydatid disease (11); 50 serum samples from Belgian blood donors who had never visited malaria-endemic countries; and the reference positive pool of 14 known serum samples from nonimmune patients with acute malaria and with IFA titers ranging from 1/320 to 1/1,280.

RESULTS

A total of 635 undocumented routine serum samples were analyzed using both IFAT and ELISA. The results are shown in Table 1. Agreement was found in 569 samples (89.6%), 464 negative and 105 positive. Disagreement was observed in 66 cases (10.4%): 53 sera (8.35%) were positive only in ELISA and 13 sera (2.05%) were positive only in IFAT.

TABLE 1
Comparison of IFAT and ELISA results obtained on 635 undocumented serum samples

IFAT *P. falciparum* Titer	ELISA *P. falciparum* $t < 2.92$ Neg.	$t \geq 2.92$ ($P \leq 0.05$) ΔABS 0–0.05	0.051–0.100	0.101–0.200	0.201–0.500	0.501–0.800	0.801–1.000	≥1.001
Neg.	464	29	18	6				
1/20	12	1	11	8	4			
1/80	1	2	4	12	13	1		
1/160				4	13	7	1	
1/320					4	4	1	2
1/640						1	1	2
>1/640							2	7

For the ELISA +/IFA− sera, ΔABS values did not exceed 0.200. On the other hand, 12 out of the 13 ELISA−/IFA+ sera were reactive only at the threshold dilution of 1/20 in the immunofluorescence test for *P. falciparum* and 5 of these had IFA titers significantly higher against the *P. vivax* antigen than against the *P. falciparum* antigen (Table 2). Ony 14 serum samples of the series presented the highest titer with the *P. vivax* antigen in the IFAT (Table 3). Furthermore, ELISA ΔABS values of these 14 samples were not strongly correlated with IFA titers obtained on the *P. vivax* antigen ($r = 0.47$, $P = 0.049$). In contrast, for the complete series of 635 samples, ΔABS values in ELISA were strongly correlated to the titers obtained in immunofluorescence using the *P. falciparum* antigen ($r = 0.80$, $P < 0.001$).

Among the 126 serum samples from patients with parasitic diseases other than malaria, 37 were positive in ELISA. Apart from the toxoplasmosis sera from Belgian residents, which were all ELISA−, all other samples came from patients who had recently been exposed to the risk of malaria and for whom the possibility of malaria infection in the past could not be excluded. The ΔABS values of these 37 ELISA+ sera showed an analogous pattern of distribution when compared to IFAT titers using the *P. falciparum* antigen (Table 4). In this series also, the correlation between ELISA ΔABS values and IFAT titers on the *P. falciparum* antigen was statistically significant ($r = 0.89$, $P < 0.001$).

The 50 serum samples from Belgian blood donors were all negative in both tests.

DISCUSSION

ELISA is generally considered to be more sensitive than corresponding IFAT. However, in-

TABLE 2
Detailed results obtained on 13 ELISA−/IFAT+ serum samples

	IFAT titer *P. falciparum*	*P. vivax*	ELISA ΔABS	*t*
Serum N. 1	1/20	Neg.	0.049	2.06
2	1/20	Neg.	0.030	1.53
3*	1/20	1/320	0.069	2.50
4	1/20	Neg.	−0.026	−0.6
5	1/20	Neg.	0.009	0.18
6	1/20	Neg.	−0.028	−1.18
7*	1/20	1/160	0.034	1.6
8	1/20	Neg.	0.002	0.35
9	1/20	Neg.	−0.306	−6.71
10*	1/20	1/320	−0.041	−2.23
11*	1/20	1/160	0.061	0.75
12*	1/20	≥1/640	−0.035	−0.94
13	1/80	Neg.	0.139	2.04

* Serum samples significantly more reactive to the *P. vivax* antigen in the IFAT than to the *P. falciparum* antigen.

TABLE 3

Detailed results obtained on 14 serum samples which were most reactive with the P. vivax *antigen in the IFAT*

	IFAT titer		ELISA	
	P. falciparum	*P. vivax*	ΔABS	*t*
Serum N. 1	Neg.	1/20	−0.013	−0.34
2	1/80	1/640	0.075	6.17*
3	1/80	1/320	0.193	12.67*
4	1/80	1/160	0.050	5.74*
5	1/20	1/320	0.069	2.5
6	1/20	1/160	0.034	1.6
7	1/80	>1/640	0.216	18.22*
8	1/20	1/320	−0.041	−2.23
9	Neg.	1/20	−0.086	−13.53
10	1/20	1/160	0.061	0.75
11	1/20	1/640	−0.035	−0.94
12	1/20	1/320	0.115	5.02*
13	Neg.	1/20	0.018	5.49*
14	1/20	1/320	0.165	7.94*

* *t* values >2.92 indicate positivity in ELISA. ELISA ΔABS values correlate better to IFAT titers against *P. falciparum* ($r = 0.60$, $P = 0.014$) than to titers against *P. vivax* ($r = 0.47$, $P = 0.049$).

creased sensitivity in serological tests often involves a decrease in specificity. Provided that the threshold dilution of specificity has been defined, the IFAT has the advantage of a visual control of the localization of fluorescence. In ELISA there is no possibility of direct control on the specificity of color development. Therefore, a maximum number of indirect controls are to be built-in.

In the present study we focused on the control of any nonspecific binding of the conjugate and on indirect control of any aspecific binding of serum antibodies to the RBC components of the crude malarial antigen.

Controls of specificity and reproducibility reduce the number of samples that can be analyzed on one plate. Results obtained with the series of 635 serum samples were analyzed by comparing the ABS values obtained with the test sera on the *P. falciparum* antigen to the ABS values obtained with a group of negative control samples on the same antigen.

Among the group of negative control samples from blood donors, one serum sample produced an excessively high reading on the *P. falciparum* antigen (0.655) but at the same time reacted strongly with the RBC control antigen (0.626). None of the other negative controls exceeded 0.350 on the *P. falciparum* antigen. ABS values obtained with the 50 serum samples on the *P. falciparum* antigen ranged from 0.055 to 0.655 with a mean of 0.226 and a SD of 0.100. Thus, the cut-off value at the 95% confidence limit is an ABS of 0.426 (mean + 2 SD of normal subjects). In that case, among the 464 sera negative both in IFAT and ELISA, 20 (4.3%) having an

TABLE 4

Comparison of IFAT and ELISA results obtained on 37 ELISA+ serum samples from patients with parasitic infections other than malaria

IFAT *P. falciparum*	ELISA *P. falciparum* ($t \geq 2.92$) ΔABS						
Titer	0–0.05	0.051–0.100	0.101–0.200	0.201–0.500	0.501–0.800	0.801–1.000	≥1.001
Neg.	2	2	1				
1/20		4	2				
1/80			2	6			
1/160				4	4		
1/320					3	1	
1/640							3
>1/640							3

TABLE 5

ABS values on P. falciparum *antigen obtained with 464 IFAT−/ELISA− sera*

ABS	ABS < 0.426	ABS − 0.426				
		0–0.050	0.051–0.100	0.101–0.200	0.201–0.500	0.501–0.800
Number of sera	444	3	2	9	5	1

ABS value >0.426 (Table 5) would have been classified positive, while among the 105 sera positive in both IFAT and ELISA, as many as 46 samples (43.8%) having an ABS value <0.426 (Table 6) would have been classified negative. These data illustrate that, despite a statistically significant correlation between ELISA ABS values on the *P. falciparum* antigen and titers in IFAT on *P. falciparum* ($r = 0.73$, $P < 0.001$), up to 66 serum samples out of 635 (10.4%) would have been misjudged in ELISA if no control antigen was used. The results also discredit the value of a cut-off point calculated on the results obtained with a group of negative controls.

Discordances of 20%–30% between IFAT and ELISA have been reported by Spencer et al.,[1] the disagreement being more frequent in specimens with low positive titers than in strongly positive samples.[2] In another study a statistically significant correlation between the two tests was observed ($r = 0.71$, $P < 0.01$), although some of the sera with IFA titers up to 1/320 were not reactive in ELISA.[6] Schapira et al. observed a significant correlation between reciprocal IFA titers and ELISA extinction values ($r = 0.34$, $P < 0.001$), but discordant results were obtained in 36% of 194 Vietnamese sera, tested in duplicate.[10] Differences in reactivity have been attributed to the fact that the two tests use different antigenic determinants and consequently determine different antibodies. Our results suggest that at least some of the observed discordances must have been due to nonspecific reactions or an inappropriate cut-off point. Indeed, even though different isolates of *P. falciparum* were used in our IFAT and ELISA procedures, discordances could be limited to 10.4% and did not affect any of the strongly positive. One serum sample which had an IFA titer of 1/80 against the *P. falciparum* antigen was classified negative in ELISA ($t = 2.04$) although the ΔABS value was >0.100. The CV calculated on the results obtained with the *P. falciparum* antigen was 11%. This might indicate that higher demands should be made on reproducibility in order to minimize the risk of false negative results.

TABLE 6

ABS values on P. falciparum *antigen, obtained with 105 IFAT+/ELISA+ sera*

IFAT titer	Number of samples ABS < 0.426	Number of samples ABS > 0.426
1/20	20	4
1/80	20	12
1/160	5	20
1/320	1	10
1/640	—	13
Total	46	59

Our results also indicate that ELISA using *P. falciparum* antigen displays a considerable degree of species-specificity, which is in agreement with the results described by Wahlgren et al.[5] The simultaneous use of a RBC control antigen with a crude *P. falciparum* antigen becomes even more important in the light of their observation that sera from patients with *P. malariae, P. ovale,* or *P. vivax* malaria reacted only weakly with the *P. falciparum* antigen, while displaying strong reactions with normal RBC.

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REFERENCES

1. Spencer, H. C., Collins, W. E., and Skinner, J. C., 1979. The enzyme-linked immunosorbent assay (ELISA) for malaria. II. Comparison with the malaria indirect fluorescent antibody test (IFA). *Am. J. Trop. Med. Hyg., 28:* 933–936.
2. Spencer, H. C., Collins, W. E., McWilson, W., Jeffery, G. M., Mason, J., Huong, A. Y., Stanfill, P. S., and Skinner, J. C., 1981. The enzyme-linked immunosorbent assay (ELISA) for malaria. III. Antibody response in documented *Plasmodium falciparum* infections. *Am. J. Trop. Med. Hyg., 30:* 747–750.
3. Tharavanij, S., Tantivanich, S., Chongsa-Nguan, M., and Prasertsiriroj, V., 1982. Comparison of various serological test results using antigens from different strains of *Plasmodium falcipa-*

rum. S.E. Asian J. Trop. Med. Pub. Hlth., 13: 174–180.

4. Voller, A., Huldt, G., Thors, C., and Engvall, E., 1975. New serological test for malaria antibodies. *Br. Med. J., 1:* 659–661.
5. Wahlgren, M., Berzins, K., Perlmann, P., and Björkman, A., 1983. Characterization of the humoral immune response in *Plasmodium falciparum* malaria. I. Estimation of antibodies to *P. falciparum* or human erythrocytes by means of micro ELISA. *Clin. Exp. Immunol., 53:* 127–134.
6. Quakyi, I. A., 1980. The development and validation of an enzyme linked immunosorbent assay for malaria. *Tropenmed. Parasit., 31:* 325–333.
7. Dutta, G. P., Srivastava, I. K., Sharma, P., Nath, A., Agarwal, S. S., and Dwivedi, S. R., 1982. Enzyme-linked immunosorbent assay test in the diagnosis of human malaria. *Indian J. Malar., 19:* 33–37.
8. Srivastava, I. K., Sharma, P., Nath, A., Agarwal, S. S., and Dutta, G. P., 1983. Enzyme-linked immunosorbent assay test with *Plasmodium knowlesi* antigen in diagnosis of malaria. *Indian J. Med. Res., 77:* 431–436.
9. Dutta, G. P., Srivastava, I. K., Sharma, P., and Agarwal, S. S., 1984. Evaluation of *Plasmodium cynomolgi* B antigen in enzyme-linked immunosorbent assay (ELISA) test for human malaria. *Indian J. Malar., 21:* 71–78.
10. Schapira, A., Fogh, S., Jepsen, S., and Strandberg Pedersen, N., 1984. Detection of antibodies to malaria: Comparison of results with ELISA, IFAT and crossed immunoelectrophoresis. *Acta Path. Microbiol. Immunol. Scand. Sect. B., 92:* 299–304.
11. Espinal T., C. A., and Olaya de Morales, P., 1984. Indirect ELISA test for malaria in blood donors. *Trans. R. Soc. Trop. Med. Hyg., 78:* 645–647.
12. Spencer, H. C., Collins, W. E., Chin, W., and Skinner, J. C., 1979. The enzyme-linked immunosorbent assay (ELISA) for malaria. I. The use of in vitro-cultured *Plasmodium falciparum* as antigen. *Am. J. Trop. Med. Hyg., 28:* 927–932.
13. Ray, K., Sharma, M. C., Sivaraman, C. A., and Rai Chowdhuri, A. N., 1983. In vitro culture as a source of *Plasmodium falciparum* antigen in micro-ELISA in malaria. *Indian J. Med. Res., 78:* 205–209.
14. Wells, L., and Ala, F. A., 1985. Malaria and blood transfusion. *Lancet:* 1317–1319.
15. Demedts, P., and Wéry, M., 1985. Indirect immunofluorescence using differentiating antigens in detection of imported malaria. *Ann. Soc. Belge Méd. Trop., 65, Suppl. 2:* 89–96.