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MALARIA SEROLOGY: PERFORMANCE OF SIX *Plasmodium falciparum* ANTIGEN EXTRACTS AND OF THREE WAYS OF DETERMINING SERUM TITERS IN IgG AND IgM-ELISA.

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SUMMARY

The study evaluated six *Plasmodium falciparum* antigen extracts to be used in the IgG and IgM enzyme-linked immunosorbent assays (ELISA), for malaria diagnosis and epidemiological studies. Results obtained with eighteen positive and nine negative control sera indicated that there were statistically significant differences among these antigen extracts (Multifactor ANOVA, $p < 0.0001$). Urea, sodium deoxycholate and Zwittergent antigen extracts performed better than did the three others, their features being very similar for the detection of IgG antibodies. Urea, alkaline and sodium deoxycholate antigen extracts proved to be better than the others for the detection of IgM antibodies.

A straight line relationship was found between the optical densities (or their respective $\log_{10}$) and the $\log_{10}$ of antibody dilutions, with a very constant slope. Thus serum titers could be determined by direct titration and by two different equations, needing only one serum dilution. For IgM antibody detections, $\log_{10}$ expression gave results that better correlated with direct titration (95% Bonferroni). For IgG antibody detections, the titer differences were not significant.

The reproducibility of antibody titers and antigen batches was also evaluated, giving satisfactory results.

KEYWORDS: Malaria; Serology; ELISA; *Plasmodium falciparum*; Antigen extracts.

INTRODUCTION

Different serological tests have been used to detect antiplasmodial antibodies. The enzyme-linked immunosorbent assay (ELISA), described by ENGVALL & PERLMANN[3], was first used in malaria serology by VOLLER et al. [19]. ELISA combines the aspects such as sensitivity, specificity and simplicity with those of the low cost and possibility of processing a large number of serum samples in automated systems. However, many factors may affect the test outcomes and these factors must be well controlled in order to validate its use in malaria serology [13]. Thus, the antigens used and the interpretation of the results, specially as regards the adopted criterion for positivity, are important steps for improving the test [18].

This study tried to evaluate six different *Plasmodium falciparum* antigen extracts, using a ELISA microplate method [14], for the detection of IgG and IgM antiplasmodial antibodies, as well as to compare three methods for determining serum titers.

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MATERIALS AND METHODS

1. Culture

Plasmodium falciparum, Adalberto strain, was assynchronically cultured "in vitro" according to TRAGER & JENSEN methodology [17].

2. Antigen preparation

Schizont forms were selected to prepare antigen extracts. For this, when the parasitemia reached 10% , erythrocytes were harvested, washed and lysed with 0.04% saponin for 20 min at room temperature [5]. The mixture was centrifuged at 11950 g at 4° C for ten minutes and washed three times in 0.01 M PBS, pH 7.2, under similar conditions. The sediment was stored at -20°C.

Ten of such collections were thawed, pooled, and aliquots of 100 mg (wet weight) sediment were treated with detergents: 1% Triton X-100 [12], 10mM Zwittergent [12], 1% sodium deoxycholate [11]; alkaline solution (0.15M NaOH) [8]; caotropic agent (8 M urea) [5,14] and saline solution (0.15 M NaCl) [1], as outlined in Figure 1. In the detergent and saline extractions, proteases inhibitors: 0.001 M PMSF (phenylmethylsulfonyl fluoride); 50 µl/ml leupeptin; and 50 µl/ml aprotinin, were added.

After extraction, the antigens were centrifuged for 60 min at 34350 g at 4°C, supernatants collected, distributed in small aliquots and kept at -70°C. Protein contents were determined by the Lowry method [10].

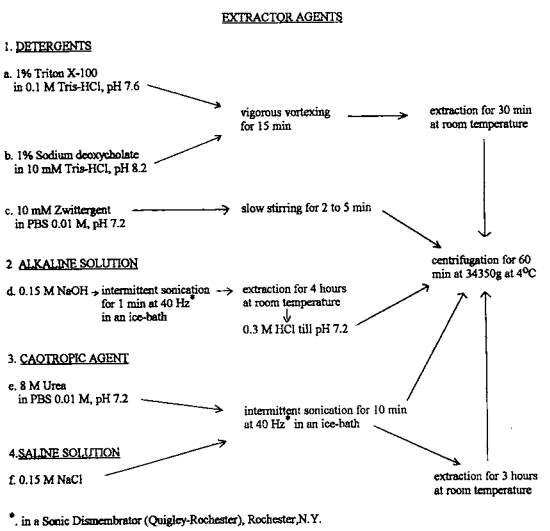

FIGURE 1 - Procedure used for the preparation of *P. falciparum* antigen extracts.

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Fig. 1 - Procedure used for the preparation of *P. falciparum* antigen extracts.

3. Sera

Twenty eight sera from patients with defined clinical and parasitologic (thick blood film positive for *P.falciparum* or *P.vivax*) diagnosis of acute malaria were collected in an endemic area (Amazonas State, Brazil) and taken as positive controls.

Nineteen sera from clinically healthy individuals living in a non-endemic area (São Paulo State, Brazil) were used as negative controls.

All sera were kept at -20°C, with an equal amount of neutral glycerin, after being tested in the indirect immunofluorescence antibody (IFA) test [4,16] and ELISA[14].

4. Immunoenzymatic assays

Microtiter plastic plate wells (Cooke) were coated, placing 100 µl (5 or 10 µg/ml) of antigen extracts in carbonate-bicarbonate buffer (0.06 M, pH 9.6), incubating for two hours at 37°C, and for 18 hours at 4°C. The plates were washed three times, five minutes each, with PBS and blocked with 200 µl of 5% skim milk, for two hours at 37°C, in a moist chamber. Sera were diluted in PBS containing 0.05% Tween 20 (PBS-T) and 2% skim milk (PBS-TM), starting from 1:50 to 1:51200, and added to each well, incubated for one hour at 37°C and washed three times in PBS-T. After a new incubation of one hour at 37°C with 100 µl of goat anti-human IgG (γ chain specific), or goat anti-human IgM (µ chain specific) horseradish peroxidase conjugate, diluted in PBS-TM, the plates were washed three times with PBS-T. Color development was done with 100 µl of a chromogen solution (0.0022 M ortophenylenediamine dihydrochloride (Sigma Chemical, Co), 0.013 M 30% hydrogen peroxide (Merck) in citrate-phosphate buffer pH 5.0). The enzymatic reaction was stopped after 30 min by adding 100 µl 2N H_2SO_4. Spectrophotometric reading was done in a Titertek Multiskan (MKII)-MCC/340 spectrophotometer at 492 nm.

Prior to use, the optimum dilution for the six antigens and two conjugates was determined by chequerboard titration. The optimum concentration of 5 µg/ml was obtained for antigen solubilized in Zwittergent, sodium deoxycholate, saline and Triton, and of 10 µg/ml, for those in NaOH and urea. The best working dilutions for anti-IgG and anti-IgM conjugates were 1/6000 and 1/2000, respectively.

Serum titers were determined by direct titration and by taking one optical density value and applying the equations A and B [2,14]

A: $$T = \log D + \frac{OD - \text{cut-off}}{k}$$

B: $$T = \log D + \frac{\log 1000 \times OD - \log 1000 \times \text{cut-off}}{k'}$$

where OD is the absorbance value observed for serum dilution D (1/100) and k and k' the slopes of dilution curves.

5. Titer reproducibility

To evaluate inter-test reproducibility of titers, duplicates of ten serum samples were titrated on different days using urea, sodium deoxycholate and Zwittergent antigen extracts in the detection of IgG and IgM antibodies.

6. Batches reproducibility

Batches of urea, sodium deoxycholate and Zwittergent antigen extracts were evaluated in terms of the differences between titers obtained with the test batches and those obtained with a reference reagent or those most commonly observed, using the control chart method, as described by HOSHINO-SHIMIZU et al.[9]. The control analysis of the batches was accomplished by doing sera titrations with panels including ten reactive and ten non-reactive sera. If the titers obtained with the reactive sera yielded a standard deviation (s) within the control limit (0.2), as well as all the non-reactive sera yielded negative results, the batch was accepted.

7. Statistical methods

Statistical calculations were made using a Statgraphics™ 5.1 (Statistical Graphic Corp., 1991) software. Serum titers (T) were transformed into $\log_{10} T^{20}$. The Multifactor Analysis of Variance (ANOVA)- Type III- (Sums of squares) was used to test differences between titers. Multiple comparison was accomplished by 95 percent Bonferroni test. Reproducibility of results (inter-test) was evaluated by linear regression (Pearson's correlation coefficient) and its significance by Student's "t" test.

The control chart method was applied to evaluate different batches of antigen extracts[9].

TABLE 1

Protein content (mg/ml) and number of assays possible to be made for Zwittergent (Zw), alkaline solution (NaOH), saline solution (Sal), Triton (Tr), urea (U) and sodium deoxycholate (SDC) antigen extracts.

	ANTIGEN EXTRACT					
PARAMETERS	Zw	NaOH	Sal	Tr	U	SDC
PROTEIN CONTENTmg/ml	3.1	4.34	1.39	1.06	6.20	3.09
NUMBER OF ASSAYS	6125	4338	2784	2110	6200	6176

RESULTS

Antigen extract performance

Table 1 shows the protein content and the number of assays possible to be made using each one of the studied antigen extracts.

To evaluate the immunological reactivity of six antigen extracts, eighteen sera from patients with acute malaria and nine from normal individuals were titrated by IgG and IgM ELISA. The stipulated cut-off of the assay corresponded to the arithmethic mean of the absorbances obtained from nine negative sera at a 1/100 dilution, plus two standard deviations (Table 2). The values > cut-off were defined as reactive, and those < cut-off, as non-reactive [7,15]. Serum titers were the highest dilutions yielding absorbance values greater than the cut-off.

TABLE 2

Limit of reactivity (cut-off) for Zwittergent (Zw), alkaline solution (NaOH), saline solution (Sal), Triton (Tr), urea (U) and sodium deoxycholate (SDC) antigen extracts in IgG and IgM ELISA.

		ANTIGEN EXTRACT					
	ANTIBODY	Zw	NaOH	Sal	Tr	U	SDC
Cut-off	IgG	0.200	0.200	0.220	0.100	0.210	0.200
	IgM	0.250	0.210	0.230	0.100	0.190	0.190

Linear regressions were calculated by plotting optical densities (or logarithms of 1000x optical densities) and logarithms of serum dilutions, comprising absorbance values of 0.1 and 2.0, and straight lines were obtained. When comparing such dilution curves with those from some other serum samples, slopes were seen to be similar, for each one of the six antigen extracts in the detection of IgG and IgM antibodies. The resulting equation was $y = a + k x$, the y-intercept (a) varying from one serum sample to another, and the slope (k) being very close for each antigen extract and each antibody class detected, except for negative sera, where the slope was often lower.

Table 3 shows the average slopes (k and k') obtained for the six antigen extracts, the standard deviations and the number of sera employed to calculate each average.

Table 4 shows the geometric mean titers (GMT) and the logarithmic mean titers (LMT) for ELISA in eighteen positive control sera as determined by direct titration and by equations A and B. In the IgG antibody detection, no significant differences could be found between the titers determined by both equations or by sera direct titration, at a level of 0.05, in Multifactor ANOVA (Table 5). In the detection of IgM antibodies, the differences were significant ($p < 0.05$) by Multifactor ANOVA, for all antigen extracts studied (Table 5). Multiple comparison, accomplished by 95 % Bonferroni test, showed significant differences between titers determined by direct titration and by equation A, for Zwittergent, saline, Triton and sodium deoxycholate antigen extracts, for IgM antibody detection. Nevertheless, no statistically significant difference was detected between titers obtained by direct titration and equation B (Bonferroni).

TABLE 3

Average slopes (k and k') and standard deviations (s) of n dilution curves for Zwittergent (Zw), alkaline solution (NaOH), saline solution (Sal), Triton (Tr), urea (U) and sodium deoxycholate (SDC) antigen extracts for the detection of IgG and IgM antibodies.

		ANTIGEN EXTRACT					
ANTIBODY	Index	Zw	NaOH	Sal	Tr	U	SDC
IgG	k(OD)	0.51	0.50	0.46	0.25	0.56	0.59
	s	0.03	0.04	0.10	0.08	0.04	0.05
	n	9	10	9	10	8	8
	k'(log)	0.38	0.40	0.38	0.38	0.37	0.39
	s	0.09	0.07	0.06	0.06	0.05	0.10
	n	8	10	10	9	5	10
IgM	k(OD)	0.33	0.34	0.23	0.11	0.31	0.29
	s	0.05	0.06	0.05	0.05	0.03	0.03
	n	7	7	9	9	9	9
	k'(log)	0.47	0.43	0.43	0.33	0.45	0.47
	s	0.06	0.06	0.05	0.08	0.09	0.04
	n	10	10	10	9	10	10

TABLE 4

ELISA geometric mean titers and logarithmic mean titers of eighteen positive control sera as determined by direct titration (T) and by equation A or B, for Zwittergent (Zw), alkaline solution (NaOH), saline solution (Sal), Triton (Tr), urea (U) and sodium deoxycholate (SDC) antigen extracts for the detection of IgG and IgM antibodies.

		ANTIGEN EXTRACT					
ANTIBODY	METHOD	Zw	NaOH	Sal	Tr	U	SDC
IgG	T	6310	3715	1585	1905	10000	8318
		3.80	3.57	3.20	3.28	4.00	3.92
	A	6918	5012	1820	2344	9333	8128
		3.84	3.70	3.26	3.37	3.97	3.91
	B	7586	4365	1950	2884	10965	9772
		3.88	3.64	3.29	3.46	4.04	3.99
IgM	T	355	933	282	251	933	661
		2.55	2.97	2.45	2.40	2.97	2.82
	A	550	1318	661	537	1380	1047
		2.74	3.12	2.82	2.73	3.14	3.02
	B	363	871	288	324	851	646
		2.56	2.94	2.46	2.51	2.93	2.81

TABLE 5

F-ratio ($F^{5\%}_{2,34} = 3.28$) and significance level for the differences (p) of eighteen positive control serum titers as determined by direct titration and by equations A or B for Zwittergent (Zw), alkaline solution (NaOH), saline solution (Sal), Triton (Tr), urea (U) and sodium deoxycholate (SDC) antigen extracts for the detection of IgG and IgM antibodies, in Multifactor Analysis of Variance (ANOVA).

		ANTIGEN EXTRACT					
ANTIBODY	Index	Zw	NaOH	Sal	Tr	U	SDC
IgG	F	0.73	1.84	0.92	1.15	0.52	0.66
	p	0.4881	0.1739	0.4080	0.3285	0.5980	0.5212
IgM	F	6.16	3.48	15.53	5.94	4.43	5.71
	p	0.0052	0.0422	<0.0001	0.0061	0.0195	0.0073

GMT and LMT for eighteen positive control sera using the six antigen extracts are shown in Table 4. Multifactor ANOVA of ELISA results (Table 6) showed significant differences between GMT obtained for IgG ($p < 0.0001$) and IgM ($p < 0.0001$) antibody detections. Mean titers of pairs of groups within each series (IgG and IgM antibody detections) were compared using the Bonferroni test, and differences between the following paired groups were found to be statistically significant. For IgG antibody detection: urea with saline, alkaline and Triton antigen extracts; sodium deoxycholate with saline, alkaline and Triton antigen extracts; Zwittergent with saline and Triton antigen extracts; saline with alkaline antigen extract. For IgM antibody detection: urea with saline, Triton and Zwittergent antigen extracts; alkaline with saline, Zwittergent and Triton antigen extracts; sodium deoxycholate with saline and Triton

TABLE 6

F-ratio ($F^{0.05}_{3,35}$ = 2.33) and significance level for the differences (p) of eighteen positive control sera with the six antigen extracts for the detection of IgG and IgM antibodies, as determined by direct titration (T) and by equations A and B, in Multifactor Analysis of Variance (ANOVA).

ANTIBODY	INDEX	T	A	B
IgG	F	22.84	12.68	26.40
	p	<0.0001	<0.0001	<0.0001
IgM	F	12.24	4.61	7.96
	p	<0.0001	0.0009	<0.0001

antigen extracts. For IgG antibodies, urea, sodium deoxycholate and Zwittergent antigen extracts gave higher GMT (significance level < 0.0001). For IgM antibodies, GMT were higher with urea, alkaline and sodium deoxycholate antigen extracts (significance level < 0.0001).

Similar analysis were performed with the titers obtained from equations A and B and the results were the same using equation B as to the IgG antibody reactivity, but for IgM antibody the only discrepancy was obtained comparing Triton and sodium deoxycholate antigen extracts (Table 6). Using equation A there was discrepancy when comparing alkaline with urea and sodium deoxycholate antigen extracts, for IgG antibody detection. For IgM antibodies, the discrepancies were: saline with urea and alkaline antigen extracts; sodium deoxycholate with saline and Triton antigen extracts.

Titer Reproducibility

The IgG and IgM ELISA were very reproducible, at the significance level of Student's "t" test ($p < 0.05$) indicating there was an association between the titer replicates obtained on two different days (Table 7). The same significance was seen when the analysis was made by means of Pearson's correlation coefficient ($p < 0.05$).

TABLE 7

Inter-test reproducibility - Student's "t" test significance level (p), linear regression calculations, Pearson's correlation coefficient (r) and probability level (p) for Zwittergent (Zw), sodium deoxycholate (SDC) and urea (U) antigen extracts for the detection of IgG and IgM antibodies, in ten serum samples titrated on two different days.

ANTIBODY	EXTRACT	y-intercept	slope	r	p	"t" test (p)
IgG	SDC	0.0021	1.0208	0.95	0.00002	0.7576
	Urea	0.9689	0.8176	0.93	0.0001	0.3715
	Zw	0.1766	0.9637	0.97	<0.0001	0.9249
IgM	SDC	0.9817	0.8239	0.92	0.00018	0.0624
	Urea	0.8374	0.8116	0.91	0.00024	0.3362
	Zw	1.2116	0.6377	0.80	0.00561	0.1753

Batches reproducibility

In assessing each reagent batch, the control limit was established as 1.5 times the arithmethic mean (s) of the standard deviations of all the batches considered acceptable. Figure 2 shows the standard deviations calculated and compares them to the control limit to decide whether or not the reagent could be accepted. The batch reproducibilities showed very similar results for the three antigen extracts, in the IgG antibody detection, whereas in the IgM, the Zwittergent antigen extract was found to be more reproducible.

DISCUSSION

ELISA is known from non-malarial contexts to have a high sensitivity, although some studies suggest that it is less efficient than other tests in detecting infected persons individually with malaria [15,18].This fact is probably due to several technical features of the test, particularly the antigens used, and the interpretation of the results, which differ according to the criterion of positivity established. The results will be greatly influenced by the quality of antigen extract used, and this, in turn, will affect the sensitivity of the assay. In this sense, six different antigen extracts were evaluated in ELISA for the detection of IgG and IgM antiplasmodial antibodies.

In terms of protein content better results were obtained with urea antigen extract; nevertheless the opti-

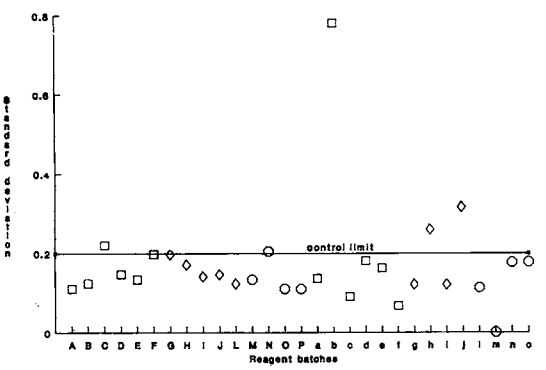

Fig. 2 - A control chart for IgG and IgM ELISA reagent batches prepared with sodium deoxycholate (SDC), urea (U) and Zwittergent (Zw). IgG ELISA: A-F (SDC), G-L (U) M-P (Zw). IgM ELISA: a-f (SDC), g-j (U), l-o (Zw).

mum coating concentration for this antigen, found by chequerboard titration was 10 µg/ml, similarly to alkaline antigen extract, and higher than the other antigens which required 5 µg/ml. The number of possible assays to be made with the urea antigen extract was similar to sodium deoxycholate and Zwittergent antigen extracts, but being these higher than the other antigens.

The apparent diagnostic efficiency of various serological methods in discriminating individuals with previous experience of malaria will depend on the criteria used for differentiating "positive" from "negative"reactions [18]. The problem with the tests is the fact that a downward shift of the cut-off point will increase sensitivity at the expense of specificity, and vice-versa [18].

Because of the linear relationship between optical densities and of antibody concentration, displaying a constant slope, serum titers are allowed to be expressed by equations A or B [2,14]. For the detection of IgG antibodies, there were no significant differences between the titers of eighteen positive control sera as determined by direct titration or by both equations, in Multifactor ANOVA ($p > 0.05$). Differences of mean titers from equations A and B, or from equation A and direct titration or from equation B and direct titration were not statistically significant for all antigen extracts in IgG antibody detection, as found in Bonferroni test. The use of Zwittergent, saline, Triton and sodium deoxycholate antigen extracts, for the IgG antibody detection, showed better titer correlations between direct titration and equation A. However, using alkaline and urea antigen extracts, titers from direct titration better correlated to those from equation B (Bonferroni). For IgM antibody detection, a significant difference was obtained between the titers from direct titration and those from both equations, for all antigen extracts employed (Multifactor ANOVA, $p < 0.05$). Multiple range tests (Bonferroni) showed differences of mean titers from direct titration and equation A, for Zwittergent, saline, Triton and sodium deoxycholate; from A and B equations, for Zwittergent, saline, urea and sodium deoxycholate. The mean titers from direct titration and equation B showed no statistically significant difference. Better correlations were found between IgM antibody titers from direct titration and equation B using all the six antigen extracts (Bonferroni).

The absorbance values obtained by ELISA are related to the amount of serum only under conditions of antigen excess[13]. For high concentration of antibody (low dilutions), the amount of antigen present on the plate becomes a limiting factor [13] and a competition effect between the target antibody and other molecules is observed[6]. At higher sample dilutions there is a straight line relationship between enzyme activity and antibody concentration[13] and the relative accessibility to the antigen improves and permits better antibody detection[6].

Negative controls or very low titered sera often yield non-parallel curves displaying lower slopes, probably due to the competition effect, but this does not invalidate the use of mathematical expressions for determining titers of positive sera.

Performance indexes of IgG and IgM ELISA were very similar with the six antigen extracts. Positivity ranged from 100% with Zwittergent, alkaline, urea and sodium deoxycholate antigen extracts to 94% with Triton and 89% with saline antigen extracts. With IgM ELISA, this index was 100% with urea, 94% with sodium deoxycholate and alkaline, 83% with Zwittergent and saline and 78% with Triton antigen extracts. Specificity index was 100% with IgG ELISA using all the six antigen extracts and with IgM ELISA, was 89% with alkaline and Triton antigen extracts and 100% with the other antigens. Thereby, the positivity here found was not a good criterion to compare the type of antigens, possibly due to the small size of serum samples studied. However, comparing the differences between titers observed by means of geometric mean titers and logarithmic mean titers in IgG ELISA, better results were obtained with urea, sodium deoxycholate and Zwittergent antigen extracts. On the other hand, in IgM ELISA, the titers were higher with urea, alkaline and sodium deoxycholate antigen extracts.

The relative usefulness of the different serological tests will depend not only on their relative sensitivity and specificity but also on the cost of labour, equipment and reagents, and on their simplicity, accuracy and reproducibility [18]. Thus, considering GMT, the yield and the number of assays possible to be made, the urea, sodium deoxycholate and Zwittergent antigen extracts were chosen to investigate inter-test and inter-batches reproducibility.

In conclusion, urea, sodium deoxycholate and Zwittergent antigen extracts can be applied as antigen to the IgG and IgM-ELISA and by performing similarly, in the diagnosis of malaria.

Serum titers provided by one dilution deviates the influence of daily variations in test sensitivity, with the possibility of correcting results according to the observed variations in the optical densities of reference sera.

The use of mathematical expression B (log dilutions x log optical densities) for quantitation of ELISA titers showed a good correspondence to the direct titration of both IgG and IgM antibodies. Otherwise, equation A (log dilutions x optical densities) was useful only for IgG antibodies. ELISA, employing equation B, is specially useful for population and individual studies since it requires only one serum dilution, besides being practical and economic.

RESUMO

Sorologia da Malária: desempenho de seis diferentes extratos antigênicos de *Plasmodium falciparum* e de três métodos de determinação de títulos de soros no teste ELISA-IgG e IgM.

Este estudo avaliou seis extratos antigênicos de *Plasmodium falciparum* para utilização no diagnóstico da malária e em estudos epidemiológicos, empregando o teste "enzyme-linked immunosorbent assay" (ELISA) para a pesquisa de anticorpos das classes IgG e IgM. Os resultados obtidos empregando dezoito soros padrão positivo e nove padrão negativo, indicaram que houve diferenças estatisticamente significativas entre os extratos antigênicos estudados (Multifactor ANOVA, $p < 0{,}0001$). Extratos antigênicos preparados com uréia, desoxicolato de sódio e Zwittergent forneceram melhores resultados que os demais e se comportaram de modo bastante semelhante, para a detecção de anticorpos IgG. Extratos antigênicos de uréia, alcalino e de desoxicolato de sódio foram os melhores na detecção de anticorpos IgM.

Devido à relação linear obtida entre as densidades ópticas (ou os $\log_{10}$ respectivos) e os $\log_{10}$ das diluições, com uma inclinação constante, os títulos dos soros foram determinados através de duas equações diferentes, que necessitam apenas de uma diluição do soro. Para anticorpos IgM, a expressão que emprega $\log_{10}$ da densidade óptica forneceu resultados que se correlacionaram melhor com a titulação (95% Bonferroni). Para anticorpos IgG, as diferenças entre resultados não foram significativas.

A avaliação da reprodutibilidade dos títulos e das partidas de antígenos forneceu resultados satisfatórios.

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