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 log 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 ENGVALM & PERLMANN, was first used in malaria serology by VOLLER et al. 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. 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.

This study tried to evaluate six different *Plasmodium falciparum* antigen extracts, using a ELISA microplate method, 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 assynchronized by culture 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 11,950 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) 6, caustropic 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 μg/ml leupeptin; and 50 μg/ml aprotinin, were added.

After extraction, the antigens were centrifuged for 60 min at 34,350 g at 4°C, supernatants collected, distributed in small aliquots and kept at -70°C. Protein contents were determined by the Lowry method 19.

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 46 and ELISA 4.

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 (λ 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 ortho-phenylenediamine dihydrochloride (Sigma Chemical, Co), 0.013 M 30% hydrogen peroxide (Merek) in citrate-phosphate buffer pH 5.0). The enzymatic reaction was stopped after 30 min by adding 100 μl 2N H2SO4. Spectrophotometric reading was done in a TiterLok Multiskan (MKII)-MCC/340 spectrophotometer at 492 nm.

Prior to use, the optimum dilution for the six antigens and two conjugates was determined by checkerboard 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 214.
A: \[ T = \log D + \frac{OD - \text{cut-off}}{k} \]

B: \[ T = \log D + \log 1000 \times OD - \log 1000 \times \text{cut-off} \]

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 Statgraphtex™ 5.1 (Statistical Graphic Corp., 1991) software. Serum titers (T) were transformed into \( \log_{10} T \). 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\(^8\).

**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 corresponds to the arithmetic 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.

<table>
<thead>
<tr>
<th>ANTIGEN EXTRACT</th>
<th>ANTIBODY</th>
<th>Zw</th>
<th>NaOH</th>
<th>Sal</th>
<th>Tr</th>
<th>U</th>
<th>SDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>0.200</td>
<td>0.200</td>
<td>0.220</td>
<td>0.100</td>
<td>0.210</td>
<td>0.200</td>
<td></td>
</tr>
<tr>
<td>IgM</td>
<td>0.250</td>
<td>0.210</td>
<td>0.230</td>
<td>0.100</td>
<td>0.190</td>
<td>0.190</td>
<td></td>
</tr>
</tbody>
</table>

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 \( y = a + kx \), 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 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.

<table>
<thead>
<tr>
<th>ANTIGEN EXTRACT</th>
<th>ANTIBODY</th>
<th>Index</th>
<th>Zw</th>
<th>NaOH</th>
<th>Sal</th>
<th>Tr</th>
<th>U</th>
<th>SDC</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
<td>k(OD)</td>
<td>0.51</td>
<td>0.50</td>
<td>0.46</td>
<td>0.25</td>
<td>0.56</td>
<td>0.59</td>
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<tr>
<td></td>
<td></td>
<td>s</td>
<td>0.03</td>
<td>0.04</td>
<td>0.10</td>
<td>0.08</td>
<td>0.04</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n</td>
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<td></td>
<td>IgG</td>
<td>k'(log)</td>
<td>0.38</td>
<td>0.40</td>
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<td>0.38</td>
<td>0.37</td>
<td>0.39</td>
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<td></td>
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<td>s</td>
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</tr>
<tr>
<td></td>
<td>IgM</td>
<td>k(OD)</td>
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<td>IgM</td>
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<td>10</td>
<td>9</td>
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### 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.

<table>
<thead>
<tr>
<th>ANTIGEN EXTRACT</th>
<th>ANTIBODY</th>
<th>METHOD</th>
<th>Zw</th>
<th>NaOH</th>
<th>Sal</th>
<th>Tr</th>
<th>U</th>
<th>SDC</th>
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<tbody>
<tr>
<td></td>
<td>IgG</td>
<td>T</td>
<td>3715</td>
<td>3715</td>
<td>1585</td>
<td>1905</td>
<td>10000</td>
<td>8318</td>
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<tr>
<td></td>
<td></td>
<td>3.80</td>
<td>3.37</td>
<td>3.20</td>
<td>3.28</td>
<td>4.00</td>
<td>3.92</td>
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<td>7586</td>
<td>4365</td>
<td>1950</td>
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<td>10965</td>
<td>9772</td>
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<tr>
<td></td>
<td></td>
<td>3.88</td>
<td>3.64</td>
<td>3.29</td>
<td>3.46</td>
<td>4.04</td>
<td>3.99</td>
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</tr>
<tr>
<td></td>
<td>IgM</td>
<td>T</td>
<td>355</td>
<td>933</td>
<td>282</td>
<td>251</td>
<td>933</td>
<td>661</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>2.45</td>
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<td>2.97</td>
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<td>2.74</td>
<td>3.12</td>
<td>2.82</td>
<td>2.73</td>
<td>3.14</td>
<td>3.02</td>
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<td></td>
<td></td>
<td>363</td>
<td>871</td>
<td>288</td>
<td>324</td>
<td>851</td>
<td>666</td>
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<td></td>
<td></td>
<td>2.56</td>
<td>2.94</td>
<td>2.46</td>
<td>2.51</td>
<td>2.93</td>
<td>2.81</td>
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</tbody>
</table>

### TABLE 5
F-ratio (F_m = 3.28) and significance level for the differences (p) of eighteen positive control sera 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).

<table>
<thead>
<tr>
<th>ANTIGEN EXTRACT</th>
<th>ANTIBODY</th>
<th>Index</th>
<th>Zw</th>
<th>NaOH</th>
<th>Sal</th>
<th>Tr</th>
<th>U</th>
<th>SDC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
<td>F</td>
<td>0.73</td>
<td>1.84</td>
<td>0.92</td>
<td>1.15</td>
<td>0.52</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p</td>
<td>0.4881</td>
<td>0.1739</td>
<td>0.0408</td>
<td>0.3285</td>
<td>0.5980</td>
<td>0.5212</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>F</td>
<td>6.16</td>
<td>3.48</td>
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<td>5.94</td>
<td>4.43</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>p</td>
<td>0.0052</td>
<td>0.0425</td>
<td>&lt;0.0001</td>
<td>0.0061</td>
<td>0.0195</td>
<td>0.0073</td>
</tr>
</tbody>
</table>

GMN 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.
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).

**Batches reproducibility**

In assessing each reagent batch, the control limit was established as 1.5 times the arithmetic 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. This fact is probably due to several technical features of the test, particularly the antibodies 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-
mum coating concentration for this antigen, found by
chequeboard titration was 10 μg/ml, similarly to alka-
line 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 sero-
logical methods in discriminating individuals with pre-
vious experience of malaria will depend on the criteria
used for differentiating "positive" from "negative" reac-
tions 14. The problem with the tests is the fact that a
downward shift of the cut-off point will increase sen-
sitivity at the expense of specificity, and vice-versa 14.

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 21,4. For the detection of IgG anti-
bodies, 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 equa-
tions, for all antigen extracts employed (Multifactor
ANOVA, p < 0.05). Multiple range tests (Bonferroni)
demonstrated differences of mean titers from direct titration
and equation A, for Zwittergent, saline, Triton and so-
dium 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 19. For high concentration of antibody
(low dilutions), the amount of antigen present on the
plate becomes a limiting factor 19 and a competition
effect between the target antibody and other molecules
is observed 4. At higher sample dilutions there is a
straight line relationship between enzyme activity and
antibody concentration 19 and the relative accessibility to
the antigen improves and permits better antibody
detection 4.

Negatives 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 dif-
fences 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 ex-
tracts.

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 antígenicos de Plasmodium falciparum e de três métodos de determinação de títulos de soros no teste ELISA-IgG e IgM.

Este estudo avaliou seis extratos antígenicos 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 dezolito soros padrão positivo e nove padrão negativo, indicaram que houve diferenças estatisticamente significativas entre os extratos antígenicos estudados (Multifactor ANOVA, p <0,001). Extratos antígenicos preparados com ureia, 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 antígenicos de ureia, 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 ótica 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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