Comparative analysis of ELISAs employing repetitive peptides to detect antibodies to *Plasmodium falciparum* sporozoites

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In the last few years, a number of different recombinant and synthetic peptides consisting of the repetitive sequence of the Plasmodium falciparum *circumsporozoite protein (NANP)$_n$ have been produced and used to develop immunoassays for the detection of antibodies against* P. falciparum *sporozoites in human sera. A comparative study of three enzyme-linked immunosorbent assays (ELISAs) that employed different (NANP)$_n$ peptides (the synthetic peptides (NANP)$_3$ and (NANP)$_{40}$ as well as the recombinant peptides R32tet32 and R32LR) was carried out using serum samples from individuals who were living in different malaria-endemic areas. The results obtained for these peptide-based ELISAs were compared with those obtained for an immunofluorescence assay (IFA) that used glutaraldehyde-fixed sporozoites. All the methods tested exhibited 100% specificity on sera from persons not exposed to malaria, good reproducibility (coeffients of variation ranged from 3% to 15% for peptide-based ELISAs), and good sensitivity. Reproducibility and sensitivity were lower for the IFA than for the peptide-based ELISAs, perhaps because of the subjective element in the interpretation of the results which is inherent in the IFA method. ELISAs based on peptides that contain a higher number of (NANP) repeats, i.e., (NANP)$_{40}$ and R32tet32 or R32LR, gave results which correlated better with each other than with those obtained with the ELISA that employed a shorter (NANP)$_3$ peptide. (NANP)$_n$-based ELISAs are relatively simple and inexpensive methods for the detection of anti-*P. falciparum *sporozoite antibodies and can readily be used in epidemiological research in the field. These assays could contribute to a better understanding of the natural history of the host–parasite relationship in malaria research.*

Malaria sporozoites possess a major surface antigen, the circumsporozoite (CS) protein, which uniformly surrounds their external coat. Despite their short persistence in circulating blood, sporozoites induce a strong immune response that is characteristically species- and stage-specific (*1*). Plasmodial CS proteins consist of tandem repeats of a number of amino acids, flanked by nonrepetitive sequences (*2*). All the monoclonal antibodies so far produced against sporozoites appear to recognize this repetitive sequence in the CS protein (*3*), as do naturally acquired antibodies from malaria-endemic areas (*4*). The repetitive domain of the *Plasmodium falciparum* CS protein consists of four amino acids (Asn-Ala-Asn-Pro = NANP) repeated several times (*5, 6*), which is well conserved in all of the isolates from different geographical regions so far investigated (*7–9*). This repetitive sequence, which has been produced by both genetic engineering methods (*10*) and chemical synthesis (*4, 11*), has been employed as the basis for the development of prototype malaria vaccines (*12–14*) and of assays for the detection of antisporozoite antibodies in human populations exposed to infection with falciparum malaria.

Until recently, circumsporozoite precipitation and immunofluorescence assay (IFA) were the only techniques available for the detection of antisporozoite antibodies in serum (*15, 16*). These techniques require a regular supply of sporozoites, which in turn demands mosquito breeding facilities—which are available in very few laboratories. A number of different recombinant and synthetic (NANP)$_n$ peptides have been produced recently and used to develop immunoassays for the detection of antisporozoite antibodies in human sera. These peptide-based methods, mainly enzyme-linked immunosorbent essays (ELISAs), have been

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used in epidemiological research, and the results obtained are similar to those obtained with IFA (*17–23*).

Since these assays employ recombinant or synthetic peptides that consist of different numbers of (NANP) repeats, it is interesting to determine whether all such peptides detect antisporozoite antibodies in the same manner. Here, we report the results of a comparative analysis of ELISAs that use different $(NANP)_n$ peptides to detect anti-*P. falciparum* sporozoite antibodies in serum samples from individuals who were living in several malaria-endemic areas. The comparative study was carried out during a workshop held in Geneva under the auspices of the UNDP/ World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

Materials and methods

Serum samples

A total of 253 serum samples were tested by each ELISA method. The set of sera consisted of the following groups: (*a*) sera from individuals who were living in malaria-endemic areas, i.e., Brazil, Burkina Faso, Cameroon, Colombia, Honduras, Kenya, Mexico, Thailand, and West Africa; (*b*) sera from 16 healthy blood donors from Geneva; and (*c*) sera from five patients with systemic lupus erythematosus and five with rheumatoid arthritis (Table 1). Sera in groups (*b*) and (*c*) were included to test the specificity of each method.

To test the sensitivity of the assays, one serum sample that was known to be positive for anti-*P. falciparum* sporozoite antibodies (obtained from P.C., a European who acquired acute *P. falciparum* malaria in the Côte d'Ivoire) was tested undiluted and prediluted at 1:2, 1:4, 1:8, and 1:16 in a human serum that was unexposed to malaria. To test the reproducibility of the assays, a pool of 30 serum samples was prepared from individuals who were living in Nigeria and known to have anti-*P. falciparum* antibodies. Serum from this pool was divided between five different sample tubes, and the same was done for a pool of serum from healthy blood donors from Geneva. Pools were used since insufficient serum was available from individuals to use single donor sources. Each sample tube was coded by Dr L. Martinez in WHO, and aliquots of 100 μl were prepared and stored at −70 °C until used.

Methods for detecting anti-*P. falciparum* sporozoite antibodies

The methods outlined below for the detection of anti-*P. falciparum* sporozoite antibodies were tested.

Table 1: **List of the serum samples that were assayed in the study**

Origin	Provided by:	No. of sera
Malaria-endemic areas		
Brazil	M. Arruda	20
Burkina Faso	J.-B. Ouedraogo	22
	J.-P. Verhave	41
Cameroon	R. Moyou	6
Colombia	M. Herrera	36
Honduras	I.G. Gallo	19
Kenya	G.M. Marangalla	16
Mexico	L. Gonzales-Ceron	12
Thailand	C. Khamboonruang	16
	R. Rosenberg	10
West Africa[a]	F. Zavala	14
Nigeria[b]	G. Del Giudice	5
Positive control[c]	G. Del Giudice	5
Malaria-nonendemic area (Geneva)		
Blood donors	G. Del Giudice	16
Negative pool[d]	G. Del Giudice	5
SLE[e] patients	G. Del Giudice	5
RA[f] patients	G. Del Giudice	5
Total		253

[a] The exact origin of these serum samples was not known.
[b] A pool of 30 serum samples that were positive for anti-*Plasmodium falciparum* sporozoite antibodies, and assayed five times.
[c] Positive control from a European who contracted *P. falciparum* malaria, assayed undiluted, and prediluted 1:2, 1:4, 1:8, and 1:16 in normal human serum.
[d] A pool of 30 serum samples from healthy blood donors from Geneva, and assayed five times.
[e] SLE = systemic lupus erythematosus.
[f] RA = rheumatoid arthritis.

• The IFA, which used glutaraldehyde-fixed *P. falciparum* sporozoites (NF54 strain) from membrane-fed *Anopheles stephensi* mosquitos, was carried out as previously described (*15*). Sera that fluoresced at a 1:40 dilution were considered to be positive.

The remaining methods were ELISAs that employed different synthetic or recombinant peptides consisting of variable numbers of the repetitive (NANP) epitope of the *P. falciparum* CS protein, as discussed below.

• The $(NANP)_3$-BSA ELISA, in which the synthetic peptide $(NANP)_3$ conjugated to bovine serum albumin (BSA) [$(NANP)_3$-BSA], was carried out as previously described (*18*), the only modification being that a horseradish peroxidase anti-human IgG antibody was employed as a probe. Serum samples were tested at a dilution of 1:40 and the results determined at $\lambda = 414$ nm using a Multiskan Titertek micro-ELISA reader.

• The R32tet32 ELISA, in which the recombinant peptide R32tet32 (consisting of the sequence $(NANP)_{15}$-NVDP $(NANP)_{15}$NVDP, plus 32 amino acids that are

encoded by a tetracycline-resistant gene read out-of-frame (tet32)), or the R32LR ELISA, in which the R32 LR recombinant lacks the last 30 amino acids of the tet32 moiety, but contains the first two, leucine and arginine (LR), was carried out as described previously (*17, 22*). Serum samples were tested at a dilution of 1:40, and the results determined at $\lambda = 414$ nm using a Multiskan Titertek micro-ELISA reader.

- The $(NANP)_{40}$ ELISA, which uses the synthetic peptide $(NANP)_{40}$, was carried out as described previously (*19, 20*). Serum samples were tested at a dilution of 1:200 and results determined at $\lambda = 492$ nm using a Multiskan Titertek micro-ELISA reader.

Definition of titration units

In order to standardize the results obtained with the different methods, a serum sample that was known to be positive for anti-*P. falciparum* sporozoite antibodies (from P.C.) was used as a positive control in each method. The absorbance obtained with this serum in each test was assigned a value of 100 titration units (TU), and all the absorbances obtained with the coded sera were proportionally adjusted to this value.

Definition of the cut-off value

After the code had been broken, the cut-off value for "positive" sera was defined as the mean +3 standard deviations of the value for the TU obtained for the 16 normal human sera included in the set of 253 samples tested. According to this procedure, the cut-off values were as follows:

— IFA = 1:40 dilution;
— $(NANP)_3$-BSA ELISA = 15.14 TU;
— R32tet32 ELISA = 13.88 TU;
— R32LR ELISA = 9.13 TU; and
— $(NANP)_{40}$ ELISA = 12.58 TU.

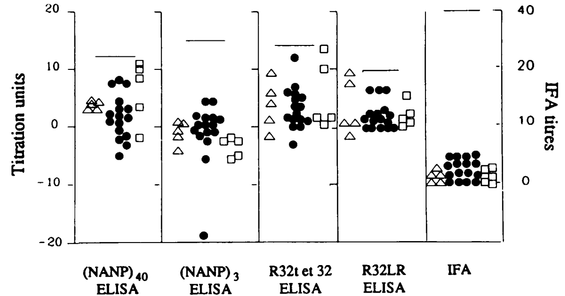
Values at or above these cut-offs were considered to be positive for anti-*P. falciparum* sporozoite antibodies.

Results

Specificity

Specificity was tested with a panel of sera from healthy blood donors from Geneva and from European patients with systemic lupus erythematosus or rheumatoid arthritis. As shown in Fig. 1, all sera from healthy blood donors gave results below the cut-off values with each method. Similarly, negative results were always obtained with sera from patients with systemic lupus erythematosus or rheumatoid arthritis, despite the presence of high titres of rheumatoid factors and other auto-antibodies. It can be concluded that the IFA and

Fig. 1. **Plots showing the specificity of the (NANP)-based ELISAs and the immunofluorescence assay (IFA) for the detection of anti-*Plasmodium falciparum* sporozoite antibodies. Serum samples from 16 healthy blood donors from Geneva (●), from five patients with systemic lupus erythematosus (△) and from five patients with rheumatoid arthritis (□) were tested. Horizontal lines represent cut-off values.**

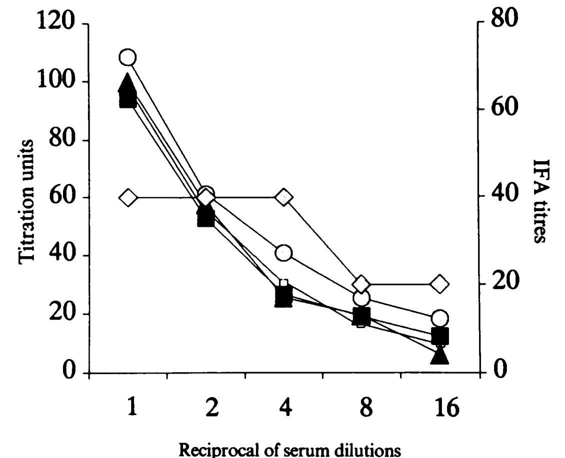


the peptide-based ELISAs for the detection of anti-*P. falciparum* sporozoite antibodies appear to be specific, since no false-positive results were obtained.

Sensitivity

Sensitivity was tested using a serum that was known to be positive for anti-*P. falciparum* sporozoite antibodies and which was progressively diluted in normal human serum. Fig. 2 shows that the $(NANP)_n$-based ELISAs

Fig. 2. **Plots showing the sensitivity of the immunofluorescence assay (IFA) and the (NANP)-based ELISAs for the detection of anti-*Plasmodium falciparum* sporozoite antibodies. A serum sample (P.C.) that was known to be positive for antisporozoite antibodies was tested undiluted and prediluted 1:2, 1:4, 1:8 and 1:16 in normal human serum. ■: $(NANP)_{40}$ ELISA; ▲: $(NANP)_3$-BSA ELISA; ○: R32tet32 ELISA; □: R32LR ELISA; and ◇: IFA.**



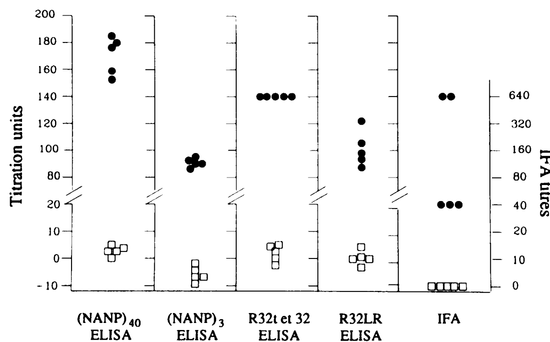
gave titration curves with similar slopes. The results for the R32tet32 ELISA were always greater than the cut-off value. In contrast, the $(NANP)_3$-BSA ELISA and the $(NANP)_{40}$ ELISA gave positive results for the first four serum dilutions, while the fifth dilution was just less than the cut-off value. Finally, serum that was undiluted or prediluted 1:2 and 1:4 was positive by IFA, but negative when prediluted 1:8 or 1:16. Thus, IFA was less sensitive than the peptide-based ELISAs in detecting anti-*P. falciparum* sporozoite antibodies.

The serum used to estimate the sensitivity of the assays was the same as that used as a positive control in each test. Fig. 2 shows that the ELISAs gave absorbances of approximately 100 TU for the undiluted sample, thus showing that the reproducibility of the results was good (see next paragraph).

Reproducibility

Reproducibility was determined with pools of sera that were positive or negative for anti-*P. falciparum* sporozoite antibodies, and the measurements repeated five times blind. The five serum samples from the positive pool and the five samples from the negative pool were always positive and negative, respectively, in the IFA and ELISAs (Fig. 3). The coefficient of variation was 15% for the R32LR ELISA, 7.4% for the $(NANP)_{40}$ ELISA, and 2.7% for the $(NANP)_3$-BSA ELISA, as determined with the pool of positive sera. The coefficient of variation could not be calculated for the R32tet32 ELISA, since the five serum samples from the positive pool always gave absorbances that were off-scale (> 2.0). Finally, the IFA exhibited a coefficient of variation of 31.3% (two of the five samples were scored as positive (1:640) and the other three as positive (1:40)), and this method therefore had the lowest reproducibility of those tested.

Fig. 3. **Plots showing the reproducibility of the immunofluorescence assay (IFA) and the (NANP)-based ELISAs for the detection of anti-*Plasmodium falciparum* sporozoite antibodies. Assays on one positive pool (●) from Nigerian and one negative pool (□) from Geneva subjects were repeated five times.**



Correlations between the results of the assays

Comparison of the results of the assays (based on the measured absorbances) using a Spearman rank correlation indicated that all the correlations were statistically significant ($P<0.001$) (Table 2). The strongest correlation was observed between the R32tet32 ELISA and the $(NANP)_{40}$ ELISA ($R=0.87$) and the weakest between the $(NANP)_3$-BSA ELISA and the $(NANP)_{40}$ ELISA ($R=0.61$). It is interesting to note that the correlation between the R32tet32 ELISA and the $(NANP)_{40}$ ELISA was similar to that between the R32tet32 ELISA and the R32LR ELISA ($R=0.86$).

Similar results were observed when the assays were compared in terms of their ability to agree or disagree, i.e., give "positive" or "negative" results (Table 3). As expected, the best agreement was between the R32tet32 ELISA and the R32LR ELISA (82.7%), followed by that between the R32tet32 ELISA and the $(NANP)_{40}$ ELISA (79.8%); the lowest was between the R32tet32 ELISA and the $(NANP)_3$-BSA ELISA (59.1%). Comparison of the results of the ELISA methods with those of the IFA indicated that the best agreement was with the $(NANP)_3$-BSA ELISA (80.8%), and the lowest was with the R32tet32 ELISA (63%). The R32tet32, R32LR, and $(NANP)_{40}$ ELISAs detected as positive (and in some instances, as highly positive) those sera from malaria-endemic areas that were scored as negative by IFA and the $(NANP)_3$-BSA ELISA. In one case, only the R32tet32 ELISA gave a high TU for a serum that was negative by the $(NANP)_{40}$ ELISA.

These results suggest that the ELISAs with the longer peptides, i.e., the R32tet32, R32LR, and $(NANP)_{40}$ ELISAs, may have a higher sensitivity than the ELISA with the shorter peptide, i.e., the $(NANP)_3$ ELISA.

*Detection of anti-*P. falciparum *sporozoite antibodies in paired mother–cord serum samples*

Sixteen paired mother–umbilical cord serum samples from Burkina Faso were included in the set of coded sera that were tested. As shown in Table 4, there was good overall agreement between the results obtained with the ELISAs. For 16 of the 32 sera, the results obtained (either positive or negative) agreed with all the assays. However, for 23 (72%) of the sera a mixture of positive and negative results were found in at least four assay methods. The mean difference in the TU

Table 2: **Results of Spearman rank correlations (*R*) for the ELISAs and IFA that were used to detect anti-*Plasmodium falciparum* sporozoite antibodies in the study**

Method	ELISA method			
	$(NANP)_3$-BSA	R32tet32	$(NANP)_{40}$	R32LR
IFA	$n=249$; $R=0.65$; ($P<0.001$)	$n=249$; $R=0.68$; ($P<0.001$)	$n=249$; $R=0.70$; ($P<0.001$)	$n=249$; $R=0.70$; ($P<0.001$)
$(NANP)_3$-BSA ELISA	—	$n=249$; $R=0.66$; ($P<0.001$)	$n=253$; $R=0.61$; ($P<0.001$)	$n=249$; $R=0.74$; ($P<0.001$)
R32tet32 ELISA	—	—	$n=249$; $R=0.87$; ($P<0.001$)	$n=249$; $R=0.86$; ($P<0.001$)
$(NANP)_{40}$ ELISA	—	—	—	$n=249$; $R=0.76$; ($P<0.001$)

Table 3: **Percentage of tests that agreed or disagreed (as expressed by whether they gave "positive" or "negative" results) for four of the assays in the study**

Method		ELISA method					
		$(NANP)_3$-BSA		R32tet32		$(NANP)_{40}$	
		+ve	−ve	+ve	−ve	+ve	−ve
IFA	+ve	*26*[a]	13	*37*	1.9	*35.1*	3.8
	−ve	6.3	*54.8*	35.1	*26*	27.4	*33.7*
$(NANP)_3$-BSA ELISA	+ve			*31.7*	0.5	*31.1*	0.5
	−ve			40.4	*27.4*	30.2	*38.2*
R32tet32 ELISA	+ve					*57.2*	14.9
	−ve					5.3	*22.6*
R32LR ELISA	+ve	*30.8*	1.4	*56.3*	15.9	*48.1*	14.4
	−ve	26.9	*40.9*	1.4	*26.4*	9.6	*27.9*

[a] The results for tests that agreed (either both positive or both negative) are shown in italics.

values of the sera from the mother–umbilical cord pairs was not statistically significant for the R32tet32 ELISA, the R32LR ELISA, the $(NANP)_3$-BSA ELISA, and the IFA (paired Student's *t*-tests); however, this difference was statistically significant for the $(NANP)_{40}$ ELISA (Table 5). This ELISA employs an anti-IgG, IgA, IgM antibody probe, whereas the other ELISAs use anti-IgG antibody probes, and it is possible that the difference could arise because antisporozoite IgM antibodies are detected by the $(NANP)_{40}$ ELISA in the serum samples from mothers, but not in those from umbilical cords.

Discussion

Although Vandenberg et al. demonstrated in 1969 the existence of an antibody response to malaria sporozoites by using the CS precipitation assay (*24*), it was not until the introduction of the more sensitive IFA by Nardin et al. (*15*) that anti-*P. falciparum* sporozoite antibodies could be detected in sera from subjects living in areas that are hyperendemic for malaria. However, because of the difficulty in obtaining the antigen used in this IFA, this technique was of limited use for epidemiological studies (*15, 16, 25, 26*).

The recent availability of synthetic and recombinant peptides from the repetitive domain of the *P. falciparum* CS protein has, however, made it possible to develop relatively simple, sensitive assays for the detection of antibodies against sporozoites. Unlike IFA, such assays require neither a source of sporozoites nor sophisticated equipment and can therefore be used readily in epidemiological research on individuals naturally exposed to malaria infection. They are

Table 4: **Results for the detection of anti-*Plasmodium falciparum* sporozoite antibodies in the 16 pairs of mother–umbilical cord serum samples assayed in the study**

		IFA[a] (reciprocal serum dilution)	ELISA method[b] (in TU)			
			$(NANP)_3$-BSA	R32tet32	$(NANP)_{40}$	R32LR
	M[c]	20	2.61	*23.94*	*16.75*	*17.68*
8	U[d]	20	9.98	*23.94*	6.27	*29.27*
	M	20	−17.26	11.27	*24.22*	*9.76*
28	U	0	−1.49	2.82	6.51	3.05
	M	*1280*	*122.20*	*140.85*	*148.55*	*122.00*
29	U	*1280*	*148.51*	*140.85*	*117.23*	*122.00*
	M	20	2.61	*33.10*	*30.36*	*20.12*
38	U	0	1.49	7.04	5.66	5.49
	M	0	0.84	*14.08*	11.08	3.05
50	U	*40*	1.21	7.04	5.06	3.66
	M	*40*	9.51	*61.97*	*30.48*	*52.44*
55	U	*160*	*21.50*	*50.70*	*28.31*	*37.20*
	M	*40*	*16.42*	*36.62*	*23.37*	*22.56*
116	U	*160*	*29.10*	*34.51*	*18.67*	*25.00*
	M	*40*	13.62	*35.92*	11.33	*21.34*
131	U	20	12.50	*28.87*	12.05	*22.56*
	M	20	4.29	*26.06*	*19.28*	*12.20*
155	U	*40*	6.06	*21.13*	8.67	*14.02*
	M	*640*	*72.11*	*77.46*	*41.93*	*85.37*
164	U	*160*	*64.83*	*73.94*	*41.93*	*70.12*
	M	*160*	*32.46*	*21.83*	*66.99*	7.93
178	U	*160*	*47.29*	*53.52*	*27.47*	*46.95*
	M	*40*	−5.22	*24.65*	*18.07*	*13.41*
290	U	0	−1.40	6.34	8.67	5.49
	M	*40*	*17.44*	*40.14*	*29.88*	*30.49*
300	U	*40*	*15.67*	*40.14*	*24.70*	*25.61*
	M	*40*	*22.57*	*47.89*	*36.75*	*41.46*
319	U	*80*	*28.17*	*59.15*	*19.28*	*51.83*
	M	0	*60.17*	*76.06*	*61.33*	*78.05*
321	U	*40*	*56.62*	*97.18*	*59.40*	*78.05*
	M	20	*18.84*	*78.87*	*47.11*	*32.32*
498	U	0	5.32	*19.72*	*14.34*	−1.83

[a] Positive results are shown in italics.
[b] Positive results (those above the cut-off values) are shown in italics.
[c] M = serum sample from the mother;
[d] U = serum sample from the umbilical cord.

also likely to have a valuable role in trials of antisporozoite vaccines.

One of the aims of the study was to compare the results obtained with ELISAs based on $(NANP)_n$ constructs as well as with those obtained with IFA, which was until recently the only method available for the detection of anti-*P. falciparum* sporozoite antibodies.

All the assays tested showed 100% specificity, based on serum samples from Geneva, since no false-positive results were obtained. It is noteworthy that the presence of high titres of rheumatoid factors and other autoantibodies in sera from individuals with systemic lupus erythematosus or rheumatoid arthritis did not interfere with the specificity of the assays. The reproducibility of the $(NANP)_n$-based ELISAs that were tested (the $(NANP)_3$-BSA, R32tet32, R32LR, and $(NANP)_{40}$ ELISAs) was good, with a coefficient of variation for positive pools of sera that ranged from 3% to 15%. However, the IFA was much less reproducible than the peptide-based ELISAs. This can be ascribed to the element of subjectivity in interpreting the result of the IFA method. When sensitivity was tested by progressively diluting a positive serum, the IFA had the lowest sensitivity, since it detected as positive only the first three dilutions of the positive control serum.

Only 63% and 69% of the results obtained with

Table 5: **Statistical comparison (paired Student's *t*-tests) of the results obtained for the assay of the 16 pairs of mother–umbilical cord serum samples in the study**

Statistical parameter		IFA (log_{10} titre)	ELISA method (in TU)			
			$(NANP)_3$-BSA	R32tet32	$(NANP)_{40}$	R32LR
Mean value	M[a]	1.53	23.33	46.92	38.59	35.63
	U[b]	1.43	27.84	41.68	25.26	33.65
Standard deviation	M	0.80	34.81	33.42	33.59	33.22
	U	0.96	38.28	37.49	28.69	33.63
t value		0.38	−1.81	1.05	4.21	0.51
Two-tail probability		0.711	0.091	0.311	0.001	0.621

[a] M = serum sample for the mother.
[b] U = serum sample from the umbilical cord.

the ELISAs that used the R32tet32 or $(NANP)_{40}$ peptides, respectively, agreed with those obtained with the IFA, whereas those for the ELISA that used the $(NANP)_3$ peptide there was 81% agreement with those for the IFA. The R32tet32 ELISA and $(NANP)_{40}$ ELISA exhibited the highest degree of agreement (80%), and both gave results that agreed less with those of the $(NANP)_3$-BSA ELISA (59% for the R32tet32 ELISA, 69% for the $(NANP)_{40}$ ELISA, and 71% for the R32LR ELISA). Several interpretations of these results are possible.

First, it appears that peptide-based ELISAs are more sensitive than the IFA in detecting anti-*P. falciparum* sporozoite antibodies in human sera. This could be due to the following technical problems inherent in the IFA procedure: sporozoite fixation with glutaraldehyde, storage of the slides, subjective interpretation of the results, etc. It is not known whether IFAs that use unfixed living sporozoites (*26*) give results that exhibit closer agreement with those obtained with peptide-based ELISAs. Alternatively, it cannot be excluded that synthetic and recombinant peptides express new or modified epitopes which may recognize larger, and in some cases non-specific, antibody populations that are not detected by IFA.

Second, the results of the ELISAs based on peptides that consist of a large number of (NANP) repeats (R32tet32, R32LR, and $(NANP)_{40}$) correlated more closely with each other than with the results of the ELISA based on the shorter $(NANP)_3$ peptide, which failed to detect as positive some sera that were positive in the other ELISAs. This could arise because the R32tet32 ELISA and $(NANP)_{40}$ ELISAs are more sensitive for the reasons outlined below.

- The R32tet32, R32LR, and $(NANP)_{40}$ peptides contain a higher number of (NANP) sequences, which could increase the sensitivity of the corresponding assays. However, in this case, the sensitivity of the $(NANP)_3$-BSA ELISA should be increased by raising the concentration of peptide used to coat the micro-titration plates.
- The R32tet32 and R32LR peptides contain two (NVDP) repeats, which may detect specific antibodies that are not detected by synthetic $(NANP)_n$-based ELISAs. However, the results obtained in the ELISAs that use R32tet32 or $(NANP)_{40}$ (which does not contain (NVDP) repeats) are very similar. Only one serum (from Colombia) was highly positive in the R32tet32 ELISA and negative in the $(NANP)_{40}$ ELISA.
- The R32tet32, R32LR, and $(NANP)_{40}$ peptides may express conformational epitopes, i.e., epitopes not simply arising from the primary structure of the amino acid sequence, which may be absent in the shorter $(NANP)_3$ peptide. This would seem to be the most likely explanation for the discrepancies in the results between the $(NANP)_3$-BSA ELISA and the R32tet32 and $(NANP)_{40}$ ELISAs. However, more research is required to define such "conformational" epitopes in the (NANP) sequence and to determine the presence and relevance (if any) of anti-(NVDP) antibodies in the sera of subjects living in areas where *P. falciparum* malaria is endemic.

$(NANP)_n$-based ELISAs for the detection of anti-*P. falciparum* sporozoite antibodies are relatively simple and inexpensive methods that are readily applicable in epidemiological research in the field. Similar methodology is now also being used to detect antibodies against *P. vivax* sporozoites (*27*). Furthermore, the results of the recent analysis of the amino acid sequence of the *P. malariae* CS protein (*28*) should soon permit the development of assays for the detection of anti-*P. malariae* sporozoite antibodies. Such assays could contribute to a better understanding of the natural history of the host–parasite relationships in

the epidemiology of malaria. Finally, the detection of antisporozoite antibodies is of potential value to malaria control programmes, since it provides an index of the degree of malaria transmission (*26*).

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Résumé

Evaluation comparative de trois ELISA employant des peptides répétitifs pour détecter les anticorps dirigés contre les sporozoïtes de *Plasmodium falciparum*

Ces dernières années, on a préparé par génie génétique ou synthèse un certain nombre de peptides constitués de la séquence répétitive $(NANP)_n$ de la protéine circumsporozoïtaire de *Plasmodium falciparum*. Ces peptides ont servi à mettre au point des méthodes immunologiques de détection des anticorps dirigés contre les sporozoïtes de *P. falciparum* dans le sérum humain. Trois méthodes de titrage immuno-enzymatique (ELISA) employant différents peptides $(NANP)_n$ (les peptides synthétiques $(NANP)_3$ et $(NANP)_{40}$ et les peptides recombinants R32tet32 et R32LR) ont fait l'objet d'une étude comparative au cours de laquelle on a analysé des échantillons de sérums d'individus vivant dans différentes régions où le paludisme est endémique. Les résultats de ces ELISA ont été comparés à ceux obtenus par une technique d'immunofluorescence (IFA) utilisant des sporozoïtes fixés par le glutaraldéhyde. Toutes les méthodes ont montré une spécificité de 100% sur les sérums de personnes n'ayant jamais été exposées au paludisme, une bonne reproductibilité (coefficient de variation compris entre 3% et 15% pour les ELISA) et une bonne sensibilité. La reproductibilité et la sensibilité ont été plus faibles pour l'IFA que pour les ELISA, peut-être en raison de l'élément subjectif inhérent à l'interprétation des résultats de la méthode IFA. La corrélation des résultats a été meilleure entre les ELISA fondés sur l'utilisation de peptides comprenant un grand nombre de séquences (NANP), c'est-a-dire $(NANP)_{40}$, R32tet32 et R32LR, qu'entre ceux-ci et l'ELISA fondé sur l'utilisation du peptide plus court $(NANP)_3$.

Les méthodes ELISA fondées sur l'utilisation des peptides $(NANP)_n$ constituent un moyen relativement simple et peu coûteux de détecter les anticorps dirigés contre les sporozoïtes de *P. falciparum* et sont facilement applicables à la recherche épidémiologique sur le terrain. Ces essais pourraient aider à mieux comprendre l'histoire naturelle de la relation hôte-parasite dans l'étude du paludisme.

References

1. **Cochrane, A.H. et al.** Immunization against sporozoites. In: Kreier, J.P., ed. *Malaria*, vol. 3. New York, Academic Press, 1980, pp. 163–202.
2. **Nussenzweig, V. & Nussenzweig, R.S.** Circumsporozoite proteins of malaria parasites. *Cell*, **42:** 401–403 (1985).
3. **Zavala, F. et al.** Circumsporozoite proteins of malaria parasites contain a single immunodominant region with two or more identical epitopes. *Journal of experimental medicine*, **157:** 1947–1957 (1983).
4. **Zavala, F. et al.** Rationale for the development of a synthetic vaccine against *Plasmodium falciparum* malaria. *Science*, **228:** 1436–1440 (1985).
5. **Dame, J.B. et al.** Structure of the gene encoding the immunodominant surface antigen on the sporozoite of the human malaria parasite *Plasmodium falciparum*. *Science*, **225:** 593–599 (1984).
6. **Enea, V. et al.** DNA cloning of *Plasmodium falciparum* circumsporozoite gene: amino acid sequence of repetitive epitope. *Science*, **225:** 628–630 (1984).
7. **Lockyer, M.J. & Schwarz, R.T.** Strain variation in the circumsporozoite gene of *Plasmodium falciparum*. *Molecular and biochemical parasitology*, **22:** 101–108 (1987).
8. **De la Cruz, V.F. et al.** Sequence variation in putative functional domains of the circumsporozoite protein of *Plasmodium falciparum*. Implications for vaccine development. *Journal of biological chemistry*, **262:** 11935–11939 (1987).
9. **Del Portillo, H.A. et al.** Circumsporozoite gene of a *Plasmodium falciparum* strain from Thailand. *Molecular and biochemical parasitology*, **24:** 289–294 (1987).
10. **Young, J.F. et al.** Expression of *Plasmodium falciparum* circumsporozoite protein in *Escherichia coli* for potential use in a human malaria vaccine. *Science*, **228:** 958–962 (1985).
11. **Ballou, W.R. et al.** Immunogenicity of synthetic pep-

tides from circumsporozoite protein of *Plasmodium falciparum. Science*, **228**: 996–999 (1985).
12. **Ballou, W.R. et al.** Safety and efficacy of a recombinant DNA *Plasmodium falciparum* sporozoite vaccine. *Lancet*, **1**: 1277–1281 (1987).
13. **Herrington, D.A. et al.** Safety and immunogenicity of a synthetic peptide malaria vaccine against *Plasmodium falciparum* sporozoites. *Nature*, **328**: 257–259 (1987).
14. **Etlinger, H.M. et al.** Assessment of a synthetic peptide-based vaccine against the sporozoite stage of the human malaria parasite *Plasmodium falciparum. Journal of immunology*, **140**: 626–633 (1988).
15. **Nardin, E.H. et al.** Antibodies to sporozoites: their frequent occurrence in individuals living in an area of hyperendemic malaria. *Science*, **206**: 597–599 (1979).
16. **Tapchaisri, P. et al.** Anti-sporozoite antibodies induced by natural infection. *American journal of tropical medicine and hygiene*, **32**: 1203–1208 (1983).
17. **Hoffman, S.L. et al.** Immunity to malaria and naturally acquired antibodies to the circumsporozoite protein of *Plasmodium falciparum. New England journal of medicine*, **315**: 601–606 (1986).
18. **Zavala, F. et al.** Synthetic peptides for the detection of humoral immunity to *Plasmodium falciparum* sporozites. *Journal of immunological methods*, **93**: 55–61 (1986).
19. **Del Giudice, G. et al.** Detection of human antibodies against *Plasmodium falciparum* sporozoites using synthetic peptides. *Journal of clinical microbiology*, **25**: 91–96 (1987).
20. **Del Giudice, G. et al.** Antibodies to the repetitive epitope of *Plasmodium falciparum* circumsporozoite protein in a rural Tanzanian community: a longitudinal study of 132 children. *American journal of tropical medicine and hygiene*, **36**: 203–212 (1987).
21. **Campbell, G.H. et al.** Detection of antibodies in human sera to the repeating epitope of the circumsporozoite protein of *Plasmodium falciparum* using the synthetic peptide $(NANP)_3$ in an enzyme-linked immunosorbent assay (ELISA). *American journal of tropical medicine and hygiene*, **37**: 17–21 (1987).
22. **Webster, H.K. et al.** Development of immunity in natural *Plasmodium falciparum* malaria: antibodies to the falciparum sporozoite vaccine 1 antigen (R32tet32). *Journal of clinical microbiology*, **25**: 1002–1008 (1987).
23. **Hoffman, S.L. et al.** Naturally acquired antibodies to sporozoites do not prevent malaria: vaccine development implications. *Science*, **237**: 639–642 (1987).
24. **Vandenberg, J.P. et al.** Protective immunity produced by the injection of X-irradiated sporozoites of *Plasmodium berghei*. V. *In vitro* effects of immune serum on sporozoites. *Military medicine*, **134** (suppl.): 1183–1190 (1969).
25. **Tapchaisri, P. et al.** Antibodies against malaria sporozoites in patients with acute uncomplicated malaria and in patients with cerebral malaria. *American journal of tropical medicine and hygiene*, **34**: 831–836 (1985).
26. **Druilhe, P. et al.** Levels of antibodies to *Plasmodium falciparum* sporozoite surface antigens reflect malaria transmission rates and are persistent in the absence of reinfection. *Infection and immunity*, **53**: 393–397 (1986).
27. **Lee, M. et al.** Interaction of Malaysian sera with *Plasmodium vivax* sporozoite antigen. *American journal of tropical medicine and hygiene*, **39**: 535–539 (1988).
28. **Lal, A.A. et al.** Structure of the circumsporozoite protein gene of *Plasmodium malaria. Molecular and biochemical parasitology*, **30**: 291–294 (1988).