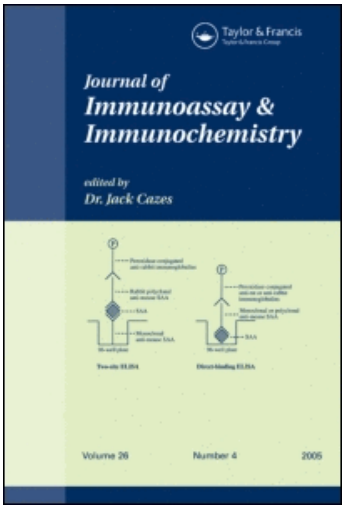

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A RAPID INHIBITION MICRO ELISA FOR DETECTING ANTIBODIES TO PLASMODIUM FALCIPARUM SPOROZOITES IN HUMAN BLOOD

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ABSTRACT

A sensitive and specific micro ELISA, named MONOPLATE ELISA, for the detection of antibodies against P. falciparum sporozoites was developed. It can be applied to many kinds of samples including serum, plasma, whole blood, eluted bloodspot and mosquito bloodmeal as well. The method makes use of a single microtiter plate and the chemically synthesized $(Asn\text{-}Ala\text{-}Asn\text{-}Pro)_{20}$ ($NANP_{20}$) antigen both as coating material and as competitive (binding) inhibitor in the samples. The specific value of each sample is obtained as the absorbance difference between the uninhibited and the fully inhibited sample. Using appropriate conditions, the results can be evaluated by simple visual inspection of the plate, without any instrument. A rapid procedure, where the incubation times for sample and conjugate are just 15 minutes, is also described. When unknown samples from a P. falciparum endemic area were tested, a close correlation was found between our results and those obtained with the only commercial ELISA kit now available (Sclavo S.p.A.). For screening purposes, as many as 48 samples per plate can be tested by this method.

(KEY WORDS: Inhibition ELISA, Rapid ELISA, Synthetic Peptide, Malaria, anti-sporozoite Antibodies, P. falciparum).

INTRODUCTION

It is well known (1,2) that the peptide NANP is present as the tandemly repeated sequence $NANP_n$ (n=37) in the circumsporozoite protein (CSP), the major surface antigen of the P. falciparum sporozoites. Chemically synthesized NANP polymers (1-6) as well as NANP repeats prepared by recombinant DNA technology (7) have been used as antigens for the detection of antisporozoite antibodies.

More recently, a micro ELISA employing a $NANP_{40}$ coated plate has been developed (5) and implemented into a commercial kit (ELISA malaria Ig) by Sclavo S.p.A., Siena (Italy) (8).

In the present study we report a specific, sensitive and rapid micro ELISA (MONOPLATE ELISA) which is based on the principle of full competitive inhibition and makes use of the synthetic peptide $NANP_{20}$.

MATERIALS AND METHODS

Chemicals, Reagents and Instrumentation

All chemicals were reagent grade. Casein, according to Hammarsten, was from Merck; Triton X-100 was from Fluka; Thimerosal, grade II, and 3,3',5,5'-tetramethylbenzidine (TMB) were from Sigma Chem. Co.; 2,2',azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), diammonium salt, was from Boehringer Mannheim GmbH; p-nitrophenylphosphate (NPP), alkaline phosphatase (AP) conjugate (Cat. No. 172-1004) and horseradish peroxidase (HRP) conjugate (No. 172-1050) of goat anti-human IgG

(H + L) were from Bio-Rad Laboratories; HRP conjugate (No. 61-204-1) of rabbit anti-mouse IgG was from ICN Immunobiologicals. $NANP_n$ polypeptides were prepared as previously described (9). Anti-NANP monoclonal antibody (MAb) was a gift from Dr. Fulvio Esposito, Università di Camerino (Italy). Flat bottom 96-well polystyrene or polyvinylchloride microtiter plates were: Dynatech M129A, M129B and M29 from Dynatech Laboratories; Nunc immunoplate IF No. 43454; Linbro No. 7638104 from Flow S.p.A. Milan, Italy; Falcon No. 3070 and Falcon Flex No. 3912 from Falcon Labware, Becton Dickinson, N.J.; Costar No. 224-0096 from Bio-Rad Laboratories; Greiner, from Sial, Rome, Italy. The automatic ELISA washer "Easy washer II" was from Sclavo S.p.A., Siena, Italy. The "Lambda Automatic Microtiter Reader" was from Perkin Elmer.

Samples

Negative serum, plasma and blood samples were obtained from healthy donors in Rome. African sera were collected in the Ouagadougou area (Burkina Faso) in July 1986, bloodspots in the Comores islands during November-December 1988. Each bloodspot consisted of 10 µl of human blood collected by finger puncture, absorbed on Whatman No. 4 filter paper, dried at room temperature and stored at -20°C in the presence of a solid desiccant until use. Just before testing, the bloodspots were eluted from the paper with the eluent buffer at room temperature for one hour. Three mosquito bloodmeal spot samples were prepared by squashing

the mosquito midgut on filter paper; the air dried spots were stored as described above for the human bloodspots.

Buffers

The following buffers were used in the Monoplate ELISA. Coating buffer: Tris buffered saline (TBS), pH 7.8, 25 mM Tris-HCl, 0.15 M NaCl. Eluent/diluent buffer: TBS, pH 7.8, containing 0.5% casein, 0.05% Triton X-100 and 0.005% Thimerosal, prepared by diluting before use a boiled four-fold concentrated stock solution stored at $4^{o}C$. Washing buffer: TBS, pH 7.8, containing 0.05% Triton X-100 and 0.005% Thimerosal, prepared by diluting before use a four-fold concentrated stock solution.

Enzyme Substrate Solutions

For the TMB substrate solution (HRP conjugate) the two solutions, A (stock solution) and B,

0.1 M citric acid containing 1 mg/ml TMB (A)

0.1 M acetate buffer, pH 5.0, containing 1.4 mM H_2O_2 (B)

were mixed (1 volume of A and 9 volumes of B) just before use; the pH of the mixture was around 4.6. The stock solution A was found completely stable after four weeks at $4^{o}C$ and gave the same results obtained with 10 mg/ml TMB in dimethylsulfoxide (DMSO), the stock solution used by Bos (10). For the ABTS substrate solution (HRP conjugate), a 0.1 M acetate buffer, pH 5.0, containing 1.1 mg/ml ABTS and 1.3 mM H_2O_2 was used. As substrate solution for the alkaline phosphatase (AP conjugate), both buffer

and NPP substrate from the Bio-Rad enzyme kit or from the Sclavo malaria kit were used.

Sclavo ELISA kit

This kit, (from Sclavo S.p.A., Siena, Italy), labelled "ELISA malaria Ig", consists of two modular 96-well Nunc plates: one plate coated with $NANP_{40}$ and postcoated with bovine serum albumin (BSA), one control plate coated only with BSA. According to the manufacturer's instructions, the following conditions were generally used: sera dilution 1:100; bloodspots dilution 1:80; AP conjugate dilution 1:50; 60 minutes for each of the three incubations (sample, conjugate, substrate); cutoff = 0.100.

Monoplate ELISA Procedure

Unless specified, all the operations were carried out at room temperature.

Coating of the plate. The plate was coated with $NANP_{20}$ by incubating overnight in a moist chamber with 0.1 ml/well of 1 /ug/ml $NANP_{20}$ solution in the coating buffer. After two washings with the coating buffer, the plate was dried at 37°C for one hour, then sealed with an adhesive cover sheet and stored at 4°C (Precoated plate). When saturating blocking was applied (not necessary for the Dynatech M129A and Linbro Flow plates) the plate, after the overnight incubation with $NANP_{20}$ and the two washings as above, was further incubated overnight with 0.2 ml/well of coating buffer containing 0.1% BSA or casein. Then the plate was washed twice with the coating buffer, dried and stored

as above. The precoated plates were stable and could be used without any loss of performance more than 2 months after coating.

Washing of the precoated plate. In order to remove loosely bound antigen, a short incubation/washing of the precoated plate with diluent buffer was included in the procedure. Therefore, 0.2 ml of diluent buffer were added to each well and discarded after a minimum of 10 minutes; a quick second washing with the same buffer was done.

Dispensing of the inhibitor $NANP_{20}$. The following three schemes, among the many possible for dispensing the samples in the plate, were used (Fig. 1). Excluding the first column (A1-H1) of the plate, reserved for the substrate solution (for blanking in the final reading of the plate), 0.01 ml/well of 0.1 mg/ml $NANP_{20}$ solution in diluent buffer, were dispensed into the rows C,D,G,H (Fig. 1A: samples tested in duplicate; reading of the plate against the substrate solution; 22 samples/plate). Alternatively, 0.01 ml/well of $NANP_{20}$ solution were put into all the wells of the rows B,D,F,H (Fig.1B: samples tested in duplicate; reading of the plate against air; 24 samples/plate). When a large number of samples had to be screened, the inhibitor solution was put into the rows B,D,F,H (Fig. 1C: samples tested in single; reading of the plate against air; 48 samples/plate). The key feature of every scheme is that the wells containing the uninhibited and the inhibited sample are adjacent to each other, thus enabling for easy evaluation of results by simple visual inspection of the plate.

Sample incubation. 0.1 ml aliquots of each diluted sample (serum and plasma diluted 1:200 if the TMB substrate was used, 1:100 if the ABTS substrate was used; bloodspot diluted 1:80) were put into four adjacent wells (sample assayed in duplicate). After 60 minutes of incubation, the plate was washed four times with 0.2 ml/well of washing buffer.

Conjugate incubation. 0.1 ml of the diluted conjugate were added to each well (except for the first column wells if the scheme of Fig. 1A was employed). After 60 minutes, the plate was washed four times with 0.2 ml/well of washing buffer.

Substrate incubation. 0.1 ml/well of TMB or ABTS (for HRP conjugate) or NPP (for AP conjugate) substrate solution were added to all wells. The TMB reaction was stopped at 10 minutes by adding 0.1 ml/well of 0.5 M H_2SO_4 solution. The ABTS reacting plate was read at 60 minutes. The NPP reaction was stopped at 60 minutes by adding 0.025 ml/well of 3N NaOH.

Evaluation of results. The plate was read for absorbance (A) at 450 nm for TMB, 405 nm for ABTS and NPP. The specific value of each sample was given by the difference:

$$A \text{ spec.} = A \text{ (uninhib.)} - A \text{ (inhib.)} = \Delta A$$

A sample was considered positive when satisfying the following two criteria:

a) $\Delta A > 0.100$, the cutoff value (see below)

b) $\Delta A / A \text{ (uninhib.)} \times 100 > 50$

i.e. we did not consider positive samples those where less than 50% of the absorbance appeared to be specific.

(A)

	1	2	3	4	5	6	7	8	9	10	11	12
A	b	1										
B	=	1										
C	=	1i										
D	=	1i										
E	=	2										22
F	=	2										22
G	=	2i										22i
H	=	2i										22i

(B)

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1										
B	1i	1i										
C	2	2										
D	2i	2i										
E											23	23
F											23i	23i
G											24	24
H											24i	24i

(C)

	1	2	3	4	5	6	7	8	9	10	11	12
A	1											
B	1i											
C	2											
D	2i											
E												47
F												47i
G												48
H												48i

Positive samples could also be detected by visual inspection of the plate. The difference in colour was particularly evident when the HRP conjugate and the TMB or ABTS substrates were used: blue colour for the unstopped TMB reaction; brilliant yellow colour for the H_2SO_4 stopped TMB reaction; green-blue colour for ABTS. Comparison between visual reading of the plate, performed by different observers, and instrumental reading, always gave identical results (as Pos./Neg. value assignement) (data not shown).

Rapid Monoplate ELISA procedure

The procedure for the rapid assay did not vary much from that for the normal assay. Higher concentrations for samples and HRP conjugate were used: sera diluted 1:100 instead of 1:200, conjugate diluted 1:500 instead of 1:2,000. TMB was used as substrate. Incubation times for sample and conjugate were 15 minutes each, so that the three incubations (sample, conjugate and substrate) lasted just 40 minutes (15+15+10) instead of the 130 minutes (60+60+10) requested by the related normal test.

FIGURE 1. Three possible ways of sample distribution in a 96-well microtiter plate in the Monoplate ELISA.

(A) Each sample tested in duplicate; reading of plate against a blank substrate (b) as zero; 22 samples per plate; i, inhibited ($NANP_{20}$) well.

(B) Each sample tested in duplicate; zeroing of the Microtiter Reader against air; 24 samples per plate; i, as in (A).

(C) Each sample tested in single; zeroing against air; 48 samples per plate; i, as in (A).

RESULTS

Choice of buffers and plate

In preliminary trials with the Dynatech M129A plate, both PBS and TBS buffers, pH 7.8, showed to be equally good coating buffers for $NANP_{20}$. TBS, pH 7.8, containing 0.5% casein, 0.05% Triton X-100 and 0,005% thimerosal, performed better as eluent-diluent buffer for samples and conjugates; TBS, pH 7.8, containing 0.05% Triton X-100 and 0.005% thimerosal performed better as washing buffer; they gave higher specific absorbance values (Δ A) for positive sera and lower nonspecific background (NSB) values, in comparison with the PBS based buffers. Therefore the TBS based buffers were chosen for the Monoplate ELISA.

The hemolysing detergent Triton X-100 in the eluent/diluent buffer did not cause any background problem when using whole blood: in fact NSB values for whole blood and its corresponding serum were practically identical. Some plates of different kinds available to us were checked in the Monoplate ELISA by using the optimized TBS buffer system. Judgement took into account both the specific values (Δ A) obtained for positive sera and the NSB values. As a result, two plates, the Dynatech M129A and the Linbro Flow, could be used successfully without blocking with BSA or casein. Other plates (Dynatech M129B and M29, Nunc IF, Greiner and Falcon Flex) gave good Δ A values for positive sera but needed BSA or casein postcoating in order to lower the too elevated NSB values (data not reported). The Dynatech M129A plate was thus selected for use in the Monoplate ELISA.

While preparing this manuscript a paper (10) appeared, confirming that the postcoating step with a blocking agent, though usually performed, is not always needed in microtiter ELISA.

Inhibition by $NANP_{20}$

In order to establish the optimal $NANP_{20}$ concentration to achieve complete inhibition of antisporozoite antibody binding to the $NANP_{20}$ coated plate, two high positive sera were tested at $NANP_{20}$ concentrations varing from 0.035 to 36 /ug/ml, i.e. from 3.9 ng to 4 /ug /well; Fig. 2 reports the inhibition curve for one serum. The minimum amount of $NANP_{20}$ which gave 100% inhibition results 1 /ug/well. This amount was thus chosen and used throughout the work.

Positivity threshold

Negative samples of 50 sera, 44 plasma and 44 whole bloods, were assayed employing either HRP conjugate (TMB and ABTS as substrates) or AP conjugate (NPP as substrate) in order to determine the positivity threshold value (cutoff) to apply to unknown samples for the estimation of the results. For both the normal and the rapid assay a cutoff value of 0.100, above the mean net absorbance ΔA plus three standard deviations, was established for all types of conjugates (HRP and AP) and samples (serum, plasma and blood).

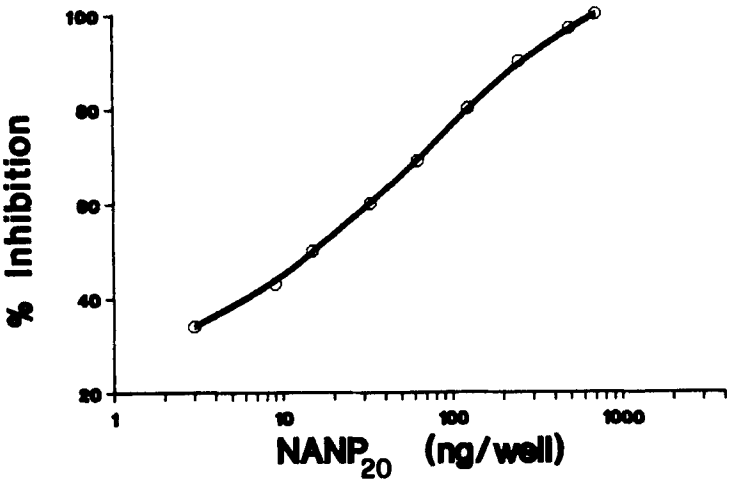

FIGURE 2. Inhibition of antisporozoite antibodies binding to the $NANP_{20}$ coated plate by increasing amounts of free $NANP_{20}$. A high positive serum (dil. 1:100), HRP conjugate (dil. 1:2,000) and ABTS as substrate were used.

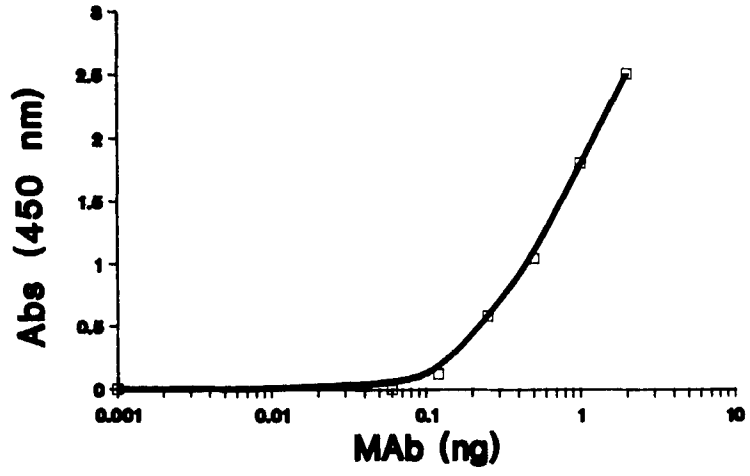

FIGURE 3. Standard curve for anti-NANP monoclonal antibody (MAb) by the Monoplate ELISA. MAb ranging from 0.0625 to 2 ng/well was dissolved in negative bloodspot eluate (1:80 blood dilution). The normal procedure (60, 60 and 10 minutes incubations) was applied using a HRP conjugate of rabbit anti-mouse IgG and TMB as substrate.

Sensitivity

To estimate the sensitivity of the Monoplate ELISA, MAb in the 0.062 - 2 ng/well range and rabbit anti-mouse IgG-HRP conjugate (diluted 1:1,000) were used. MAb solutions were prepared by dissolving MAb in a negative blood pool obtained from bloodspots of 6 African individuals, eluted together at 1:80 dilution. The results (Fig. 3) show that as low as 0.125 ng of MAb (0.1 ng/μl of blood) is the minimum amount of antibodies detectable by this assay.

Assay variation

In order to determine the assay variation, a pool of several positive sera, whose ΔA value was around 1.000, was repeatedly assayed (four times on different days) by using the HRP conjugate and TMB as substrate in the normal Monoplate ELISA. The intra-plate and plate-to-plate coefficients of variation (CV%) were 7.0 and 14.1, respectively.

African Sera and Bloodspots

Previously untested African sera were assayed by the normal Monoplate ELISA, using both the HRP conjugate (TMB or ABTS as substrates) and the AP conjugate (NPP as substrate), as well as by the Sclavo ELISA Kit. Results are reported in Table 1. A good correlation can be noticed between the data of the two methods. Absolute concordance (as positive/negative results) exists between the Monoplate ELISA using the HRP conjugate and the Sclavo kit (first, second and fourth columns). The HRP conj/TMB

TABLE 1

Results for African Sera Assayed by Two Methods: (a) Monoplate ELISA (HRP conjugate, TMB and ABTS as substrates; AP conjugate, NPP as substrate); (b) Sclavo kit (AP conjugate, NPP as substrate). Sera dilution was 1:100, except for the HRP conj./TMB ELISA (1:200). Conjugates dilutions: 1:2,000 for the Monoplate ELISA; 1:50 for the Sclavo kit (according to the manufacturer's instructions).

Sera	Monoplate ELISA			Sclavo Kit
(No.)	HRP conjugate (TMB)	HRP conjugate (ABTS)	AP conjugate (NPP)	AP conjugate (NPP)
	ΔA_{450}	ΔA_{405}	ΔA_{405}	ΔA_{405}
NC	0.010	-0.029	0.005	0.002
PC	1.500	0.970	0.310	1.450
23	0.654	0.317	0.124	0.439
24	0.302(+)	0.122(+)	0.082(-)	0.209(+)
26	0.326(+)	0.140(+)	0.080(-)	0.366(+)
29	0.478	0.321	0.114	0.841
33	2.465	1.502	0.508	2.105
34	0.S.	2.086	1.276	0.S.
37	1.000	0.781	0.336	0.841
38	0.123(+)	0.098(±)	0.070(-)	0.085(-)
40	2.635	2.091	0.848	2.461
43	1.765	1.009	0.392	1.835
58	0.353(+)	0.123(+)	0.064(-)	0.259(+)
61	0.057	0.015	0.016	0.035
65	0.029	0.048	0.014	0.036
74	0.S.	2.632	1.384	2.091
81	0.002	-0.020	0.006	0.005
84	0.S.	0.S.	1.546	0.S.
89	1.168	0.724	0.276	0.753
94	1.296	0.695	0.258	1.124
104	2.000	1.143	0.590	1.844
113	0.537	0.140	0.132	0.335

NC and PC, negative and positive control sera; 0.S., out of scale; (+) (-) (±), positive, negative and borderline (only discordant sera are indicated); cutoff= 0.100.

assay (first column) shows the greater sensitivity, considering the high ΔA values obtained with half amount of sample (sera dilution 1:200; 1:100 for the other assays). A lower sensitivity is noticed for the assay with the AP conjugate (third column), where many positive samples (No. 24,26,38 and 58) appear negative.

In order to determine the recovery of positivity from a bloodspot, simulated bloodspot samples were prepared by absorbing and drying at room temperature on Whatman No. 4 filter paper either (a) equal volumes of a positive serum and pelletted human red cells or (b) one volume of a positive serum and two volumes of a negative whole blood. After seven days at $4^{\circ}C$ in a desiccator, the spots were eluted for one hour at room temperature, at a 1:100 dilution for positive serum, and tested by Monoplate ELISA using the HRP conjugate and ABTS as substrate. The mean bloodspot/serum net absorbance ratios obtained for the two types of simulated samples were 0.85 and 0.87 respectively.

Bloodspot samples from 20 African individuals were tested by the normal Monoplate ELISA (HRP conjugate and TMB or ABTS as substrate) and by the Sclavo ELISA kit. The results (Table 2) show a very good correlation (positive/negative results) between the two methods. The only discordance concerns the sample No.104, which is low positive (cutoff = 0.100) only by the Sclavo kit. Unfortunately, no retesting was possible due to lack of further sample.

TABLE 2

Results for Bloodspot Samples Assayed as for Table 1, with the Omission of the AP conj./NPP Monoplate ELISA. Blood dilution, 1:80.

Bloodspots	Monoplate ELISA		Sclavo Kit
(No.)	HRP conjugate		AP conjugate
	(TMB)	(ABTS)	(NPP)
	ΔA_{450}	ΔA_{405}	ΔA_{405}
NC	0.011	0.002	0.010
PC	1.400	0.550	0.347
104	0.059(-)	0.020(-)	0.121(+)
105	0.037	-0.013	-0.028
107	0.032	-0.019	0.051
108	0.006	-0.004	-0.017
109	0.042	0.001	-0.004
110	0.064	0.016	0.053
111	0.171	0.101	0.151
112	0.311	0.123	0.177
113	1.394	0.553	1.176
115	1.654	0.879	1.075
116	0.375	0.135	0.161
117	0.012	0.019	-0.040
118	0.052	0.034	0.002
119	0.048	0.026	-0.002
120	0.069	0.041	0.050
121	0.009	0.015	0.013
122	0.022	0.019	0.060
123	0.010	0.018	n.d.
126	0.050	0.012	0.036
127	0.635	0.541	0.307

NC and PC, negative and positive control sera; n.d., not determined; (+) (-), positive and negative (only discordant sera are indicated); cutoff= 0.100.

Mosquito bloodmeal spots

The three dried spots were eluted together for one hour at 4°C with 0.6 ml of eluent buffer containing 1 mM phenylmethylsulfonilfluoride (PMSF) as proteinase inhibitor. The eluate was assayed by the Monoplate ELISA using the HRP conjugate and ABTS as substrate. A specific value $\Delta A = 0.300$, well beyond the positivity threshold, was obtained, thus proving the applicability of this ELISA to such kind of samples.

Rapid Monoplate ELISA

Preliminary experiments (data not reported) proved that the rapid Monoplate ELISA was possible only by using a high quality HRP conjugate and TMB as substrate: with the ABTS substrate (HRP conj.) or the AP conj./NPP substrate many low positive samples resulted negative.

A number of sera and bloodspots, already tested by the normal Monoplate ELISA and the Sclavo kit, were then assayed also by the rapid Monoplate ELISA. The specific values (ΔA) obtained are reported in Table 3, where the values by the normal assay are also reported for comparative purposes. A complete correlation (positive/negative results) for both sera and bloodspots between the rapid and the normal assay can be observed.

Epidemiological results

In a preliminary study, the frequency of individuals positive for anti-NANP antibodies was analyzed in an area endemic

TABLE 3

Comparison of the Results obtained for Sera and Bloodspots by the "Rapid" and "Normal" Monoplate ELISA. Sera dilutions: 1:100 (Rapid assay); 1:200 (Normal assay). Blood dilution: 1:80 for both assays. HRP conjugate dilutions: 1:500 (Rapid assay); 1:2,000 (Normal assay). Substrate, TMB. (Normal assay values taken from Tables 1 and 2).

Sera	ΔA_{450}		Bloodspots	ΔA_{450}	
(No.)	Rapid assay	Normal assay	(No.)	Rapid assay	Normal assay
NC	0.013	0.005	NC	0.020	0.011
PC	1.100	1.500	PC	1.080	1.400
23	0.478	0.654	104	0.027	0.059
24	0.334	0.302	105	0.007	0.037
26	0.364	0.326	107	0.009	0.032
29	0.703	0.478	108	0.001	0.006
33	1.992	2.465	109	0.024	0.042
34	2.277	O.S.	110	0.030	0.064
37	1.035	1.000	111	0.158	0.171
38	0.202	0.123	112	0.197	0.311
40	2.470	2.635	113	1.806	1.394
43	1.885	1.765	115	1.493	1.654
58	0.391	0.353	116	0.137	0.375
61	0.054	0.057	117	0.001	0.012
65	0.043	0.029	118	0.019	0.052
74	2.426	O.S.	119	0.016	0.048
81	-0.017	0.001	120	0.033	0.069
84	2.544	O.S.	121	-0.002	0.009
89	0.891	1.168	122	0.003	0.022
94	1.109	1.296	123	0.026	0.010
104	1.698	2.000	126	0.027	0.050
113	0.488	0.537	127	0.288	0.635

NC and PC, negative and positive control sera; cutoff = 0.100.

TABLE 4

Detection of Antisporozoite Antibodies in Bloodspot eluates from 481 Individuals living in an Endemic Area (Comores) by the Normal Monoplate ELISA (HRP conjugate and TMB as substrate).

Subjects	Age (yrs)	No. positive/ no. tested (%)
African individuals	<5	0/5 (0)
from endemic area	5-10	116/248 (26.8)
(Comores Islands)	11-20	37/72 (51.4)
	21-30	38/49 (77.5)
	31-40	38/47 (80.1)
	>40	45/60 (75.0)

for P. falciparum malaria. 481 individual samples were randomly selected among a collection of 2541 blood samples, representative of all age and sex groups, done in 21 rural villages of the three Comores Islands. The specimens, collected during Nov-December 1988 by finger puncture, were stored dried on filter paper at -20°C for a period of 15 months before testing. The frequency of positive subjects increased with age, reaching 80% in individuals more than 30 years old (Table 4).

A more complete study of the epidemiological situation in Comores Islands will be reported elsewhere (12).

DISCUSSION

The indirect micro ELISA for antibody detection is now a well established technique. It has been applied by several

researchers for the detection of P.falciparum antisporozoite antibodies in human sera (5,6,13). All these and other studies employ a classical ELISA using two microtiter plates, one coated with the antigen and the other with an inert protein like BSA. The use of two plates implies at least two potential disuniformities, one due to the plate itself and the other to the adsorbed material, possibly resulting in both false negative and false positive values.

The method described in the present paper overcomes these problems by abolishing both causes of risk: only specific binding is taken into account for each sample, i.e. the one that can be inhibited by a saturating amount of the same antigen used for the coating ($NANP_{20}$); a single coating operation (no postcoating blocking) is done with $NANP_{20}$; the inhibited and uninhibited samples are in the same plate, adjacent to one another. Besides avoiding comparison between different plates, this enables the operator to evaluate the results "by eye", without use of any instrument. An additional cause of potential troubles, blocking, is also eliminated in the Monoplate ELISA (attention should be paid to using the right plate, e.g. Dynatech M129A). A 1/ug/ml $NANP_{20}$ solution gave the best results for coating of the plate; comparable results were obtained when $NANP_n$ polymers with different number of repeats ($20 < n < 50$) were used (data not shown). 1 /ug/well (9 /ug/ml) of the same peptide was sufficient to completely inhibit the highest positive sera available to us. It

should be noticed that $NANP_n$ polymers can be prepared in large quantities with a very efficient and simple procedure (9) and that the amount employed in the present test, though higher than the one used in more classical $NANP_n$ (or similar antigens) based assays, in no way limits the potential widespread applicability of the Monoplate ELISA. The optimized normal procedure of the present method makes use of the HRP conjugate and TMB as substrate, though the ABTS substrate may also be used. In these conditions, a very high sensitivity is obtained, i.e. 0.1 $/$ug of antibody/ml of whole blood.

A rapid test for screening purposes was also developed: the incubation times for sample, conjugate and substrate were 15, 15 and 10 minutes, respectively (instead of the 60, 60 and 10 minutes for the normal assay). In this case, it was necessary to use a high quality, affinity purified, HRP conjugate and the more sensitive TMB substrate; optimal dilutions were 1:100, 1:80 and 1:500 for sera, blood and conjugate, respectively.

As the only commercially available Kit for P. falciparum antisporozoite antibody detection was the Sclavo "ELISA malaria Ig" (Sclavo SpA, Siena, Italy), we took it as a standard for comparison with our Monoplate ELISA: as can be seen from Tables 1 and 2, a very good correlation of positive/negative results exists. This is not surprising, since both tests are based on the same antigen, the synthetic polypeptide $NANP_n$, though fractions

with different average molecular weights are used in the two cases ($NANP_{20}$ in our Monoplate ELISA, $NANP_{40}$ in the Sclavo kit).

The epidemiological significance of the Monoplate ELISA (performed according to the scheme of Fig.1C) was established by analysis of 481 African bloodspot samples from the Comores islands area. The frequency of positive subjects increased with age, reaching 80% in individuals more than 30 years old. This finding is in agreement with previous studies conducted in other P. falciparum endemic areas (6,14) and demonstrates that bloodspot analysis is as effective as serum analysis for the evaluation of antisporozoite antibody positivity in populations living in endemic areas. Given the easiness of sampling in the former case, application of this assay (and/or similar ones) to epidemiological studies should prove very useful.

Finally, as each sample has its own control in the form of the inhibited well, it is possible, for mass screening purposes, to dispense with the duplicate readings and to test as many as 48 samples per plate. If one considers the economical constraints of malaria control programs in many underdeveloped countries, this possibility looks especially attractive.

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