TWO-SITE SANDWICH ELISA FOR DETECTION OF *PLASMODIUM VIVAX* BLOOD STAGE ANTIGENS USING MONOCLONAL AND POLYCLONAL ANTIBODIES

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Abstract. Two systems of sandwich enzyme-linked immunosorbent assay (ELISA), a two-site monoclonal antibody sandwich ELISA MAb-MAb sandwich ELISA) and a two site polyclonal-monoclonal antibody sandwich ELISA (PAb-MAb sandwich ELISA) for the detection of *Plasmodium vivax* antigens were developed. The assays showed good correlation with the level of parasitemia when tested against serially diluted *P. vivax* parasites ($r = 0.937$, and 0.997 for MAb-MAb and PAb-MAb sandwich ELISA, respectively), with the ability to detect as few as 6.68 parasites/10^6 erythrocytes and 2.69 parasites/10^3 erythrocytes, respectively. The MAb-MAb sandwich ELISA was specific, since it was positive only with *P. vivax*-infected erythrocytes from vivax malaria patients and negative when erythrocytes from 34 healthy individuals and 30 falciparum malaria cases were tested. In contrast, cross-reaction was found in the PAb-MAb sandwich ELISA when the plates were coated with polyclonal IgG and tested against the serially diluted *P. falciparum* SO strain antigen prepared from *in vitro* cultures. Comparison between the two systems of two-site sandwich ELISA showed that the MAb-MAb sandwich ELISA was superior to the PAb-MAb sandwich ELISA : (1) it gave a higher sensitivity when tested with serially diluted *P. vivax* antigen preparations from vivax malaria patients; (2) it gave a higher specificity when tested with the SO strain of *P. falciparum* from *in vitro* cultures. (3) it gave a lower absorbance value when tested with erythrocytes from healthy individuals. All 281 cases of vivax malaria already proven by microscopic examination were positive by MAb-MAb sandwich ELISA. However, the assay showed a poor correlation with parasitemia in infected individuals examined by microscopy ($r = 0.117$).

INTRODUCTION

Specific diagnosis of malaria is usually by visual microscopic detection of malaria parasites in stained blood films. This method is satisfactory in terms of sensitivity and specificity and permits species differentiation of the parasites. Microscopic examination can also provide information about the viability of any parasite present in peripheral blood and such knowledge may be helpful when the response to treatment is being assessed (WHO, 1986). However, microscopic examination, even by an expert microscopist, is time-consuming, especially when the parasites are infrequent in blood or absent at the time of testing. An additional difficulty is the diagnosis of mixed infections of *P. falciparum* and relatively smaller percentages of ring stage *P. vivax,* the identification of which is often missed. As a consequence, there is a need to develop alternative method to diagnosis malaria cases, especially those with low grade parasitemia, in order to supplement and perhaps in certain situations to replace microscopic examination.

In *P. vivax,* the detection and identification of sporozoites by an ELISA using monoclonal antibodies (MAbs) raised against *P. vivax* sporozoites has been reported (Wirtz *et al,* 1985). We have reported a very sensitive MAb-based immunoradiometric assay (IRMA) capable of detecting as few as 0.24 blood stage parasites per 10^7 RBC. The assay is specific for *P. falciparum* and was shown to be correlated with parasitemia in either hospitalized patients or infected individuals in a malaria endemic area (Khusmith *et al,* 1987, 1988). However, this development was not applicable in the field because of the isotope requirement. The objective of the present study was to develop a two-site sandwich ELISA for the detection of *P. vivax* antigens in blood using the specific MAbs produced in our laboratory.

MATERIALS AND METHODS

Subjects

Patients with vivax malaria: Two hundred and eighty-one vivax malaria patients admitted to the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Bangkok, from September 1985 to April 1989 were studied. Blood samples were obtained from all patients during the pretreatment period.

Patients with falciparum malaria: Included in the study were 30 patients with falciparum malaria admitted to the Hospital for Tropical Diseases from June to October 1986.

Healthy controls: Thirty-four healthy persons residing in Bangkok, where malaria is not endemic were studied. They denied travelling to any malaria endemic area during the past 2 years and hence would be most unlikely to have been exposed to malaria during the time of study.

Blood samples

Three milliliters of blood were collected in screw-capped tubes containing 20 units heparin, from which thick and thin blood films were made, stained with Giemsa and examined by light microscopy. The number of infected erythrocytes/10^3 erythrocytes was determined by counting the number of parasitized cells in 10^3 erythrocytes in thin blood smears. For light infections, the number of parasites in thick blood films was counted and expressed per 200 white blood cells (WBC). The parasite count was further expressed as the number per 10^6 erythrocytes using the formula:

$$\text{No. parasites/}10^6 \text{ RBC} = \frac{\text{No. parasites/200 WBC} \times 7{,}500 \times 10^6}{\text{No. RBC/}\mu l \times 200}$$

This formula was based on the observation that a Thai patient with malaria has an average WBC count of 7,500 per μl of blood (T Harinasuta, personal communication). The number of erythrocytes was determined by extrapolation from a standard curve which shows a linear correlation between hematocrit values and the number of erythrocytes. The formula used for this conversion was $Y = 0.907657 + (0.0982103\ X)$ where Y = number erythrocytes ($\times 10^6$) per μl of blood and X = hematocrit value expressed in percent.

The blood was centrifuged at $500g$ for 10 minutes at room temperature to remove the plasma, and the remaining packed cells were washed twice by centrifugation with 0.01 M phosphate-buffered saline (PBS), pH 7.2 and stored at -70°C until use. The packed cells were thawed once and treated with 9 volumes of 0.01 M PBS, pH 7.2 containing 0.05% Tween 20 and 0.05% Nonidet P-40 (BHD Chemicals Ltd, Poole, England) (PBST-NP40) for 10 minutes at room temperature.

Standard antigen preparation

P. vivax antigen: P. vivax infected red blood cells were concentrated using a two step procedure comprising an initial removal of WBC by passing through a column of a mixture of sulfoethyl cellulose (SEC) and sephadex G-25 followed by Percoll gradient centrifugation (SEC-G-25-percoll) (Tharavanij et al, 1987). Before use, the infected RBC number was adjusted to achieve a similar concentration of the control packed RBCs and then lysed in 9 volumes of PBST-NP40 for 10 minutes at room temperature followed by serial dilution.

P. falciparum antigen: The SO strain of P. falciparum isolated from a Thai patient and grown continuously in vitro was used. The culture was harvested at the time when parasitemia reached 8%, and containing all blood stages. The cells were washed 5 times in 0.01 M PBS, pH 7.2 by centrifugation at $500g$ at room temperature for 10 minutes. The packed cells were then treated with 9 volumes of PBST-NP40 for 10 minutes at room temperature, and stored at -70°C. Before use the P. falciparum infected red cell extract was serial diluted with the red cell diluent.

Control antigen: Normal red blood cells from healthy group O individuals were washed 5 times in 0.01 M PBS, pH 7.2 by centrifugation at $500g$ for 10 minutes at room temperature and the plasma removed. The washed normal packed red blood cells were lysed in 9 volumes of PBST-NP40 for 10 minutes at room temperature and then used as control antigen and as diluent for the standard parasite antigens.

Preparation of anti P. vivax antibody

MAb: The McPV7 MAb prepared from the corresponding hybridoma culture supernatant was used. This MAb was produced by fusion of

Sp2/0 myeloma cells with spleen cells from a BALB/c mouse immunized with erythrocytic stages of *P. vivax* as described previously (Khusmith *et al*, 1984). Based on the immunofluorescent reactivities demonstrated with *P. falciparum* (Khusmith *et al*, 1984), the McPV7 MAb belonged to group I which reacted with uniformly bright generalized staining to all blood stages. This MAb reacted with a common blood stage antigen of Mr 30,000 shared by almost all isolates 92.57% reactive (unpublished data).

PAb: A rabbit was immunized intramuscularly with 4 doses of 0.5 ml of approximately 10^8 erythrocytic forms of *P. vivax* comprising of not less than 50% of schizonts with an equal volume of Freund's complete adjuvant initially and Freund's imcomplete adjuvant subsequently at an intervals of three weeks. The animal was bled one week after the last injection. The serum was then inactivated at 56°C for 30 minutes and absorbed twice with an equal volume of packed group AB erythrocytes from healthy individuals, incubated at 37°C for 30 minutes and at 4°C overnight, followed by centrifugation at 500g for 10 minutes at room temperature. The IgG fraction was purified by protein A Sepharose CL-4B affinity chromatography by the method advised by the manufacturer.

Enzyme labeling of anti-*P. vivax* monoclonal IgG (MIgG)

IgG fractions from MAb against *P. vivax* were prepared by protein A-Sepharose CL-4B (Pharmacia) chromatography. The enzyme labeled MIgG was prepared by a one-step glutaraldehyde method (Avrameas, 1969). Briefly, 2 mg IgG, suspended in 1 ml of 0.01 M PBS, pH 7.2 mixed with 5 mg of alkaline phosphatase enzyme (Type VII-S, Sigma) with an activity of at least 1,000 units per mg protein at room temperature. The mixture was dialysed against 2 l of 0.01 M PBS, pH 7.2 at 4°C overnight with one change. Thereafter, 10% glutaraldehyde (Sigma) was slowly added to yield a final concentration of 0.2% (v/v), stirred gently and allowed to incubate for 2 hours at room temperature. The mixture was dialysed against 0.01 M PBS, pH 7.2 containing 1 mM $MgCl_2$ at 4°C overnight with 2 changes, followed by extensive dialysis with several changes of 0.05 M Tris-HCl buffer pH 8.0 containing 1 mM $MgCl_2$ and 0.02% sodium azide. The conjugate was diluted to 4 ml with 0.05

M Tris-HCl buffer pH 8.0 containing 1% bovine serum albumin fraction V (BSA, RIA grade, US Biochemical Coorporation) and 0.02% sodium azide, aliquoted and stored in the dark at 4°C.

Two-site sandwich ELISA

Wells of 96 well flat bottom micro-ELISA plates (Costar, USA) were each coated with 150 µl of 10 µg/ml MIgG and PIgG for MAb-and PAb-MAb sandwich ELISA, respectively, followed by incubation at 37°C for 3 hours and 4°C overnight. The unbound MIgG or PIgG were washed 5 times with PBST-0.05% BSA and the non-reactive sites were saturated with 200 µl of PBST-1% BSA for 1 hour at room temperature. The plates were thoroughly washed with PBST-0.5% BSA to which 100 µl of either the erythrocyte preparations to be tested or standard antigen in various dilutions was added to each well, followed by incubation at room temperature for 2 hours. After washing, 100 µl of appropriate dilution of alkaline phosphatase labeled-MIgG in PBST-0.5% BSA containing 10% inactivated normal human serum was added and the plates were incubated for another 2 hours at room temperature. After being washed three times with PBST-0.5% BSA, 100 µl of the enzyme substate solution (p-nitrophenyl phosphate (PNPP), Sigma 104) was added. The reaction was stopped with 50 µl 3 N NaOH, after 30 minutes and the OD was measured by ELISA reader (Titertek Multiskan, MCC/340, Flow laboratories) at 405 nm. The tests were done in duplicate. The control normal RBC and *P. falciparum* infected RBC were tested as negative and positive references in each plate.

RESULTS

Sensitivity and specificity of two-site sandwich ELISA

The MAb-and PAb-MAb sandwich ELISA performed on blood samples from 34 healthy individuals showed that the mean value of optical densities (mean OD ± 1 SD) were 0.008 ± 0.008, and 0.037 ± 0.008, respectively. Since the accuracy of the ELISA reader (Titertek Multiskan MCC/340) and the micro ELISA plate (Costar) recommended by the manufacturer is 0.05 and 0.005, respectively, the precise recommended cut-off level should include

these error values. Therefore, the test sample was considered positive if its OD was equal to or above the mean OD + 2 SD of 0.024 for the MAb-MAb, and 0.053 for the PAb-MAb sandwich ELISA plus the error values obtained from the ELISA reader and micro ELISA plate of 0.055 (equivalent to 0.079, and 0.108, respectively). These cut-off levels were used to determine the sensitivity of a two-site sandwich ELISA when various dilutions of standard *P. vivax* infected erythrocytes were allowed to react with anti-plasmodium antibodies. The sensitivity was 6.68 parasites/10^6 erythrocytes and 2.69 parasites/10^3 erythrocytes with MAb-MAb and PAb-MAb sandwich ELISA, respectively (Fig 1).

The specificity of the assays was determined in a serially diluted suspension of *P. falciparum*-infected erythrocytes from *in vitro* cultures with an initial parasitemia of 8%. The binding in the MAb-MAb sandwich ELISA was below the cut-off level in all concentrations of the parasites. In contrast, the binding activity in the PAb-MAb sandwich ELISA increased when higher concentrations of parasites were tested (Fig 2).

MAb-MAb sandwich ELISA in clinical specimens

The blood from vivax malaria patients collected during the pretreatment period was tested by the
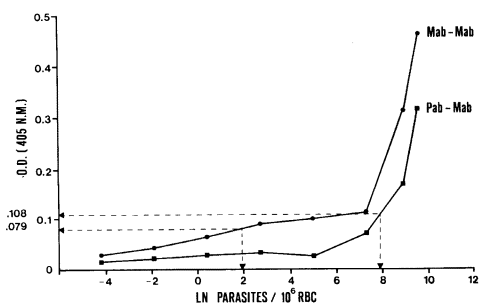

Fig 1—Sensitivity of MAb-(●——●) PAb-MAb (■——■) in a serially diluted suspension of *P. vivax* infected-erythrocytes from patients. Based on the cut-off OD (---------) of 0.079 and 0.108 for MAb- and PAb-MAb sandwich ELISA, respectively, the sensitivity was 1.9 and 7.9 ln parasites/10^6 RBC, which equivalent to 6.68 parasites/10^6 RBC and 2.69 parasites/10^3 RBC for MAb-and PAb-MAb sandwich ELISA, respectively.
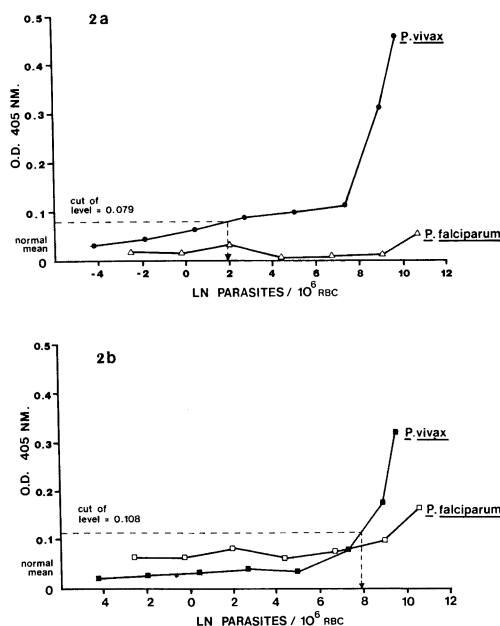

Fig 2—Specificity of a two site sandwich ELISA. 2a) Specificity of the MAb-MAb sandwich ELISA determined in a serially diluted suspension of *P. vivax* (●——●) and of *P. falciparum* infected erythrocytes from *in vitro* culture (△——△). Binding activity with *P. falciparum* was below the cut-off level in all concentrations of parasites. 2b) Specificity of PAb-MAb sandwich ELISA determined in a serially diluted suspension of *P. vivax* (■——■) and *P. falciparum* (□——□) from *in vitro* cultures. Binding activity was below the cut-off level except at the parasite concentration of 80,000 parasites/10^6 RBC.

MAb-MAb sandwich ELISA, and the results were compared with those found by microscopic examination. All 281 of vivax malaria cases proven by microscopic examination were positive by MAb-MAb sandwich ELISA. In addition all 34 healthy controls and 30 falciparum malaria cases were negative (Fig 3). Thus, the sensitivity, specificity and positive predictive values were each 100%. In addition, the yellow color produced by the reaction of the alkaline phosphatase enzyme with its PNPP substrate in assay of *P. vivax* blood samples could be distinguished by eye from the pale color-to-colorless reactions in assays of falciparum and healthy controls. When parasitological counts for all 281 samples positive by micro-
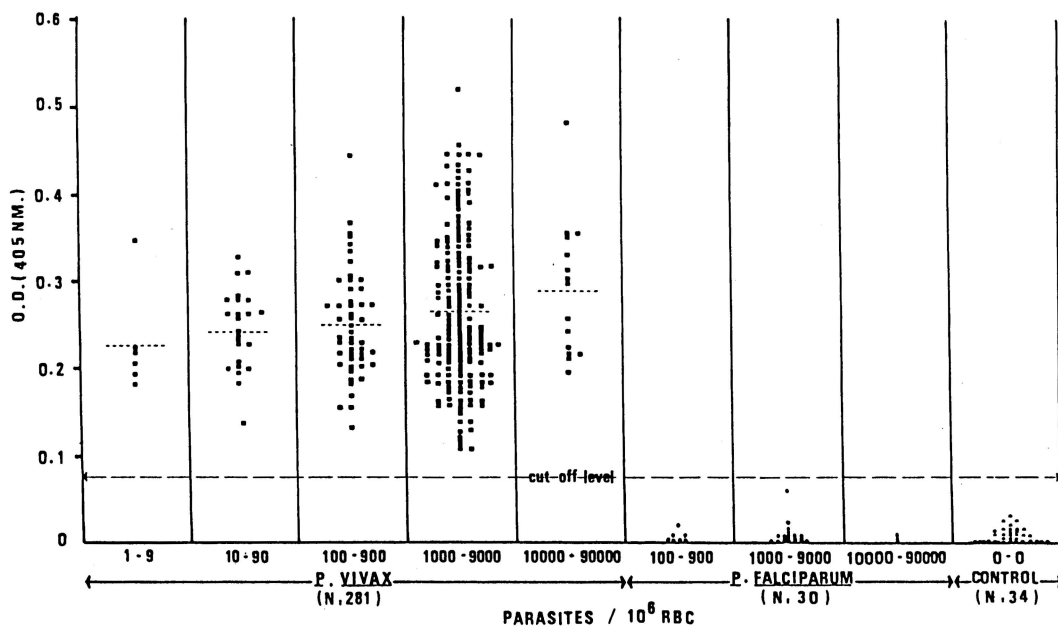
Fig 3—Scatter diagram of OD values from the MAb-MAb sandwich ELISA for detection of *P. vivax* antigen in 281 vivax malaria cases. Controls are 34 healthy individuals and 30 falciparum malaria cases.

scopic examination were plotted against the optical densities determined by MAb-MAb sandwich ELISA, a very poor correlation was demonstrated (r = 0.117). However, the sandwich ELISA showed good correlation with parasitemia when tested against *P. vivax*-infected erythrocytes in a serially diluted preparation (r = 0.937).

Reproducibility of MAb-MAb sandwich ELISA

For assays of high precision the results must be read photometrically. Since the precision of the ELISA reader given by the manufacturer is 2.5% or ± 0.05, difference in absorbance (OD) between duplicate blood samples lower or equal to 0.05 can be considered as the same reading. The reproducibility of the optical densities among 281 vivax malaria cases determined by duplicate testing was 93.95%.

DISCUSSION

Using *P. vivax*-infected erythrocyte serially diluted preparations from vivax malaria patients,

the MAb-MAb sandwich ELISA proved to be reproducible and capable of detecting *P. vivax* at a level of 6.68 parasites/10^6 erythrocytes, a degree of sensitivity close to that achieved by microscopic examination of thick blood films (Meuwissen, 1981). This level of sensitivity was similar to that previously reported for the detection of *falciparum* antigens (Avraham *et al*, 1981, 1983; Mackey *et al*, 1982; Frazen *et al*, 1984; Barker *et al*, 1986), namely 1-10 parasites/10^6 erythrocytes. However, the PAb-MAb sandwich ELISA showed a very low sensitivity, at 3.92 parasites/10^3 erythrocytes. A factor contributing to the lower sensitivity of the PAb-MAb sandwich ELISA is the relatively low antibody titer of the rabbit immune serum (1 : 640 IFA).

Our assay was specific, since it was positive with 281 cases of vivax malaria and negative when erythrocytes from 34 healthy individuals and 30 falciparum malaria cases were tested. No false positive or false negative reactions were observed in this assay under the carefully controlled laboratory conditions using the cut-off level of 0.079. The MAb-MAb sandwich ELISA showed a very low non-specific background reaction using blood

from healthy individuals residing in non-malaria endemic area. However, the cut-off level should be reconsidered when the test is applied to detect malaria parasites in endemic areas where soluble malaria antigen would be present in the plasma during and for some time after infection, as well as during drug suppression. Thus, higher backgrounds can be expected if whole blood is tested in malaria endemic areas. In the present study, this problem was overcome by using washed packed red blood cells, a more idealized situation than can be attained in the field.

All 281 vivax malaria cases already proven by microscopic examination were positive by ELISA. The parasite count in these specimens tested ranged from 7 to 42,000 parasites/10^6 erythrocytes. However, there was no correlation (r = 0.117) between the ELISA positivity and degree of parasitemia. In contrast this assay showed a good correlation (r = 0.937) when tested in a serially diluted *P. vivax* antigen preparation. Possible reasons for the non-correlation in the non-correlation in the patient series are : (1) The developmental stage variation of parasites found among the blood specimens of vivax malaria which lead to different quantitative and qualitative estimates of protein contents. In *P. falciparum*, the relatively good correlations between RIA or ELISA positivity and level of parasitemia (Mackey *et al*, 1980; Khusmith *et al*, 1987, 1988), probably reflects the dominance of the single (ring) stage in the circulation, although DNA probes for the detection of *P. falciparum* in blood specimens show a relatively low correlation between the degree of hybridization and parasitemia (Boonsaeng *et al*, 1989). (2) The antigenic diversity of *P. vivax* blood stage parasites as shown by several studies using a panel of MAbs in Western blot analysis (Andrysiak *et al*, 1986; Udagama *et al*, 1987). (3) The binding capacity of the MAb to parasite antigen. The MAb obtained from clone McPV7 which reacted with a single antigenic band (Mr 30 kDa) by Western blot analysis was used both as a coating antibody and as antibody probe for the detection of *P. vivax* antigens. If the epitope(s) on this antigen vary qualitatively or quantitatively in different isolates this will affect the ability to bind MAb.

This assay should be regarded as semi-quantitative. The yellow colored ELISA reaction product in positive cases can be clearly distinguished visually. Thus, this assay can be performed even if an ELISA reader is not available in the field. More appropriately, however, the assay needs to be translated into a rapid format for field application.

ACKNOWLEDGEMENTS

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