

# TWO-SITE PAN-SPECIES MONOCLONAL ANTIBODY ELISA FOR DETECTION OF BLOOD STAGE MALARIA ANTIGEN

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**Abstract.** A two-site pan-species monoclonal antibody sandwich ELISA (MAB-MAB ELISA) was developed to detect both *Plasmodium vivax* and *P. falciparum* antigens in whole blood. In this assay, the plates were coated with pan-species MAb 3F9 and another pan-species MAb M26-32 conjugated with alkaline phosphatase was used for detection of bound antigen. The sensitivity of this assay was 5, 10 and 10 parasites per 10<sup>6</sup> erythrocytes for cultured *P. falciparum*, patient-derived *P. vivax* and *P. falciparum*, respectively. The coincidence rates for this assay were 93% (93/100) with healthy individuals and 92.5% (49/53) with microscopically conformed vivax and falciparum malaria cases. After two weeks treatment 77.7% (14/18) of *vivax* malaria were still positive by this assay but with diminished level of reactivities.

## INTRODUCTION

Microscopic examination of thick and thin blood smears for malaria parasite detection is time-consuming, even by expert malariologists, especially in malaria endemic areas where the parasitemia and incidence are low. A sensitive and simple method for malaria diagnosis in these areas is needed to supplement or even to replace microscopic examination. Several monoclonal antibody (MAb) based enzyme-linked immunosorbent assays (MAB-ELISA) or radioimmunoassays (MAB-RIA) for detection of blood stage *Plasmodium falciparum* antigen have been developed (Avidos *et al*, 1987; Khusmith *et al*, 1987, 1988; Dubarry *et al*, 1990). Only a few studies, with varying sensitivity and specificity, on detection of blood stage *Plasmodium vivax* antigen have been reported (Wang *et al*, 1987; Li *et al*, 1991; Katzin *et al*, 1991). A polyclonal antibody/pan species monoclonal antibody ELISA (PAb-MAB-ELISA) for detection of both *P. vivax* and *P. falciparum* antigen in blood has been evaluated with the ability to detect as few as 1 parasite per 10<sup>5</sup> erythrocytes and a coincidence rate of 90.7% for microscopically confirmed *P. vivax* and *P. falciparum* malaria cases and 95% for healthy individuals (Gao *et al*, 1991).

We report here a modified two-site pan-species monoclonal antibody ELISA (MAB-MAB ELISA)

for detection *P. vivax* and *P. falciparum* antigen in whole blood of malaria cases.

## MATERIALS AND METHODS

### Blood samples

Blood samples were collected from (i) 46 vivax malaria cases admitted to the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Bangkok from January to May 1992; (ii) from 7 falciparum malaria cases, 2 of them from the Hospital for Tropical Diseases and the other 5 from Wuxi Hospital, Jiangsu Province, China; (iii) 100 healthy persons residing in Bangkok, where malaria is not endemic, which were used as controls. Thick and thin blood films were made for parasitological examination and 20 µl of heparinized blood was spotted on filter paper, air dried and stored at 4°C until use. Blood from the same group of patients (i) was collected again 2 weeks after treatment.

### Pan-species MABs and MAB conjugate

Hybridoma cell lines M26-32 and 3F9 (Li *et al*, 1984) were kindly provided by Professor Liu Er-xiang, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Beijing. Each of

these cell lines produces a pan-species MAb which recognises all malaria parasite species tested. MABs M26-32 and 3F9 were prepared as ascitic fluids in BALB/c mice and concentrated by ammonium sulphate precipitation. MAB M26-32 was conjugated with alkaline phosphatase (Sigma type VII-s) by the two-step glutaraldehyde as described by Voller and Bidwell (1986).

### Polyclonal antibody (PAb)

Rabbit anti-*P. cynomolgi* PAb was prepared as described previously (Gao *et al*, 1990). The titer of the PAb as determined by counter immunoelectrophoresis was greater than 1 : 128.

### MAb-MAb ELISA

96-well microplates (Immulon II, Dynatech Laboratories, USA) pretreated with glutaraldehyde were coated with MAB 3F9 (3 µg/ml in phosphate-buffered saline (PBS), 200 µl/well) and kept at 4°C overnight. The plates were washed 3 times with 0.02M Tris-HCl buffer pH 7.4 containing 0.075% tween-20 before use. The blood spots on filter paper each solubilized in 450 µl of 1% triton x-100 for 30 minutes at room temperature; then 200 µl were added to each well followed by incubation for 1 hour at 37°C. After washing, an appropriate dilution of MAB M25-32/alkaline phosphatase conjugate (diluted with PBS containing 0.075% Tween-20 and 3% non-fat milk) was added (200 µl/well) and incubated for 1 hour at 37°C. The plates were washed again and 200 µl/well of substrate solution [p-nitrophenyl phosphate (PNPP), Sigma 104] were added for 30 minutes at 37°C. The reaction was stopped with 3N NaOH (50 µl/well). The optical density (OD) at 405 nm was recorded using an ELISA reader (Titertek Multiskan Mcc/340 USA). The tests were done in duplicate. The normal RBC and *P. falciparum* infected RBC from *in vitro* culture were also tested as negative and positive references in each plate.

### PAb-MAb ELISA

The test was performed as described previously (Gao *et al*, 1991). Briefly, the PAb-MAb ELISA procedure is similar to the MAB-MAB-ELISA, but the plates were coated with rabbit anti-*P. cynomolgi* PAb, followed by lysed blood samples, then M26-32 MAB; subsequently the reaction was

detected by peroxidase-labeled anti-mouse IgG conjugate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA), using 3,3',5,5'-tetramethyl-benzidine (TMB, Sigam) as substrate. The OD was recorded at 450 nm.

The results obtained were expressed as follows:

$$\text{OD increase(\%)} = \frac{\text{OD of test sample} - \text{OD of negative reference}}{\text{OD of negative reference}} \times 100$$

## RESULT

### Sensitivity of MAb-MAb ELISA

The assay performed on blood samples from 100 healthy individuals showed that the mean OD increase ( $\pm$  SD) above background was 8.5%  $\pm$  15.8% (mean OD  $\pm$  SD = 0.138  $\pm$  0.02). A sample was considered positive if the increase in OD value was above 40% (mean OD increase + 2 SD). This cut-off level was used to determine the sensitivity when various dilutions of cultured *P. falciparum* infected RBC and whole blood samples from vivax and falciparum malaria patients were tested. The sensitivity was 5, 10 and 10 parasites per 10<sup>6</sup> erythrocytes with cultured *P. falciparum*

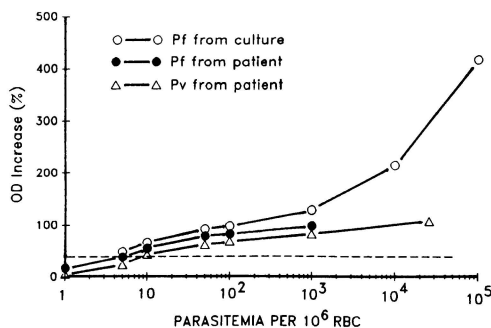


Fig 1—Sensitivity of MAb-MAb ELISA in detecting *P. falciparum*-infected erythrocytes from *in vitro* culture (○—○), *P. falciparum*-infected erythrocytes (●—●) and *P. vivax*-infected erythrocytes (△—△) from malaria patients. Based on the cut-off level of OD increase value 40%, the sensitivity was 5, 10 and 10 parasites per 10<sup>6</sup> erythrocytes with cultured *P. falciparum* infected erythrocytes and with *P. vivax* and *P. falciparum* malaria patient materials respectively.

infected RBC, and with *P. vivax* and *P. falciparum* malaria patient material, respectively (Fig 1).

### Specificity of MAb-MAb ELISA

The assay was performed on blood from 53 microscopically confirmed *P. vivax* and *P. falciparum* malaria cases. The mean OD increase ( $\pm$  SD) was  $99.2\% \pm 43.8\%$  (mean OD  $\pm$  SD =  $0.263 \pm 0.06$ ). There was a statistically significant difference in OD increase between microscopically confirmed clinical malaria cases and healthy individuals ( $p < 0.01$ ). Using a 40% OD increase as cut-off level, 49 cases gave positive results and the coincidence rate with microscopic examination was 92.5% (49/53). The assay was performed on *P. vivax* malaria blood samples before and after treatment of the patients with antimalaria drugs. The results in Fig 2 show that before treatment 94.4% (17 of 18) of patients were positive and two weeks after treatment, 77.7% (14 of 18) of patients were still positive with the MAb-MAb ELISA, but with decreased titers.

### Comparison of MAb-MAb and PAb-MAb ELISA

Forty-five clinical *P. vivax* malaria cases and 99 healthy individuals were tested with the two systems and the results were compared with microscopic examination (Table 1). Similar results were obtained, namely 92.9% (92/99) and 94.3% (94/99) coincidence with healthy individuals by MAb-MAb and PAb-MAb ELISA, respectively and 93.3% (42/45) coincidence with microscopically confirmed *P. vivax* malaria cases by both MAb-MAb and PAb-MAb ELISA.

### DISCUSSION

Two pan-species monoclonal antibodies, M26-32 and 3F9 were used in this assay. They differ from

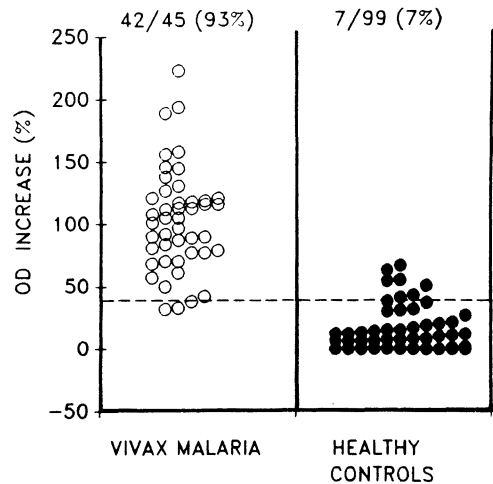


Fig 2—Scatter diagram of OD increase value from McAb-McAb sandwich ELISA for detection of *P. vivax* antigen in 45 vivax malaria cases. Controls are 99 healthy individuals.

other MABs which react with species-specific antigens of *P. falciparum* or *P. vivax*, some of which may fail to react with antigens of same parasite isolates from different endemic areas because of antigen diversity (Knowles *et al*, 1984; McBride *et al*, 1984). Previous studies showed that MAb M26-32 could immunoprecipitate a number of protein antigens of cultured *P. falciparum*, and by indirect immuno-fluorescence assay (IFA), it could react with *P. vivax*, *P. ovale* and isolates of *P. falciparum* from different areas of the world as well as with mouse and monkey malarias (Li *et al*, 1987). MAb 3F9 could at least react with antigens of *P. falciparum* and *P. vivax*. Structural analysis of the core epitope recognized by M26-32 shows this to be a tetrapeptide (NKND) with redun-

Table 1

Comparison of two-site MAb and PAb-MAb-ELISA for detection of *P. vivax* antigen in blood samples from vivax malaria cases and from healthy individuals.

Microscopic examination	Two-site MAb ELISA		PAb-MAb ELISA	
	+	−	+	−
<i>P. vivax</i> (n = 45)	42 (93%)	3 (7%)	42 (93%)	3 (7%)
Normal (n = 99)	7 (5%)	92 (92.4%)	5 (5%)	94 (95%)

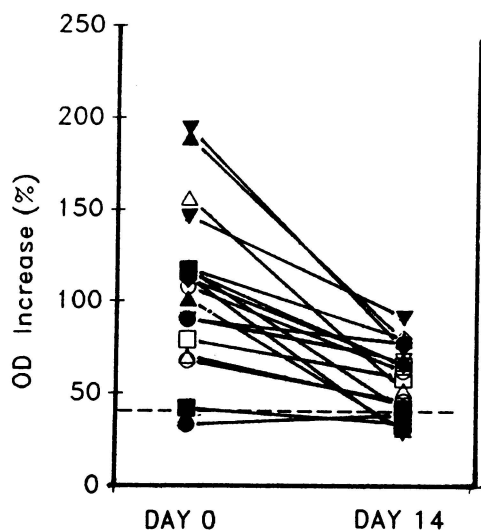


Fig 3—The OD increase value in blood from vivax malaria patients before and 2 weeks after anti-malaria treatment compared with healthy controls.

dancy in the two end positions; this set of analog sequences is known to be present in many malaria antigen sequences (Cheng *et al*, 1991), thus explaining its pan-species nature and the degree of sensitivity obtained in the assay. Thus MAbs M26-32 and 3F9 could detect antigens of both *P. falciparum* and *P. vivax* infected erythrocytes, and also circulating antigens in plasma or serum so that either whole blood or plasma/serum may be used in the MAb-MAb ELISA.

In the present study, the specificity of this assay was 93% for healthy individuals and 92.4% for *P. vivax* and *P. falciparum* malaria cases. Two weeks after treatment, 77.7% of *P. vivax* malaria cases were still positive by this ELISA, which is similar to the result obtained for *P. falciparum* malaria by specific MAb-MAb RIA (Khusmith *et al*, 1987). The sensitivity were 5, 10 and 10 parasites per  $10^6$  erythrocytes from cultured *P. falciparum* and from *P. vivax* and *P. falciparum* malaria cases, respectively. This degree of sensitivity can be increased if a MAb conjugate of greater specific activity (unpublished data) is used. The MAb-MAb ELISA involves one less step than the PAb-MAb ELISA and it is not necessary to prepare specific

polyclonal antibody which is difficult to standardize. Therefore the MAb-MAb ELISA should be more economic, time-saving and easy to standardize than the PAb-MAb ELISA. In addition, the use of filter paper collected whole blood facilitates performance in the field. The previous study (Gao *et al*, 1991) showed that these filter papers could be kept at room temperature for two months and for more than six months at 4°C

For more rapid diagnosis, the MAb-MAb ELISA may be modified to provide a pan-species MAb based dot-ELISA or dot immunogold assay. In addition, the assay may be modified by using a combination of pan-species MAb and species-specific MAbs in order to permit differentiation between *P. falciparum* and *P. vivax* infections. Such further developments would allow field use of a pan-species MAb based set of assays for individual case detection as well as for epidemiologic analysis.

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