DETECTION OF BLOOD STAGE ANTIGENS OF *PLASMODIUM VIVAX* BY SANDWICH ELISA USING PAN-SPECIES MONOCLONAL ANTIBODIES AND POLYCLONAL ANTIBODIES

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Abstract. This paper reports an improved PcAb-McAb-ELISA test to detect blood stage *Plasmodium vivax* antigen in which the plates were coated with rabbit anti-*P. cynomolgi* polyclonal antibody to capture the antigens in test samples and two monoclonal antibodies, M26-32 and 3F9, were added together to react with the captured antigens. The coincidence rate with this test was 93% with microscopically confirmed *P. vivax* cases, 97% with normal samples, 95% with microscopically negative fever cases from non-endemic areas and 86% from endemic areas, respectively. The sensitivity was greater than 1 parasite/10^5 RBC.

INTRODUCTION

Since the introduction of enzyme-linked immunosorbent assay (ELISA) for the diagnosis of *Plasmodium falciparum,* (Mackey *et al,* 1982) a number of monoclonal antibody-ELISA and dot-ELISA for the detection of parasitic antigens have been described (Londner *et al,* 1988, Fortier *et al,* 1987). But antigen-based tests for detection of *P. vivax* are less advanced than those for *P. falciparum* (Londner *et al,* 1987; Dubarry *et al,* 1990; Wang *et al,* 1987).

We have been engaged in developing a monoclonal antibody-ELISA for detecting antigens of blood stage *P. falciparum* and *P. vivax* since 1984. Different test systems, including inhibitory-ELISA and sandwich ELISA have been tried (Yang *et al,* 1987; Gao *et al,* 1990). In 1988, we developed an anti-*P. falciparum* polyclonal antibody-double monoclonal antibody sandwich ELISA (PcAb-McAb-ELISA). It was found that the sensitivity and specificity of this method for *P. falciparum* were high, but it was not satisfactory for *P. vivax* (Gao *et al,* 1990).

Recently, we have modified this method for detecting *P. vivax* infection under field conditions.

MATERIALS AND METHODS

Blood samples: All blood samples for PcAb-McAb-ELISA were collected on filter papers. Blood samples from normal individuals were collected from 128 city inhabitants in non-endemic areas of China (Wuxi and Fuzou). Blood samples of fever cases were collected from 299 suspected malaria patients in local township hospitals in malaria endemic areas of Jiangsu, Fujian and Hainan provinces and from 95 patients in city hospitals in a non-endemic area of China (Wuxi). Reference samples were prepared from normal RBC from the blood bank in Wuxi city.

Monoclonal antibodies: Monoclonal antibodies M26-32 and 3F9 were obtained from Professor Liu Erxiang (Li *et al,* 1984).

Preparation of rabbit anti-*P. cynomolgi* PcAb: Blood samples infected with *P. cynomolgi* were collected from Rhesus monkeys *(M. mulatta sinica)* at a parasitemia rate of 10%, washed with PBS (pH 7.4, 0.01 M) to remove plasma and then passed through a Waterman CF-11 cellulose column to remove WBC according to Ruenwongsa's (1985) method. The cells were lysed by adding 0.1% saponin-NS (8 times volume of the

pellet). Then the mixture was centrifuged at 10,000 rpm for 10 minutes. The pellet was washed 3 times with PBS, suspended in an equal volume of PBS and then sonicated for 1.5 minutes. The sonicate was centrifuged at 10,000 rpm for 10 minutes to remove insoluble material. The supernatant was used for immunizing rabbits (New Zealand) according to Yu He's method (Yu *et al*, 1982). When the titers (counter immunoelectrophoresis) were over 1:128, sera containing anti-*P. cynomolgi* antibodies were collected and stored at -20°C.

Test procedure: 40-well microplates were coated by adding 200 μl of a 1:64 dilution of rabbit anti-*P. cynomolgi* PcAb in 0.02M PBS, pH 7.4 to each well and stored at 4°C overnight. Then the plates were washed 3 times with 0.02M Tris-HCl buffer, pH 7.4 containing 0.05% Tween-20. Each test sample on filter paper was first washed with pH 7.4 PBS for 10 minutes to remove serum and then solubilized in 450 μl of 1% Triton X-100 for 45 minutes at room temperature. To each well was added 200 μl test sample, followed by incubation for 1 hour at 37°C. Following 3 washings in Tris-HCl buffer, an equal volume of an appropriate dilution of McAb mixture of M26-32 and 3F9 was added to the plates (200 μl/well), then incubated at 37°C for 1 hour. After washing, an appropriate dilution of rabbit anti-mouse IgG conjugated with horseradish peroxidase was added, 200 μl/well, and incubated for 1 hour at 37°C. The plates were washed again, 200 μl/well of substrate solution was added and incubated at room temperature for 30 minutes. The composition of the substrate solution was 0.2 mg/ml TMB (tetramethylbenzidine) in 0.1M sodium acetate-citric acid buffer, pH 5.0 containing 0.03% H_2O_2. After stopping the reaction with 2M sulfuric acid (50 μl/well), the optical density (OD) at 450nm was recorded using a microplate reader (Model GXM 201). All samples were tested in duplicate and each plate contained a reference sample (2 wells).

Threshold determination: For threshold determination, 128 normal blood samples were tested. The results obtained were expressed as follows: OD increase (%) = (OD value of test sample−OD value of reference sample)/OD value of reference sample × 100%. According to the distribution of OD increase in 128 control samples, with P95 as the upper limit of normal, an increase of OD value by ≥35% compared with the reference samples was considered to be positive.

RESULTS

Altogether 299 fever cases in endemic areas were examined for *P. vivax* and *P. falciparum* infections using both microscopic examination and PcAb-McAbs-ELISA. Comparison of the results of these two methods is shown in Table 1. 76.3% were positive for *P. vivax* or *P. falciparum* infection by both PcAb-McAbs-ELISA and microscopy and 14.4% were negative by both methods. Thus, in 90.7% of the samples the PcAb-McAbs-ELISA and microscopy gave the same results. In 9.3% of the samples the results of the two methods were different.

The results of PcAb-McAbs-ELISA with microscopically confirmed positive fever cases are shown in Table 2. The coincidence rate with *P. vivax* averaged 93% and there was no statistically significant difference in coincidence rate between samples from Jiangsu, Hainan and Fujian provinces. The coincidence rate was lower with *P. falciparum* than with *P. vivax* in samples from the same areas in Hainan province.

The results of PcAb-McAbs-ELISA with microscopically negative samples are shown in Table 3. There was no statistically significant difference in coincidence rate between the normal samples and microscopically negative fever cases from non-endemic areas. But the coincidence rate with microscopically negative fever cases from endemic areas was lower than those from non-endemic areas and normal samples and the difference was statistically significant ($u = 2$, $p < 0.05$).

Six microscopically confirmed positive cases (2 *P. vivax* and 4 *P. falciparum*) after treatment with Artemether for two days turned out to be negative by microscopy but remained positive by PcAb-McAbs-ELISA.

To determine the sensitivity of parasite detection, the PcAb-McAbs-ELISA was carried out on blood from *P. vivax* and *P. falciparum* cases with 0.5-1% parasitemia, serially diluted with RBC of blood donors free from malaria. The results shown in Fig 1 suggest that this test is sensitive enough to detect 1 parasite/10^5 RBC.

Table 1

Comparison of results from microscopic examination and PcAb-McAbs-ELISA.

	Microscopic examination		
	Positive	Negative	Total
PcAb-McAbs-ELISA			
Positive	228 (76.3%)	7 (2.3%)	235 (78.6%)
Negative	21 (7.0%)	43 (14.4%)	64 (21.4%)
Total	249 (83.3%)	50 (16.7%)	299 (100%)

Table 2

Results of PcAb-McAbs-ELISA with microscopically positive fever cases.

Species	Source of samples	No. of samples	OD increase (%) (X ± SD)	No. of positive	Coincidence rate (%)
P. vivax	Jiangsu	47	59.5 ± 30.3	41	87
	Fujian	94	47.6 ± 15.2	88	94
	Hainan	41	53.5 ± 16.0	40	98
	Total	182	52.2 ± 20.8	169	93
P. falciparum	Hainan	67	51.8 ± 17.2	59	88

Table 3

The results of PcAb-McAbs-ELISA with microscopically negative samples.

	No. of samples	OD increase (%) (X ± SD)	No. of negatives	Coincidence rate (%)
Normal samples	128	21.9 ± 13.6	124	97
Fever cases from non-endemic areas	95	18.9 ± 11.3	90	95
Fever cases from enedmic areas	50	22.6 ± 19.0	43	86

DISCUSSION

The purpose of this study was to compare the sensitivity and specificity of the PcAb-McAbs-ELISA with that of microscopic examination for diagnosis of *P. vivax* malaria under field conditions.

In this technique, anti-*P. cynomolgi* polyclonal antibody was used instead of anti-*P. falciparum* polyclonal antibody to coat the wells of the microplate to increase the capture of *P. vivax* antigens in test samples (Gao *et al,* 1990). Two monoclonal antibodies, M26-32 and 3F9, were used together to react with more epitopes of the antigens captured in the wells than would be the case for a single MAb. The results showed that out of 182 microscopically positive cases, 169 cases were positive by this method (coincidence rate 93%). Although this method using pan-species specific McAbs can not distinguish *P. falciparum* from *P. vivax* infection it is promising for use where only *P. vivax* is endemic. On the other hand, the use of Pan-species McAbs allows rapid identification of any malaria infection, following which species can be determined by microscopy of positive cases. Thus approach would reduce the load of slide examination in the field.

Using microscopy as the standard, this method gave a false negative rate of 7% for *P. vivax,* a false positive rate of 3% for normal samples, a

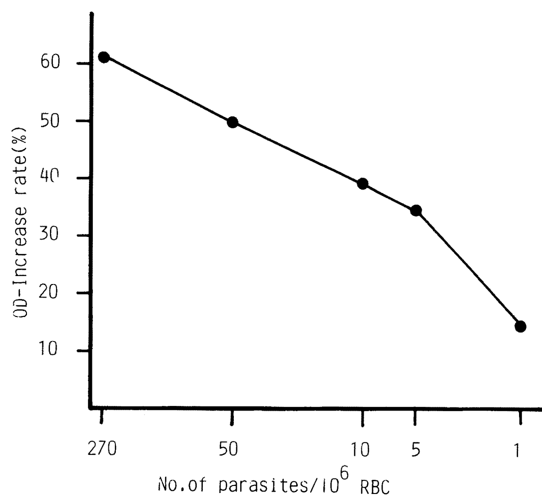

Fig 1—PcAb-McAbs-ELISA results obtained from microscopically confirmed *P. vivax* and *P. falciparum* cases serialy diluted with normal RBC; shown are the average of 4 *P. vivax* and 2 *P. falciparum* cases.

false positive rate of 5% for fever cases from non-endemic areas and of 14% for those from endemic areas. For normal samples and fever cases from non-endemic areas, the false positives could possibly be due to nonspecific binding of the antibodies to blood components, or to other infectious organisms found in the blood. In addition to the above, the high 'false positive' rate observed in fever cases from endemic areas could be due to a very low parasitemia which is very difficult to detect by microscopy, or due to parasite antigen residue not yet cleared from blood in patients that have just been treated. But in this study the duration of the time that test remains positive after treatment was not determined.

In these studies, test samples were collected on filter paper and kept at room temperature for two months as an extreme test of the assay's usefulness under field conditions. Clearly further analysis of the best conditions for sample handling is required to maximize sensitivity. The manipulation is simple and is suitable for large scale operation so it is a potential tool for epidemiological investigation in *P. vivax* endemic areas.

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