

# DETECTION OF *P. VIVAX* ANTIGENS IN MALARIA ENDEMIC POPULATIONS OF NEPAL BY ELISA USING MONOCLONAL ANTIBODIES RAISED AGAINST THAI ISOLATES

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**Abstract.** An indirect enzyme linked immunosorbent assay (ELISA) using monoclonal antibody (MAb) originated from the native Thai isolates of *P. vivax* (McPV1) and the polyclonal antibody (PAb) raised against Nepali isolates of *P. vivax* was developed for detection of *P. vivax* antigens in red cell lysates. The assay was specific (100%) since it was positive only with *P. vivax*-infected erythrocytes and was negative when erythrocytes from 40 healthy individuals from malaria non-endemic areas and 40 *P. falciparum* infected erythrocytes were tested. When the assay was applied to 203 vivax blood samples already proven by microscopic examination collected from Dhanusha district of Nepal, and using the cut-off level of the mean optical density (OD) (0.144) of 40 healthy individuals who had been living in malaria-endemic areas (0.073) + 2 SD (0.016), the assay could detect 189/203 samples, indicating the sensitivity of the test was 93.1% with a detection limit of erythrocytes of 240 parasites/10<sup>6</sup> erythrocytes. In addition, the assay was negative when 40 blood samples with fever of unknown origin, collected from the same malaria-endemic areas, were tested. However, there was a significant correlation between OD values and parasitemia ( $r = 0.649$ ;  $p = 0.018$ ). The results indicate that MAb-PAb indirect ELISA using MAb raised against Thai isolates of *P. vivax* as the coating antibodies, and polyclonal antibodies raised against local Nepali isolates as the detecting antibody, could detect *P. vivax* antigens with high degrees of sensitivity and specificity. Furthermore, it seems that the McPV1 MAb raised against Thai isolates of *P. vivax* could recognize the antigens of Nepali isolates in a wide range of blood samples.

## INTRODUCTION

Malaria remains a major global public health problem. It causes 300 to 500 million cases of acute illness, with more than a million deaths, each year, and contributes to an ever-widening gap in prosperity between endemic countries and the malaria-free world (WHO, 2003). Besides falciparum malaria, which is responsible for most severe and often fatal disease, *P. vivax* infection is an increasing public health problem in Asia, and South and Central America, which have been associated with high levels of mortality, probably indirectly through the presence of other diseases

and conditions of poor health (Mendis *et al*, 2001). In Nepal, more than 70% of the total population is at risk of malaria with varying numbers of Malaria Parasite Incidence Rate/1,000 (Department of Health Services, 1998/1999). Specific diagnosis of malaria is usually by visual microscopic detection of malaria parasites in stained blood films. Although the method is satisfactory in terms of sensitivity, and specificity and permits species differentiation of malaria parasites, microscopic examination, even by an expert microscopist, is time-consuming, especially when the parasites are infrequent in blood, absent at the time of testing, or in mixed infections. As a consequence, several alternative methods have been developed to diagnose both falciparum and vivax malaria cases, especially those with low-grade parasitemia, in order to supplement and perhaps, in certain situations, to replace microscopic examination. *P. falciparum* specific monoclonal antibodies (MAbs)

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have been used to develop immunological assays for detection of *P. falciparum* antigens by immunoradiometric assay (IRMA) (Khusmith *et al.*, 1987, 1988), and ELISA (Lim *et al.*, 1992; Namsiripongpun *et al.*, 1993; Taylor and Voller, 1993; Tanpradist *et al.*, 1995). For *P. vivax* infections, an IgM MABs which reacted with blood stages of *P. vivax*, was used in a cell-ELISA to detect parasites in the blood with 100% specificity for *P. vivax* (Bracho and Perez, 1996). Likewise, an immunofluorescent test based on the specific MAB was developed and used for detection of parasitized erythrocytes in blood in Venezuela (Perez *et al.*, 1995).

At present, no single technique has been described that is clearly superior to microscopic examination. However, increasing evidence indicates that specific detection of antigen markers with specific monoclonal antibodies is potentially suited to overcome most of the problems of by thick and thin smear examinations. Thus, a two-site monoclonal antibody (MAB) sandwich ELISA (MAB-MAB sandwich ELISA), using MAB raised against *P. vivax* Thai isolates, could detect *P. vivax* antigens in blood samples with a detection limit of 6.68 *P. vivax* parasites/10<sup>6</sup> RBC, and a specificity of 100% (Khusmith *et al.*, 1992). However, in that study, the optical density value was shown to have a poor correlation with parasitemia in infected individuals examined by microscopy. In this study, we developed another version of ELISA using a MAB produced against Thai isolates of *P. vivax*, and polyclonal antibody raised against Nepali isolates of *P. vivax* to detect malaria parasite antigens in the blood of people living in malaria endemic areas in southern Nepal.

## MATERIALS AND METHODS

### Subjects

**Patients with vivax malaria.** Two hundred and three blood samples from microscopically-confirmed *P. vivax*-positive patients, aged between 1-82 years, were collected from Godar Health Post, Dhalkebar Health Post and Lalghad Health Post of Dhanusha district, Janakpur Zone, Nepal, between July 2001 and May 2002. Written informed consent was obtained before blood was drawn. Ethical clearance was approved by the

Nepal Health Research Council, with an official approval letter.

***P. falciparum* patients.** Included in the study were 40 patients with falciparum malaria proven by microscopic examination, collected from the same endemic areas between March and May, 2002.

### Healthy controls from non-endemic area.

Forty healthy adults residing in Kathmandu, where malaria is not endemic formed the control group. They had no history of malaria and denied traveling to any malaria-endemic areas during the past two years; hence, they would be most unlikely to have been exposed to malaria during the time of the study.

**Healthy controls from endemic areas.** Forty adult healthy individuals residing in malaria-endemic areas of Dhanusha district were studied.

**Patients with fever of unknown origin (FUO) other than malaria infections.** Forty adult patients with fever of unknown origin (FUO) for more than two weeks, collected from malaria-endemic areas, proven microscopically-negative for malaria, were included.

### Blood samples

Two to three ml of blood were collected in screw-capped tubes containing 10 µl of 10% EDTA per ml, from which thick and thin blood films were made, stained with Giemsa and examined by light microscope. The collected blood samples were centrifuged at 1,000 rpm for 10 minutes at room temperature to remove the plasma. The remaining packed red cells were aliquoted and stored at -20°C until used. All samples were transported to Kathmandu in dry ice. For testing, the packed red blood cells were treated with 9 volumes of 0.01 M phosphate buffered saline (PBS), pH 7.4 containing 0.05% Tween 20 and 0.5% Nonidet P-40 (BHD Chemical, Poole, England) (PBST-NP40) for 10 minutes at room temperature.

### Preparation of anti-*P. vivax* polyclonal antibodies (PABs)

A healthy male rabbit was immunized intramuscularly with 3 doses of 0.5 ml of approximately 3.6 x 10<sup>7</sup> *P. vivax* infected erythrocytes

(Nepali isolates) previously concentrated by Percoll gradient centrifugation using the method described by Khusmith *et al* (1992) with an equal volume of Freund's complete adjuvant (Difco Laboratories, Michigan, USA) initially, and Freund's incomplete adjuvant subsequently, at intervals of three weeks. The blood was taken one week after the last injection. The serum was separated and inactivated at 56°C for 30 minutes. The immunoglobulin was concentrated by saturated ammonium sulphate (Voller and Bidwell, 1986).

#### **Preparation of anti-*P. vivax* monoclonal antibodies (MABs)**

The McPV1 MAB prepared from the corresponding hybridoma culture supernatant was kindly provided by Prof Srisin Khusmith, Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Thailand. This MAB was produced by fusion of Sp 2/0 myeloma cells with spleen cells from BALB/c mice immunized with erythrocyte stages of *P. vivax* Thai isolates (Khusmith *et al*, 1984). The McPV1 MAB belonged to group I, which reacted with uniform bright generalized staining of all blood stages. This MAB reacted with a common blood-stage antigen of Mr 30,000, shared by almost all Thai isolates.

#### **MAB-PAb indirect ELISA**

An indirect ELISA method was developed as described by Khusmith *et al* (1992), with some modifications. Wells of 96-well flat-bottomed micro-ELISA plates (Costar, USA) were each coated with 100 µl of 10 mg/ml McPV1 MAB, followed by incubation at 37°C for 2 hours and further incubation at 4°C overnight. The unbound MAB was washed 3 times with PBS Tween-20 (PBST 0.05%) and the non-reactive sites were saturated with 200 ml of PBST-2.5% milk 1 hour at room temperature. The plates were thoroughly washed with PBST to which 100 µl of test and control erythrocyte lysates were added per well, followed by incubation for 2 hours at 37°C. After washing, 100 µl of 10 µg/ml of anti-*P. vivax* PAb were added and incubated for 1 hour at 37°C. The plates were washed again with PBST and 100 µl of goat anti-rabbit IgG labeled with HRP conjugate (Dako Company, Denmark, 1:2000) was added to each well and incubated at 37°C for another hour. After washing, the enzyme substrate solution, Ortho-

phenylenediamine (OPD) (Dako Company, Denmark) in citrate buffer at pH 5.0, was added, and the reaction was stopped with 50 µl of 0.5 M H<sub>2</sub>SO<sub>4</sub> after 30 minutes at room temperature. The OD was measured by ELISA reader (MTP-32 Microplate Reader, Corona Electric) at 492 nm.

#### **Data analysis**

SPSS 10.0 for Windows was used for statistical analysis of the data. Pearson's correlation was used to estimate the correlation between the number of parasites/10<sup>6</sup> erythrocytes and the OD values (492 nm) of the developed MAB-PAb-based indirect ELISA method.

## **RESULTS**

#### **Sensitivity and specificity of MAB-PAb indirect ELISA**

The MAB-PAb indirect ELISA using MAB raised against *P. vivax* Thai isolates as coating antibody and PAb raised against Nepali isolates was performed on blood samples from 40 healthy individuals from non-malaria, and 40 from endemic areas. The results showed that the mean value optical densities among individuals from endemic areas (0.073) was higher than for individuals from non-malaria areas (0.043) (Fig 1), with SD of 0.003 and 0.008, respectively. Therefore, we decided to use the mean OD among the individuals from endemic areas to establish the cut-off level. Since the accuracy of the ELISA reader (MTP-32 Microplate Reader, Corona Electric) is 0.05, and the accuracy of the micro ELISA plate (Costar, USA) recommended by the manufacturer is 0.005, the precise recommended cut-off level should include these error values. Therefore, the test sample was considered positive if its OD was  $\geq$  an OD of 0.144 [mean OD + 2 SD (0.016) + the error values obtained from the ELISA reader and plate, of 0.050 and 0.005, respectively]. These cut-off levels were used to determine the sensitivity of the MAB-PAb indirect ELISA. The specificity of the assay was determined when tested with *P. falciparum* blood lysate that was shown 100% specific, since all falciparum blood samples were negative (Fig 2).

#### **MAB-PAb indirect ELISA in clinical samples**

The pre-treatment blood from vivax malaria patients was tested by MAB-PAb indirect ELISA,

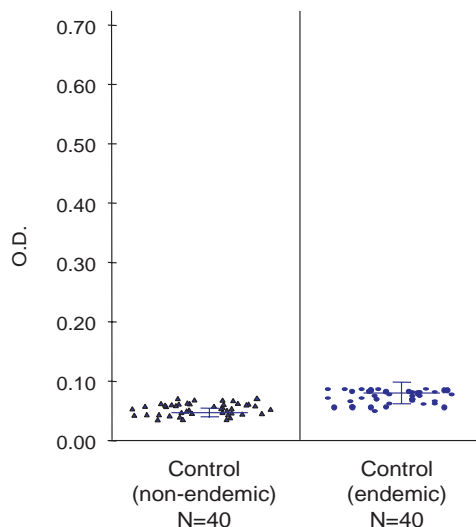


Fig 1—Scatter diagram showing the mean OD  $\pm$  1SD of blood samples collected from healthy controls who had been living in malaria-endemic and non-malaria areas.

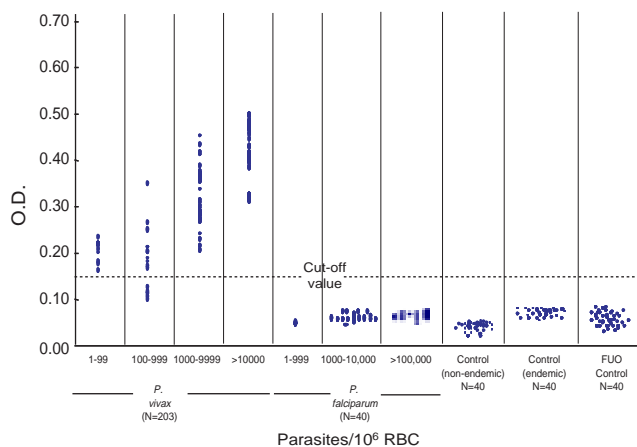


Fig 2—Scatter diagram showing the cut-off OD value (0.144) of 40 healthy controls collected from malaria-endemic areas. The OD values of 14 of 203 *P. vivax* samples were below the cut-off value; hence they were considered negative. Included were 40 healthy individuals from non-malaria areas, 40 F/UO, and 40 falciparum malaria cases from an endemic area.

and the results compared with those found by microscopic examination. Among 203 samples tested, 189 vivax samples were positive by the established MAb-PAb indirect ELISA (Fig 2). In addition, when 40 samples of F/UO patients were

tested, all were negative. Thus, the sensitivity, specificity, and positive predictive values were 93.1, 100, and 94.5%, respectively. In addition, the orange-brown color produced by the reaction of peroxidase enzyme with its OPD substrate in the assay of *P. vivax* samples could be distinguished by the naked eye from the pale color-to-colorless reactions in the assay of *P. vivax* lysates and healthy controls. Based on the parasitemia in the test blood samples (range 6-32,920 parasites/ $10^6$  erythrocytes), the established MAb-PAb indirect ELISA could detect as low as 240 parasites/ $10^6$  erythrocytes, and a significant correlation was observed ( $r = 0.649$ ;  $p = 0.018$ ).

### Reproducibility of MAb-PAb indirect ELISA

For assays of high precision, the results must be read photometrically. Since the precision of the ELISA reader given by the manufacturer was 2.5%, the difference in absorbance (OD) between duplicate blood samples  $\leq 2.5\%$  can be considered as the same reading. The reproducibility of the optical densities among 203 vivax malaria cases determined by duplicate testing was within 2.5%, hence all samples were considered as the same reading.

### DISCUSSION

An indirect enzyme-linked immunosorbent assay (ELISA), using monoclonal antibody (MAb) originated from native Thai isolates of *P. vivax* (McPV1), and polyclonal antibody (PAb) raised against Nepali isolates of *P. vivax*, was developed to detect *P. vivax* antigens in red cell lysates. The assay was specific (100%), since it was positive only with *P. vivax*-infected erythrocytes, and was negative when erythrocytes from healthy individuals from non-endemic malaria areas and from *P. falciparum*-infected erythrocytes were tested. The MAb-PAb indirect ELISA showed a very low non-specific background reaction using blood from healthy individuals. However, higher backgrounds were observed in the blood of healthy individuals in malaria-endemic areas in Dhanusha district, southern Nepal were used. As recommended previously, (Khusmith *et al*, 1992), to evaluate the sensitivity of the test, the cut-off level should be considered when it is applied. Therefore, the cut-off level for the blood of



healthy individuals, who had been living in malaria-endemic areas was used. When the assay was applied to 203 vivax malaria cases already proven by microscopic examination, collected from Dhanusha district of Nepal, the assay could detect 189/203 samples, indicating that the sensitivity of the test was 93.1%. The parasite count in the specimens tested ranged from 6-32,920 parasites/10<sup>6</sup> erythrocytes, and the detection limit was 240 parasites/10<sup>6</sup> erythrocytes. However, there was a significant correlation between OD values and parasitemia ( $r = 0.649$ ;  $p = 0.018$ ). In addition, the assay was negative when blood samples with fever of unknown origin, collected from the same malaria endemic areas, were tested. However, the sensitivity and the detection limit of the present assay was lower than that reported previously using monoclonal antibody that recognized protein at the same molecular weight by MAb-MAb sandwich ELISA (Khusmith *et al*, 1992). This showed that all vivax malaria cases proven by microscopic examination were positive (100% sensitivity), with parasite detection as low as 6.68 parasites/10<sup>6</sup> erythrocytes. In addition, the MAb-MAb sandwich ELISA was proven to be reproducible. This differential sensitivity might be due to (i) antigenic diversity of *P. vivax* blood-stage antigens, as shown by several studies using a panel of MAbs by Western blot analysis (Udagama *et al*, 1987; Khusmith *et al*, 1998a,b), and (ii) the binding capacity of the MAb to the parasite antigen. The MAb obtained from clone McPV1, which reacted with a single antigenic band (Mr. 30 kDa) by Western blot analysis, was used as capture antibody. If the epitopes on this antigen vary qualitatively or quantitatively in different isolates, this will affect the ability to bind the monoclonal antibodies. Although the monoclonal antibodies raised against Thai isolates could recognize the common antigens on Nepali strains, some epitopes might be different.

The results obtained indicate that MAb-PAB indirect ELISA using MAb raised against Thai isolates of *P. vivax* as the coating antibodies, and polyclonal antibodies raised against local Nepali isolates as the detecting antibody could detect *P. vivax* antigens with high degrees of sensitivity and specificity. Furthermore, it seems that the McPV1 MAb raised against Thai isolates of *P. vivax* could

recognize the blood-stage antigens of Nepali isolates in a wide range of blood samples. Therefore, such monoclonal antibody could be used further to develop a rapid test for detection *P. vivax* in field isolates elsewhere. However, an assay with higher sensitivity should be developed.

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