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AGENCY FOR INTERNATIONAL DEVELOPMENT  
WASHINGTON, D.C. 20548

DATE:

8/4/88

MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18  
FROM: AID/SCI, Victoria Ose *VO*  
SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No.

5.232

5.232

PR m'd 11/5/82

Attachment

11

## PROGRESS REPORT TO USAID

5. 232

Contract No. 93G-5542-9-00-5078-00

X

Title of project : Antigenic diversity of Plasmodium vivax

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Time period covered : March 1986-August 1986

## SCIENTIFIC PROGRESS

Following progress has been made:

1. Cloning of hybrids secreting anti-P. vivax antibodies.
  2. Characterization of monoclonal antibodies (MABs) by indirect fluorescent antibody technique.
  3. Successful concentration of P. vivax infected red blood cells (RBCs) minimally contaminated with leucocytes from malaria patients.
  4. Establishment of short term culture of P. vivax.
  5. Initiation of more fusion.
1. Cloning of hybrids secreting anti-P. vivax antibodies.

In our previous report, 28 of 385 hybrids secreted anti-P. vivax antibodies. We have now cloned these hybrids by limiting dilution and eventually obtained 46 clones. The antibodies derived from these clones were collected either from the supernate from culture in RPMI-1640 medium with 10% fetal bovine serum or from the ascitic fluid of pristin primed BALB/c mice.

## 2. Characterization of MABs.

based on classification previously described (Khusmith et al., 1984), our MABs could be divided into group I, II, III and V. The number of monoclones and their IFA patterns is given in table 1. Appearances of their IFA staining are shown in figures 1-4. We have not yet determined the molecules to which these MABs react. Some of these MABs may react to the same antigenic determinants. As soon as we have acquired sufficient quantity of antigens, we shall then perform immunoblot to identify these molecules.

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Table 1. Monoclones and their IFA staining patterns

Group	Staining patterns	Number of monoclones
I	Bright generalized staining of all blood stages	25
II	Strong reactions with whole merozoites or their organelles	12
III	The surface membranes of merozoites with cluster of grapes appearance	1
IV	Strong reaction with surface of trophozoites and schizonts	8
Total		46

### 3. Successful concentration of P. vivax infected cells.

In our previous report, our preparation of P. vivax infected red blood cells (RBCs) were invariably contaminated with leucocytes. We have now successfully developed a technique to remove leucocyte contamination and obtain almost leucocyte-free preparation of P. vivax infected (RBCs).

A one ml aliquot of heparinized whole blood from a patient with vivax malaria was passed through a column containing an equal volume of sulfoctylcellulose and Sephadex G-25 previously equilibrated with phosphate buffer pH 7.5 (680 mg KH<sub>2</sub>PO<sub>4</sub>, 40.9 ml of 0.1 N NaOH, 807.3 mg MgCl<sub>2</sub>·6H<sub>2</sub>O and H<sub>2</sub>O to make 100 ml). The column was then washed with approximately 40 ml of the buffer until the column was free from RBCs. The eluate was centrifuged, the supernate removed and the cell sediment restored to 50% hematocrit. The cell suspensions from several tubes were pooled and two ml each was layered on top of discontinuous gradients comprising successive layers of two ml each of 75%, 60%, 50% and 40% of Percoll in the same buffer. After centrifugation at 2,600 rpm for 10 min at 20 deg C, two and some time three bands were obtained. The first and second bands were localized in the layers of 50% and 60% Percoll and the third band in the interface of 60% and 75% Percoll. The cells in the first and second bands were pooled and called 'upper band', whereas the third band was called 'lower band'. The smears were made from the blood prior to passage through the column and after Percoll gradient centrifugation, stained with Giemsa and the result examined by light microscopy.

The study was performed in 10 patients, in five of whom the cell fractions in upper and lower bands were pooled. In these patients, the parasites were enriched from the initial mean parasitemia (+ S.D.) of 6 6  
15.4±6.5 per 10 RBC to 5112±2125 per 10 RBC (table 2), an enrichment of 331.9 folds. In three patients (PV050, PV052 and PV054), only ring stage

were recovered after enrichment. In the remaining five patients in whom the parasites from the upper and lower bands were separated, it is shown (table 3) that the parasites were concentrated from the initial parasitemia of 15.4±6.6 to 2935±2387 in the upper band and 5006±3184 in the lower band an enrichment of 255 and 325 fold respectively. The proportion of parasite stages in the upper and lower bands was not well defined, since ring and trophozoite stages were found overlapping in both bands. Nevertheless if the trophozoites were present, they were found in higher proportion (e.g. PV051) in the upper than in the lower bands. The mean (+ S.D.) parasite recovery rate in 8 patients (table 4) is 57.6±31.4 (range 9.6-87.8).

Table 2. Enrichment of *P. vivax* infected red blood cells from the blood of malaria patients in whom parasites in upper and lower bands were pooled.

Patient	Before enrichment								After enrichment							
	4				Differential				4				Differential			
	Parasite stage/10 <sup>6</sup> rbc				count/10 <sup>6</sup> rbc				Parasite stage/10 <sup>6</sup> rbc				count/10 <sup>6</sup> rbc			
R	T	S	Total	PMN	L	Eo	Total	R	T	S	Total	PMN	L	Eo	Total	
PV050	4	7	0	11	6	1	0	7	5540	0	0	5540	0	0	0	0
PV052	18	0	0	18	1	0	0	1	8200	0	0	8200	0	0	0	0
PV054	7	1	0	8	5	0	0	5	2260	0	0	2260	0	0	0	0
PV057	14	1	0	15	9	4	0	13	4000	750	0	4750	0	10	0	0
PV058	22	3	0	25	5	0	0	5	4340	470	0	4810	9	0	0	0
Total	13	2.4	0	15.4	5.2	1	0	6.2	4868	244	0	5112	0	2	0	0
	+7.5	+2.8		+6.6	+2.9	+1.7		+4.4	+2201	+348		+2125		+4.5		

4

Table 3. Enrichment of *P. vivax* infected red blood cells from malaria patients in whom parasites from upper and lower bands were separated.

Patient	Before enrichment								After enrichment															
									Upper band								Lower band							
	Parasite stage per 10 <sup>4</sup> rbc				Differential count/10 <sup>4</sup> rbc				Parasite stage per 10 <sup>4</sup> rbc				Differential count/10 <sup>4</sup> rbc				Parasite stage per 10 <sup>4</sup> rbc				Differential count/10 <sup>4</sup> rbc			
	R	T	S	Total	PMN	L	Eo	Total	R	T	S	Total	PMN	L	Eo	Total	R	T	S	Total	PMN	L	Eo	Total
PV051	14	4	0	18	4	0	0	4	1710	4520	0	6230	0	1	0	1	3820	2940	0	5760	0	1	0	1
PV053	16	0	0	16	1	0	0	1	2520	0	0	2520	0	0	0	0	1170	0	0	1170	0	0	0	0
PV055	28.5	1	0	29.5	3.5	2	0	5.5	7180	80	0	7260	0	0	0	0	8540	0	0	8540	0	0	0	0
PV056	13	4	0	17	0	3	0	3	4310	140	0	4450	0	2	0	2	2260	0	0	2260	0	0	0	0
PV059	8	0	0	8	4	1	0	5	2100	0	0	2100	0	0	0	0	7300	0	0	7300	0	0	0	0
Total	15.9	1.3	0	17.7	2.5	1.2	0	3.7	3564	948	0	3935	0	0.6	0	0.6	4418	0	0	5006	0	0.1	0	0.2
	-7.6	+2.0		-7.7	+1.9	-1.3		+1.8	-2252	+1997	0	+2387		-0.9		-0.9	+328			-3184		+0.45		-0.45

Table 4. Parasite recovery rate from eight patients with vivax malaria after enrichment.

Patient	Before enrichment			After enrichment	
	Number of parasites per 1 ml of blood ( $\times 10^6$ )	Volume of blood (ml)	Total number of parasites ( $\times 10^6$ )	Number of parasites recovered	Percent recovery
PV050	11.1	1	11.1	9.75	87.8
PV051	14.1	5	70.5	29.3	41.6
PV052	9.45	4	37.8	32.9	87.0
PV053	12.2	5	61.0	5.85	9.6
PV054	3.15	6	18.9	12.6	66.7
PV057	4.8	6	28.8	18.5	64.2
PV058	18.3	3	54.9	9.6	17.5
PV059	2.7	6	16.2	13.96	86.2
		Total	37.4 +22.4	16.56 +9.75	57.6 +31.4

4. Establishment of short term culture of P. vivax parasites.

The continuous supply of P. vivax infected red blood cells is most needed in this study to be used in the production of MABs and for their screening and characterization especially by the IFA and immunoblot technique. Though the continuous in vitro culture technique for P. vivax has not been established, short term cultivation has been successfully developed in several laboratories. In Thailand, Brockelman et al. (J. Parasitol. 32:76, 1962) have recently devised a new culture medium, the SCMI 612, in which as high as 99% of ring-infected red blood cells developed synchronously to gametocytes. With this lead, we therefore attempted to culture this parasites using initially the SCMI 612 medium. We shall attempt also to devise other culture medium by supplementing the RPMI-1640 with MPT2, G412 and hypoxanthine. In 3 patients in whom the culture in SCMI 612 had been attempted, it was found (table 5) that in one patient (PV044) the parasites developed up to day 6 after completing only one cycle of in vitro growth and then died on day 9. In two other patients (PV045 and PV046) the parasites had maturation arrest and died on day 6 and day 4 of culture respectively. Though we did not have much success in our initial attempts, we had been able to prepare slides for IFA to screen our MABs. We shall try again to culture these parasites in order to provide us with materials for characterization of our MABs.

Table 5. Growth of P. vivax parasites on different days of culture in SCMI 162 medium.

Patient	Percent Parasitemia							
	d0	d2	d4	d5	d6	d7	d8	d9
PV044	0.3 (T 20% R 20%)	0.3 (S 70% T 30%)	0.6 (T 50% R 50%)	-	0.7 (R 90% T 10%)	-	0.02	die
PV045	0.7 (mostly T)	0.4 (mostly T)	0.1	0.02	die			
PV046	0.1 (R 30% T 70%)	0.02 (mostly T)	die					

R = ring, T = trophozoite, S = schizont

5. Initiation of more fusion. Two more BALB/c mice have been immunized with P. vivax parasites and the fusion will be made during the next two, three weeks.

Patterns of indirect immunofluorescent antibody (IFA)  
staining produced by monoclonal antibodies on acetone-fixed blood  
films of P. vivax

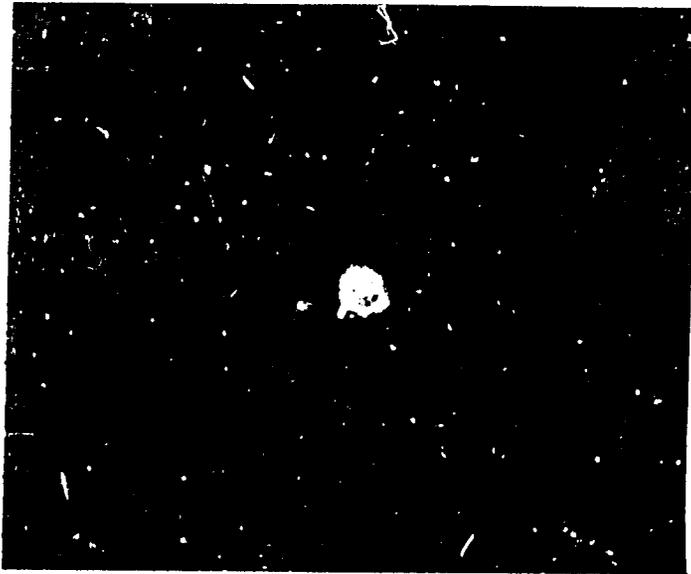


Fig. 1 Staining with group I antibodies. Bright generalised  
staining of schizonts, rings is shown.



Fig. 2 Staining with group II antibodies showing reaction with whole intraschizont merozoites (fig. 2a) and free merozoites as well as organelles (probably rhoptries) of intraschizont merozoites (fig. 2b).

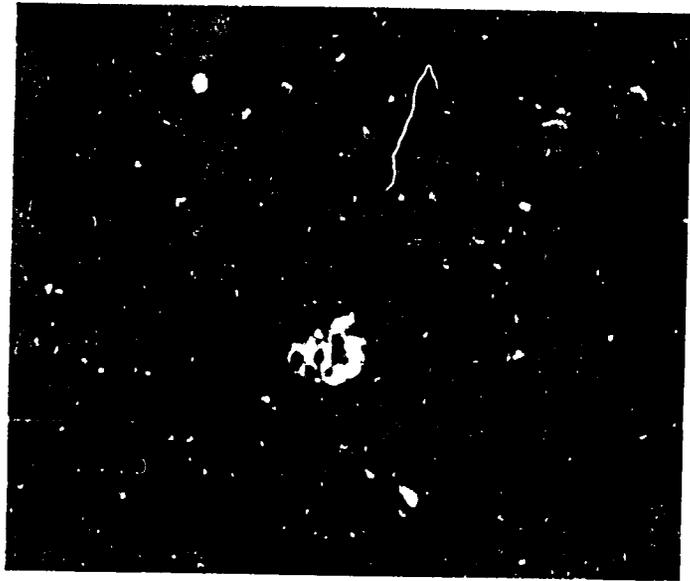


Fig. 3 Staining with group III antibodies showing reaction with the perimeters of intra-schizont merozoites making an appearance of a cluster of grapes.

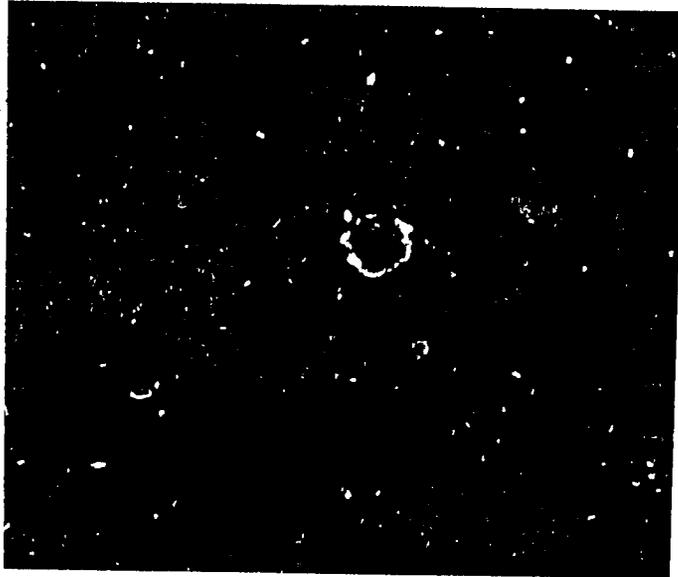


Fig. 4 Staining with group V antibodies reaction (mottling)  
with the surface of the schizonts or tryphozoites.

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WASHINGTON, D.C. 20548

DATE: \_\_\_\_\_

8/4/88

MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18  
FROM: AID/SCI, Victoria use *ru*  
SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. 5. 232  
*PR # 5*

Attachment

5. 232  
XN-0BA-541-A

PROGRESS REPORT TO USAID

PROGRESS REPORT NO. 5

"ANTIGENIC DIVERSITY OF P. VIVAX

A RESEARCH PROJECT

USAID/PSTC PROGRAM

GRANT NO. 936-5542-G-00-5078-00

SUBMITTED BY

SRISIN KHUSMITH

SAVANAT THARAVANIJ

DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

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AUG 03 1988

13

ANTIGENIC DIVERSITY OF PLASMODIUM VIVAX

## Project Profile

Grant No. : 936-5542-G-00-5078-00  
Program : Program on Science and Technology Cooperation  
Project Title : Antigenic diversity of *P. vivax*  
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Total Project Budget : U.S. \$ 150,000  
Project Duration : September 1985 - February 1988  
Reporting Period : September 1987 - February 1988

## Overall Objectives

1. To raise and characterize monoclonal antibodies against blood stages of *P. vivax*
2. To determine whether there is antigenic diversity among different strains of *P. vivax*
3. To investigate whether distribution of different antigenic types is related to their geographical locations
4. To demonstrate whether the strain recrudescence after anti-malaria drug therapy is the same or different from those causing initial clinical symptoms

## Plans of work

- 1) Production of monoclonal antibodies against *P. vivax*
- 2) Characterization of monoclonal antibodies against *P. vivax*
  - 2.1 IFA
  - 2.2 SDS-PAGE and Western blot analysis
- 3) Preliminary screening of monoclonal antibodies and select them for establishment of a panel of monoclonal antibodies for strain specificities
- 4) Typing of *P. vivax* isolates using several monoclonal antibodies and demonstrate their antigenic diversity
  - 4.1 Isolates from patients admitted to the Hospital for Tropical Diseases
  - 4.2 Isolates from *P. vivax* cases from malaria endemic areas in Thailand
- 5) Demonstration whether there is any antigenic disparity between parasites causing initial clinical symptoms and those causing recrudescence

## Scientific Progress

Following progress has been made :

1. Characterization of anti-*P. vivax* monoclonal antibodies.
  - 1.1 Patterns of immunofluorescence
  - 1.2 SDS-PAGE and Western blot analysis
  - 1.3 Immunoglobulin class and subclass of a panel of monoclonal antibodies
  - 1.4 Species cross-reactivity
2. Establishment of a panel of monoclonal antibodies for investigation the antigenic diversity of *P. vivax*.
3. Collection and preparation of *P. vivax* slides from vivax malaria patients attending malaria clinics in endemic areas.
4. To explore the degree of polymorphism that prevailed in a series of *P. vivax* isolates obtained from infected patients.
5. Calculation the frequencies of several parasite antigens in the samples.
6. To examine the distribution of antigenic variation and geographic distribution.
7. Preparation of *P. vivax* antigens for immunofluorescent test and Western blot analysis.

## 1. Characterization of anti-*P. vivax* monoclonal antibodies

### 1.1 Patterns of immunofluorescence

In three fusions using various isolates of *P. vivax* from different geographical locations in Thailand, 1175 hybrid cultures were obtained of which 494 secreted antiplasmodial antibodies detectable by indirect immunofluorescence assay. Since our hybridomas were raised against a mixture of developmental stages of the parasite, the antibodies produced by them were expected to recognize a range of different antigens.

A number of different indirect immunofluorescence assay (IFA) reactivity patterns were distinguished during the primary screening, and 80 hybrids producing antibodies representing five patterns were successfully cloned.

Individual monoclonal antibodies (McAbs) produced characteristic patterns of indirect immunofluorescence staining on acetone-fixed smears of *P. vivax*. The pattern produced by any given antibody was the same on all parasite isolates which reacted with that McAb regardless of whether culture supernatants or ascitic fluids were used.

Based on the immunofluorescence reactivities demonstrated with *P. falciparum* (Khusmith et al., Asian Pacific J. Allergy and Immunology 2:91-94, 1984). Five staining patterns were recognized and the antibodies were classified into five corresponding groups (Table 1). These characteristic IFA patterns are indicative of stage specificity of the IFA reaction and possible location of the reactive antigen(s) either on the parasite or on or in the infected erythrocytes.

Table 1. Monoclones and their IFA staining patterns from 3 fusions

Group	Staining patterns	Number of monoclones
I	Bright <i>generalized</i> staining of all blood stages	67
II	Strong reactions with merozoites as well as organelles	21
III	Strong reactions with the surface membranes of merozoites with cluster of grape appearance	5
V	Strong reactions with the surface of schizont and trophozoites	16
VII	Strong irregular staining of internal components of trophozoites	46

## 1.2 SDS-PAGE and Western blot analysis

Electrophoresis was carried out in a vertical slab gel apparatus (Bio-Rad, U.S.A.) using the method of Laemmli and Favre (1973) (Nature 227: 680-685). A 4% acrylamide stacking gel and a 10% acrylamide separating gel were used. Samples containing various amounts of *P. vivax* blood stages in RPMI-1640 medium were heated at 100° C for 3 minutes in the presence of sodium dodecyl sulphate (SDS) and 2-mercaptoethanol before loading onto the gel. Each lane contained  $5 \times 10^6$  infected red blood cells. The electrophoresis was made at 30 mA per slab gel for 3-4 hours until the tracking dye reached the bottom of the gel. After electrophoresis the gels were cut and subjected to protein staining, or electroblotted onto a 0.45  $\mu$ m nitrocellulose membrane (Bio-Rad Laboratories, California, U.S.A.) according to the method of Towbin et al., (1979) (Proc. Nat. Acad. Sci. 76: 4350-4354). After blotting the unreacted sites on the membranes were blocked by soaking the strips in phosphate buffered saline (containing 3% gelatin and 0.02% sodium azide (PBS-GEL)) at 4° C for 16 hours. The blots were treated with 2 ml of monoclonal antibody or supernatant of positive clones at 26° C for 2 hours with gentle rocking. The strips were then washed 5 times with PBS containing 0.05% Tween 20 and then treated with  $^{125}$ I labeled anti-mouse immunoglobulin (Amersham, England) ( $1 - 5 \times 10^5$  cpm/ml in PBS-BSA) at 26° C for 30 minutes with rocking. The nitrocellulose strips were washed as above, dried and autoradiographed on Kodak X-Omat RP films (Eastman Kodak Co., N.Y., U.S.A.) at -70° C.

Results of the antigen analysis by SDS-PAGE and the results of the Western blot analysis of selected 58 monoclonal antibodies with variable IFA reactivities are shown in Fig. 1 and Table 2, respectively.

Fig. 1 SDS-PAGE analysis of *P. vivax* antigens obtained from infected blood



24

Fig. 2 Western blot analysis of the molecules of *P. vivax* antigen

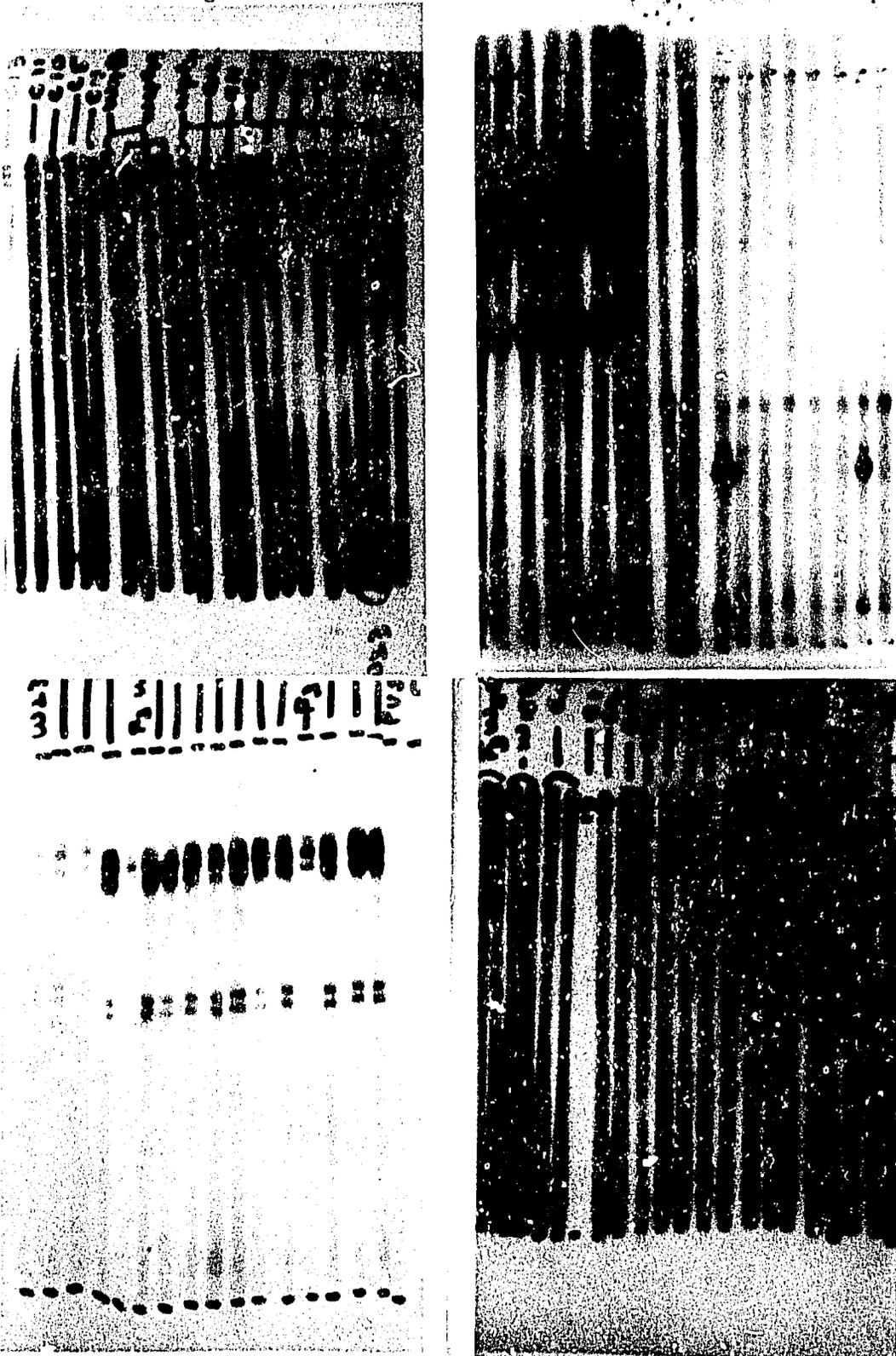


Table 2. Western blot analysis of the molecules of *P. vivax* antigen reacting with a panel of 58 anti-*P. vivax* monoclonal antibodies

Monoclonal antibodies	Number of monoclonal antibodies tested	Molecule of <i>P. vivax</i> antigens in kilodalton to which monoclonal antibodies react
Group I	21	PV15, PV28, PV30, PV36, PV39, PV85, PV95, PV100, PV115, PV200, PV>200
Group II	17	PV30, PV35, PV41, PV68, PV75, PV90, PV100, PV110, PV135, PV200
Group III	5	PV30, PV57, PV67, PV75, PV190
Group V	7	PV36, PV39, PV85, PV95, PV115
Group VII	8	PV30, PV85

### 1.3 Immunoglobulin class and subclass of 58 anti-P. vivax antibodies

A technique modified from Beyer (1984) (J. Immunol. Methods, 67:79-87) was used. Briefly, strips (9.5 x 0.6 cm) of nitrocellulose membrane (33 BA88, Schleicher & Schuell, Dassel, West Germany) were soaked in Tris buffered saline pH 7.5 (TBS) and dried on absorbent paper for 5 min. Three ul of monoclonal antibodies was spotted to each strip and dried. The strips were incubated together in 100 ml of TBS containing 0.2% gelatin for 30 min, rinsed twice in TBS and each strip was individually incubated for two hours in five ml of appropriately diluted anti-human IgG1, IgG2a, IgG2b, IgG3, IgG4 and IgM (Serotec, Blackthorn, Beccater, England). After incubation, the strips were rinsed in TBS and then incubated for one hour in appropriately diluted peroxidase-protein A conjugated solution (Sigma Chemicals, St. Louis, Missouri). After washing in TBS, the strips were transferred to a solution of 4-chloro-1-naphthol (Sigma) and incubated further for 10-30 min. The strips were rinsed in water to stop the reaction and the result read with naked eyes.

Results of the isotyping of monoclonal antibodies to blood stages of P. vivax are shown in table 3.

Table 3. Isotyping of the 58 *P. vivax* monoclonal antibodies

Immuno- fluorescent patterns	Number of monoclonal tested	Isotypes					
		IgG1	IgG2a	IgG2b	IgG3	IgG4	IgM
Group I	21	12	2	-	1	-	6
Group II	17	17	-	-	-	-	-
Group III	5	3	1	-	-	-	1
Group V	7	5	-	-	2	-	-
Group VII	8	5	-	-	-	-	2

#### 1.4 Species cross-reactivity

Eleven strains of *P. falciparum* were obtained from acutely infected individuals and those with cerebral malaria. These isolates were cryopreserved in our laboratory since 1980 as shown in Table 4.

Table 4. Eleven isolates of *P. falciparum*

Isolate	Malaria	Date obtained
S0	acute	February 18, 1980
H5	acute	June 28, 1983
H11	acute	November 7, 1983
K1	acute	July 7, 1983
BW2	acute	-
SN3-5	acute	-
I1	cerebral	November 7, 1983
R5	cerebral	July 8, 1983
R1	cerebral	May 5, 1983
R6	cerebral	July 11, 1983
QD7	cerebral	November 8, 1983

The cryopreserved parasites was thawed out immediately at 37°C for 30 sec. The parasites were centrifuged at 500 x g for 5 min at room temperature and the supernatant was discarded. The cell sediment was washed with an equal volume of saline solution (3.5% NaCl) and then washed at least twice with RPMI 1640 medium. The parasites were recultured by adding

washed uninfected AB erythrocytes and RPMI 1640/10% AB serum to obtain a final concentration of 5% haematocrit, 0.1% parasitaemia and then incubated in a candle jar at 37°C (Trager & Jensen 1976, Science, 193: 673-675).

The species specificity of monoclonal antibodies was determined by an indirect fluorescent antibody test using acetone-fixed infected blood as antigen (Khusmith et al., 1984, Asian Pacific J. Allerg. Immunol., 2: 91-95). Blood smears were made from washed asynchronously grown strains of *P. falciparum* and stored at -70°C in sealed containers with silica gel until required. The results are shown in Table 5.

Table 5. Reactivities of strains and species specific monoclonal antibodies with different strains of *P. vivax* and *P. falciparum* strains

Monoclonal antibodies <i>P. vivax</i> (MPV)	IFA patterns with <i>P. vivax</i>	Strain distribution of MPV		Cross reactivities with <i>P. falciparum</i>
		No positive strains/ total strains tested		No positive strains/ total strains tested
MPV1-MPV18, MPV55-MPV57	Group I	3/10 - 10/10		0/11
MPV19-MPV35	Group II	5/10 - 8/10 <sup>a</sup>		0/11
MPV36, MPV38, MPV54, MPV58	Group III	3/10 - 9/10 <sup>a</sup>		0/11
MPV39-MPV45	Group V	5/10 - 8/10		0/11
MPV46-MPV53	Group VII	6/10 - 10/10		0/11

<sup>a</sup> Only 9 isolates of *P. vivax* tested have schizont stage

2. Establishment a panel of monoclonal antibodies for investigation the antigenic diversity of *P. vivax*

Among 155 anti-*P. vivax* monoclonal antibodies. A panel of 58 McAbs that recognized different molecules of antigens and hence different IFA patterns were selected.

Table 6. Characteristics of *P. vivax* asexual erythrocytic stages McAbs

McAb <sup>a</sup>	Isotype	IFA pattern	Cross reactivity <i>P. falciparum</i>	Isolated specificity (%) <sup>b</sup>	Mr (10 <sup>3</sup> )
MPV1	IgG1	I	-	100	30, 85
MPV2	IgG1	I	-	100	30, 85
MPV3	IgG1	I	-	100	30
MPV4	IgG3	I	-	40	100
MPV5	IgG1	I	-	90	30
MPV6	IgG1	I	-	30	100
MPV7	IgG1	I	-	90	30
MPV8	IgG1	I	-	90	30
MPV9	IgG1	I	-	90	30
MPV10	IgM	I	-	30	>200
MPV11	IgG1	I	-	80	36, 39, 85, 95, 115
MPV12	IgG1	I	-	50	100, 135, >200

a = monoclonal antibodies

b = ten isolates were initially tested

McAb <sup>a</sup>	Isotype	IFA pattern	Cross reactivity P. falciparum	Isolated specificity (%) <sup>b</sup>	Mr (10 <sup>3</sup> )
MPV13	IgM	I	-	60	30
MPV14	IgM	I	-	50	135, 200
MPV15	IgM	I	-	80	35, 85
MPV16	IgM	I	-	90	100, 135, >200
MPV17	IgM	I	-	80	36, 39, >200
MPV18	IgG1	I	-	90	>200
MPV55	IgG2a	I	-	40	15, 28
MPV56	IgG2a	I	-	30	28
MPV57	IgG1	I	-	30	200
MPV19	IgG1	II	-	100	67, 75
MPV20	IgG1	II	-	100	35, 41
MPV21	IgG1	II	-	100	41, 88, 75, 90
MPV22	IgG1	II	-	100	110, 200
MPV23	IgG1	II	-	100	200
MPV24	IgG1	II	-	100	11, 90, 110, 200
MPV25	IgG1	II	-	100	200
MPV26	IgG1	II	-	100	110
MPV27	IgG1	II	-	100	30, 50
MPV28	IgG1	II	-	80	39
MPV29	IgG1	II	-	50	95

a = monoclonal antibodies

b = ten isolates were initially tested

52

McAb <sup>a</sup>	Isotype	IFA pattern	Cross reactivity P. falciparum	Isolated specificity (%) <sup>b</sup>	M <sub>r</sub> (10 <sup>3</sup> )
MPV30	IgG1	II	-	90	115
MPV31	IgG1	II	-	100	30
MPV32	IgG1	II	-	60	36
MPV33	IgG1	II	-	80	30, 50, 85
MPV34	IgG1	II	-	100	50, 85
MPV35	IgG1	II	-	80	115, 200
MPV36	IgG2a	III	-	90	30, 68, 100
MPV37	IgG1	III	-	100	95
MPV38	IgM	III	-	30	100
MPV54	IgG1	III	-	100	85
MPV58	IgG1	III	-	90	30, 41, 68
MPV39	IgG1	V	-	80	85, 95
MPV40	IgG1	V	-	50	36
MPV41	IgG1	V	-	70	115
MPV42	IgG3	V	-	50	36
MPV43	IgG3	V	-	50	36
MPV44	IgG1	V	-	80	85, 95
MPV45	IgG1	V	-	60	115
MPV46	IgG1	VII	-	60	85
MPV47	IgG1	VII	-	70	85

a = monoclonal antibodies

b = ten isolates were initially tested

McAb <sup>a</sup>	Isotype	IFA pattern	Cross reactivity P. falciparum	Isolated specificity (%) <sup>b</sup>	Mr (10 <sup>3</sup> )
MPV48	IgG1	VII	-	100	30
MPV49	IgG1	VII	-	100	30
MPV50	IgGM	VII	-	70	85
MPV51	IgGM	VII	-	60	85
MPV52	IgG1	VII	-	90	30
MPV53	IgG1	VII	-	90	85

a = monoclonal antibodies

b = ten isolates were initially tested

### 3. P. vivax antigen slides for indirect fluorescent antibody test (IFA)

Blood smears were made from :

1. P. vivax infected cells obtained from patients with vivax malaria (PV001-PV300) admitted to the Hospital for Tropical Diseases, Bangkok. These patients were from different areas of malaria including :
  - 1.1 Chanthaburi province
  - 1.2 Kanjanaburi province
  - 1.3 Trat province
  - 1.4 Tak province
  - 1.5 Ratburi province
  - 1.6 Chachoengsao province
  - 1.7 Prachin Buri province
  - 1.8 Chumpon province

- 1.9 Prachuap Khiri Khan province
- 1.10 Nakhon Sawan province
- 1.11 Chon Buri province
- 1.12 Surat Thani province
- 1.13 Nong Khai province
- 1.14 Nan province
- 1.15 Phet Buri province
- 1.16 Rayong province
- 1.17 Phitsanulok province
- 1.18 Samut Sakhon province
- 1.19 Nakhon Ratchasima province
- 1.20 Nakhon Si Thammarat province
- 1.21 Buriram province
- 1.22 Nakhon Nayok province
- 1.23 Uthai Thani province
- 1.24 Phetchabun province
- 1.25 Chiangrai province
- 1.26 Ubonrajithani province
- 1.27 Krabi province
- 1.28 Lop Buri province
- 1.29 Narathiwat province
- 1.30 Nakhon Ratchasima province
- 1.31 Kumphaeng Phet province
- 1.32 Udon Thani province
- 1.33 Uttaradit province
- 1.34 Lamphun province
- 1.35 Mae Hong Son province
- 1.36 Burma

The blood from patients was washed 5 times with RPMI-1640 without serum and the cell sediment resuspended in the same medium, followed by smear on clean glass slides, quickly dried in air, kept for a day at room temperature placed in a sealed plastic envelope together with silica gel and stored at -70°C until used.

2. Concentrated P. vivax infected red cells obtained from patients with vivax malaria (PV001-PV300). Enrichment of P. vivax infected RBCs was achieved by using SEC-G-25-Percoll method described recently (Tharavanij et al., Southeast Asian J. Trop. Med. Pub. Hlth., 18: 39-43). The concentrated P. vivax infected cells were washed 3 times with PBS pH 7.2 and the resuspended sediment, were smeared on clean glass slides, quickly dried in air, kept for a day at room temperature, placed in a sealed plastic envelope together with silica gel and stored at -70°C until used.

3. P. vivax infected cells obtained from patients attending malaria clinics in Umphur Mee Sariang, Chiangmai province (CMPV1-CMPV50). Thin blood smears were made directly from the patients on clean glass slides, quickly dried in air, kept for a day at room temperature, placed in a sealed plastic

envelop together with silica gel and sent immediately to the laboratory in Bangkok. The slides were then stored at  $-70^{\circ}\text{C}$  until used.

#### 4. Demonstration of antigenic diversity in *P. vivax*

A panel of 58 McAbs were used to demonstrate considerable antigenic diversity in 300 primary isolates of *P. vivax* collected from 35 provinces around Thailand including one isolate from Burma using an immunofluorescent test. The indirect fluorescence using acetone-fixed infected blood as antigen was used according to the technique previously described (Khusmith et al. 1984, Asian Pacific J. Allerg. Immunol., 2:91-95). Smears were made from washed infected blood of patients with *P. vivax* and stored at  $-70^{\circ}\text{C}$  in sealed containers with Silica gel until required. The IFA was carried out at  $37^{\circ}\text{C}$  in a humid chamber. After fixation in acetone ( $-20^{\circ}\text{C}$ ) each well was treated with 10 ul of listed monoclonal antibodies for 2 hours at  $37^{\circ}\text{C}$ , the slides were washed vigorously and reacted further for another hour with fluorescein isothiocyanate conjugated goat anti-mouse IgG, A, M (Pasteur Institute, France). After washing and drying the slides were mounted in a mounting medium containing 4.5% N propyl gallate in 0.2 M Tris in glycerol and examined under a fluorescent microscope.

The percentages of parasites reacting with particular monoclonal antibodies were estimated by assessing the proportions of malaria pigment granules (as observed by normal transmitted

white light) which were associated with fluorescent parasites (as observed by incident UV light). To exclude inter-observer error one person read all the IFA slides. Positive reactivities of these McAbs with an isolate were scored (+ to +++) depending on the intensity of the fluorescence, and in each case the proportion of parasite giving a positive reaction was noted.

Steps taken to ensure the validity of results :

1. A single batch of all monoclonal antibodies was used for the entire investigation.
2. At least 4 different isolates were processed for the IFA and assayed simultaneously.
3. The assay was standardized from one batch of isolates to another by including a previously tested isolate in each batch.

Reactivities of these monoclonal antibodies with 300 different parasite isolates obtained from patients from 35 provinces in Thailand including one isolate from Burma revealed a high level of antigenic diversity of this parasite. About 10 monoclonal antibodies reacted with epitopes that were represented in more than 90% of parasite isolates, and therefore, appeared to be relatively conserved among different isolates. The remaining monoclonal antibodies reacted with only 30% to 80% of parasite isolates.

5. Calculation the frequencies of several parasite antigens in the samples

The data are currently under analysis.

## 8. The distribution of antigenic variation and geographic distribution

The data are currently under analysis. Our preliminary result showed that the antigenic diversity of *P. vivax* occurs even in the same area in Thailand.

## 7. Preparation of *P. vivax* antigens for immunofluorescent test and Western blot analysis

Enrichment of *P. vivax* infected RBCs was achieved by using SEC-G-25-Percoll method described recently. The specimens were either processed immediately or stored overnight at 4° C.

A one ml aliquot of heparinized whole blood was passed through a column an equal volume of sulfoethyl cellulose and Sephadex G-25 previously equilibrated with phosphate buffer pH 7.5. The column was then washed with approximately 40 ml of the buffer until the column was free from RBCs. The eluate was centrifuged, the supernate removed and the cell sediment restored to 50% hematocrit. The cell suspensions from several tubes were pooled and two ml each was layered on top of discontinuous gradients comprising successive layers of two ml each of 75%, 60%, 50% and 40% of Percoll in the same buffer. After centrifugation at 2,800 rpm for 10 min at 20° C. Two and sometime three bands were obtained. The first and second bands were localized in the layers of 50% and 60% Percoll and the third band in the interface of 60% and 75% Percoll. The cells in the first and second bands were pooled and called "upper band", whereas the third band was called "lower band". The smears were made from the blood prior to passage through the column and after

Percoll gradient centrifugation, stained with Giemsa and the result examined by light microscopy.

Cells in the upper and lower consisted of both trophozoites and ring stages with more mature trophozoites and a relatively higher percentage of trophozoites in the upper band. Enrichment of *P. vivax* infected red blood cells from the blood of 160 malaria patients were summarized in table 7.

Table 7. Parasite yields after enrichment

Patient	Parasite count ( x 10 <sup>7</sup> )		
	Upper band	Lower band	Total
PV141-PV142	1.60	15.00	16.60
PV143	ND	ND	ND
PV144	0.99	1.20	2.19
PV145-PV147	0.02	6.20	6.22
PV148	1.50	12.00	13.50
PV149-PV150	168.00	170.00	338.00
PV151	14.00	12.00	26.00
PV152-PV154	4.50	5.50	10.00
PV155	12.00	18.00	30.00
PV156	ND	ND	15.00
PV157	1.80	5.30	7.10

ND = not done

Patient	Parasite count ( x 10 <sup>7</sup> )		
	Upper band	Lower band	Total
PV158-PV161	19.00	25.00	44.00
PV162-PV163	1.30	2.60	3.90
PV164	0.34	4.50	4.84
PV165	1.60	9.40	11.00
PV166	1.70	1.50	3.20
PV167	1.60	4.30	5.90
PV168	2.80	3.40	6.20
PV169-PV171	ND	ND	102.00*
PV172	7.00	1.10	8.10
PV173	1.10	0.94	2.04
PV174	30.00	59.00	89.00
PV175-PV176	ND	ND	32.00
PV177	1.80	36.00	37.80
PV178	TB	3.70	3.70
PV179	32.00	35.00	67.00
PV180-PV181	13.00	18.00	31.00
PV182	0.65	0.78	1.43
PV183-PV184	1.20	9.20	10.40
PV185	21.00	12.00	33.00
PV186	18.00	28.00	46.00

\* PV169-PV171 = 3 tubes, each fraction A + fraction B = 34.00  
 ND = not done

41

Patient	Parasite count ( x 10 <sup>7</sup> )		
	Upper band	Lower band	Total
PV187-PV189	7.50	15.00	22.50
PV190-PV192	6.20	12.00	18.20
PV193-PV194	10.00	13.00	23.00
PV195-PV197	12.00	32.00	44.00
PV198-PV199	4.60	10.00	14.60
PV200-PV201	6.30	14.00	20.30
PV202-PV204	34.00	57.00	91.00
PV205	21.00	74.00	95.00
PV206	1.80	1.80	3.60
PV207-PV208	9.40	49.00	58.40
PV209-PV213	8.70	37.00	45.70
PV214-PV216	2.00	14.00	16.00
PV217-PV219	22.00	40.00	62.00
PV220	24.00	71.00	97.00
PV221-PV224	14.00	73.00	87.00
PV225-PV226	3.40	21.00	24.40
PV227	2.40	15.00	17.40
PV228	12.00	71.00	83.00
PV229-PV231	12.00	31.00	43.00
PV232	4.00	17.00	21.00
PV233-PV235	10.00	110.00	120.00
PV236-PV239	28.00	260.00	288.00

Patient	Parasite count ( x 10 <sup>7</sup> )		
	Upper band	Lower band	Total
PV240-PV241	14.00	190.00	204.00
PV242-PV244	TB	150.00	150.00
PV245-PV248	7.20	100.00	107.20
PV249-PV250	6.30	23.00	29.30
PV251-PV252	9.90	35.00	44.90
PV253-PV256	15.00	260.00	275.00
PV257	2.50	12.00	14.50
PV258-PV259	ND	ND	27.00
PV260-PV261	1.80	2.50	4.30
PV262-PV263	4.20	280.00	284.20
PV264	4.20	2.90	7.10
PV265-PV267	2.90	10.00	12.90
PV268-PV269	8.30	28.00	36.30
PV270-PV272	TB	39.00	39.00
PV273-PV275	7.80	14.00	21.80
PV276-PV279	12.00	49.00	61.00
PV280-PV283	5.50	100.00	105.50
PV284-PV287	ND	ND	48.00
PV288-PV289	2.60	10.00	36.00
PV290-PV291	10.00	21.00	31.00
PV292-PV293	3.10	5.50	8.60

TB = tube was broken during experiments  
 ND = not done

45

Patient	Parasite count ( x 10 <sup>7</sup> )		
	Upper band	Lower band	Total
PV294-PV296	11.00	21.00	32.00
PV297	4.40	20.00	24.40
PV298-PV300	5.40	28.00	33.40

### Conclusion

We are now in the stage of data analysis to investigate whether distribution of different antigenic types is related to their geographic locations or not. The blood samples are now being collected from the vivax patients before and after recrudescence in order to demonstrate whether the strain recrudescence after anti-malaria drug therapy is the same or different from those causing initial clinical symptoms using a panel of monoclonal antibodies.

45