รายงานวิจัยฉบับสมบูรณ์

การตรวจหา Common epitopes ของ Duffy Binding Protein II ที่กระตุ้นให้เกิดภูมิคุ้มกันต่อต้านเชื้อ *Plasmodium vivax* สายพันธุ์ที่ระบาดในกลุ่มคนไข้ไทยเพื่อนำไปสู่การผลิต DBPII vaccine

โดย

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เมษายน พ.ศ. 2560

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มหาวิทยาลัยมหิดล

(บทคัดย่อ)

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ชื่อโครงการ: การตรวจหา Common epitopes ของ Duffy Binding Protein II ที่กระตุ้นให้เกิดภูมิคุ้มกันต่อต้านเชื้อ *Plasmodium vivax* สายพันธุ์ที่ระบาดในกลุ่มคนไข้ไทยเพื่อนำไปสู่การผลิต DBPII vaccine

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บทคัดย่อ

Plasmodium vivax Duffy Binding Protein II (PVDBPII) มีบทบาทสำคัญในการจับกับโมเลกุลตัวรับเพื่อการบุกรุกเข้าสู่เซลล์ reticulocyte ดังนั้น PvDBPII จึงเป็นโมเลกุลที่มีความสำคัญและมีศักยภาพสูงในการผลิตวัคซีนต่อต้านโรคมาลาเรียชนิด *Plasmodium vivax* แต่อย่างไรก็ตามการกลายพันธุ์และการเกิดความหลากหลายของโมเลกุล PVDBPII เป็นอุปสรรคที่สำคัญในการผลิตวัคซีนป้องกันโรคมาลาเรีย ในการศึกษาครั้งนี้จึงได้ทำการวิเคราะห์การตอบสนองของแอนติบอดีจำเพาะต่อหลายสายพันธุ์ของแอนติเจน PvDBP หรือ (Broadly antibody response) ที่พบได้บ่อยในกลุ่มคนไข้ไทยคือ สายพันธุ์ PvDBL-TH2, -TH4, -TH5, -TH6 และ -TH9 โดยได้ผลิตโปรตีนลูกผสมเพื่อนำไปผลิต antisera จำเพาะต่อโปรตีนเหล่านี้และนำ antisera ไปประยุกต์ใช้ศึกษาการเกิด cross-reactivity โดยการทดสอบ ELISA depletion และนำไปศึกษาการเกิด broad inhibition การจับกันระหว่าง PvDBL-TH variants และเม็ดเลือดแดงโดยการทดสอบ *in vitro* COS7 erythrocyte inhibition binding assay นอกจากนี้การศึกษาครั้งนี้ยังได้ศึกษาคุณสมบัติ immunogecity ของ PvDBL-TH ในคนไข้มาลาเรียอีกด้วย ผลการทดลองพบว่า antisera จำเพาะต่อ PvDBL-TH สามารถกระตุ้นให้ระบบภูมิคุ้มกันสร้างแอนติบอดีที่มีคุณลักษณะในการจับข้ามสายพันธุ์ของ PvDBL-TH variants โดย anti-PvDBL-TH sera ที่ถูกดูดซับแอนติบอดีจำเพาะต่อ PvDBL-TH2 ไม่สามารถจับกับโปรตีนสายพันธุ์อื่นๆของ PvDBL-TH variants ได้ และ anti-PvDBL-TH2 ยังสามารถยับยั้งข้ามสายพันธุ์ได้อีกด้วยเมื่อทดสอบการยับยั้งของ PvDBL-TH variants ณ ความเข้มข้นของแอนติบอดีที่ยับยั้ง PvDBL-TH2 ได้ 50% (Inhibition concentration 50%) ผลการทดลองนี้บ่งชี้ให้เห็นถึงการเกิด cross-reactivity ของ anti-PvDBL-TH sera โดยแอนติบอดีสามารถจับกับ conserve epitopes ของ PvDBL-TH variants ได้ แต่อย่างไรก็ตามการศึกษานี้ยังตรวจพบ strain-specific antibody จำเพาะต่อ PvDBL-TH variants อีกด้วย anti-PvDBL-TH5 สามารถจับกับโปรตีน PvDBL-TH variants อื่นๆได้สูงอย่างมีนัยสำคัญทางสถิติหลังจากทำการดูดซับแอนติบอดีจำเพาะต่อ PvDBL-TH5 ออก นอกจากนี้ anti-PvDBL-TH5 sera ยังมีประสิทธิภาพในการยับยั้งการจับของ PvDBL-TH variants ได้แตกต่างกันอย่างนัยสำคัญทางสถิติอีกด้วย การศึกษาในครั้งนี้จึงสามารถสรุปได้ว่า strain-transcending antibody ที่มีประสิทธิภาพในการยับยั้งทุกสายพันธุ์ของ DBPII สามารถกระตุ้นให้ผลิตขึ้นมาได้ทั้งในสภาวะที่มีการติดเชื้อและจากการ immunization ดังนั้นการผลิต DBPII-based vaccine จึงควรมุ่งเป้าไปที่ conserved epitopes เพื่อกระตุ้นให้ร่างกายสร้าง broadly neutralizing antibody ในการป้องกันการติดเชื้อ PvDBL-TH ของทุกสายพันธุ์

คำหลัก: พลาสโมเดียมชนิดไวแว็กซ์ Duffy Binding Protein II-TH variants (DBL-TH) Strain-transcending antibodies

Abstract

Project Code: TRG5880217

Project Title: Characterization common epitope of Duffy binding protein II associated with inhibition of erythrocyte binding among *Plasmodium viv*ax variant haplotypes: Approach to DBPII vaccine design

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Project Period: 2 July 2015 – 2 July 2017

Abstract

Plasmodium vivax Duffy binding protein ligand domain (DBPII) is an important candidate vaccine for antibody-mediated immunity against vivax malaria. The antigenic variation of DBPII is a major challenge for development of a broadly effective against vivax malaria. The present study was undertaken to investigate the presence of broadly antibody response against polymorphic PvDBL-TH antigens. The most common five variant strain of DBPII in Thai malaria endemic areas was expressed, and cross reactivity of anti-DBPII antibody responses were measured in antisera by ELISA depletion assay. The broadly inhibition of antibody against PvDBL-TH biding to erythrocytes was determined by *in vitro* COS7 erythrocyte inhibition binding assay. In this study, we found that the polymorphic haplotypes of PvDBL-TH variants was able to induce cross-reactivity antibody responses to heterologous PvDBL-TH variants. No significantly binding to heterologous PvDBL-TH variants after sera depletion with homologous antigens was observed. The evaluation of cross-inhibition activity of anti-PvDBL-TH2 sera showed no significant difference of inhibition against all PvDBL-TH variants binding. However, we also found a strain-specific antibody against PvDBL-TH variants. Anti-DBL-TH5 depleted sera showed a significant variation of the binding to all heterologous DBL variants. At 50% inhibition concentration of anti-PvDBL-TH5 body, there was a significantly difference of inhibition against all PvDBL-TH variant binding to erythrocytes. Together, our study demonstrated that the polymorphic haplotypes of PvDBPII could induce antibody responses in natural infection. Both strain-transcending antibody as well as strain-specific antibody against some polymorphic PvDBL-TH variants and against homologous alleles were elicited in response to PvDBL-TH variants. The finding target epitopes of strain-transcending neutralizing antibodies responses to PvDBL-TH antigens will be useful for vaccine development in malaria-endemic areas of Thailand and neighboring countries

Keywords: *Plasmodium vivax,* Duffy binding protein II-TH variants (DBL-TH), Strain-transcending antibodies

Executive summary

Plasmodium vivax Duffy binding protein ligand domain (DBPII) is an important candidate vaccine for antibody-mediated immunity against vivax malaria. The challenge for effective vaccine development is to overcome variation in the DBPII domain, which is a form of immune evasion. Our previous studies identified important differences in quantitative and quanlitative responses of naturally acquired anti-DBP in individual patients living in Thai endemic areas. The target epitopes of naturally acquired inhibitory antibody contain polymorphic residues that alter antibody recognition and reduce sensitivity to antibody inhibition. Amino acid sequence analysis identified 12 variant residues that were common among Thai DBPII haplotypes. The polymorphic patterns were defined into 9 haplotypes (Thai DBL-1, -2, -3, etc...). A study association of Thai DBPII polymorphisms with the functional inhibition of anti-DBPII monoclonal antibodies against a panel of Thai DBL variants showed that antibody recognized variant epitope among Thai haplotypes. Interestingly, there was broadly inhibition of anti-DBPII monoclonal antibody in response against Thai DBPII-erythrocyte binding, indicating conserved epitopes were the target of anti-DBPII neutralizing. The immune efficacy of a DBPII vaccine will depend on the specificity of anti-DBPII antibodies induced and that it is preferable to optimize responses to conserved epitopes for broadly neutralizing protection against *P. vivax*. Therefore, the goal of the project is to evaluate broadly antibody responses against a panel PvDBL-TH variants in animal model and in natural infection. The common PvDBL-TH variants was expressed and used human plasma in individual *P. vivax* exposure subjects and mice antisera upon DBPII immunization to evaluate broadly inhibition in protection against Thai PvDBPII-erythrocyte binding for application of the antibodies to the mapping target epitope of anti-DBPII neutralizing antibody.

A study of cross-reactivity showed both strain-transcending antibody as well as strain-specific antibody against some polymorphic PvDBL-TH variants and against homologous alleles were elicited in response to PvDBL-TH variants. We found no significantly binding to heterologous PvDBL-TH variants after sera depletion with homologous antigen. The evaluation of cross-inhibition activity of anti-PvDBL-TH2 sera showed no significant difference of inhibition against all PvDBL-TH variants binding. This data supported the ability of DBPII variants in induction of strain-transcending immunity. However, we also found the strain-specific antibody against PvDBL-TH variants. Anti-DBL-TH5 depleted sera showed a significant variation of the binding to all heterologous DBL variants. At 50% inhibition concentration of anti-PvDBL-TH5 body, there was a significantly difference of inhibition against all PvDBL-TH variant binding to erythrocytes. Importantly, our study demonstrated that all PvDBL-TH variants had high antigenicity during *P. vivax* infection. The high seroprevalence of PvDBL-TH variants, >97% was found in PvDBL-TH2, followed by PvDBL-TH6.

Together, our study demonstrated that the polymorphic haplotypes of PvDBL-TH variants was able to induce cross-reactivity antibody responses to heterologous PvDBL-TH variants. Therefore, a protective PvDBPII-based vaccine development should base on the common DBP variant strains in malaria-endemic areas. The finding target epitopes of strain-transcending neutralizing antibodies responses to DBL-TH

antigens will be useful for vaccine development in malaria-endemic areas of Thailand and neighboring countries.

Experiments (การดำเนินงานวิจัยตามวัตถุประสงค์)

1. Blood sample preparation

To study immunity to polymorphic DBL-TH haplotypes at malaria clinics in Chumphon province Southern part of Thailand, three subcohorts was designed in the study (Figure 1). Between July 2011-July 2012, one hundred three plasma samples were collected from acutely infected *P. vivax* patients for screening of anti-DBPII responses and evaluation of strain-transcending inhibition against DBL-TH variants. In July 2014-Dec 2014, forty blood samples were collected to study the association of DBL-TH polymorphisms and broadly inhibitory activity of anti-DBPII antibodies in individuals with *P. vivax* exposure. The individuals were assigned for DBPII sequence analysis and testing of inhibitory function of antibodies against Thai DBPII haplotypes. Three blood spots were collected on filter paper from each consenting *P. vivax* patient for preparation of parasite isolates. Parasite genomic DNA was extracted with a QIAamp DNA mini kit (Qiagen, Valencia, CA, USA). In July 2014- July 2015, serological responses to polymorphic strain of DBL-TH, the longevity of anti-DBL-TH antibody responses and inhibitory function against polymorphic DBL-TH antigens bind to human erythrocyte in P. vivax exposure was studied. Plasma sample from symtomatic (n=40) and after recover from *P. vivax* infection at 3 months (n=24), 9 months (n=16) and 12 months (n=15) were carried out for ELISA assay and COS7 erythrocyte inhibition binding assay.

The confirmation of *P. vivax* infection was performed by microscopic examination of thin and thick Giemsa-stained blood smears. Blood samples for malaria-naïve controls were obtained from 35 healthy volunteers who live in Bangkok and had no history of exposure to Plasmodium parasites. Acute P. vivax-infected volunteers who registered at Malarial Clinics and naïve control subjects were asked for informed consent under the protocol approved by the Ethic Committee on Human Rights Related to Human Experimentation, Mahidol University (MUIRB2012/079.2408).

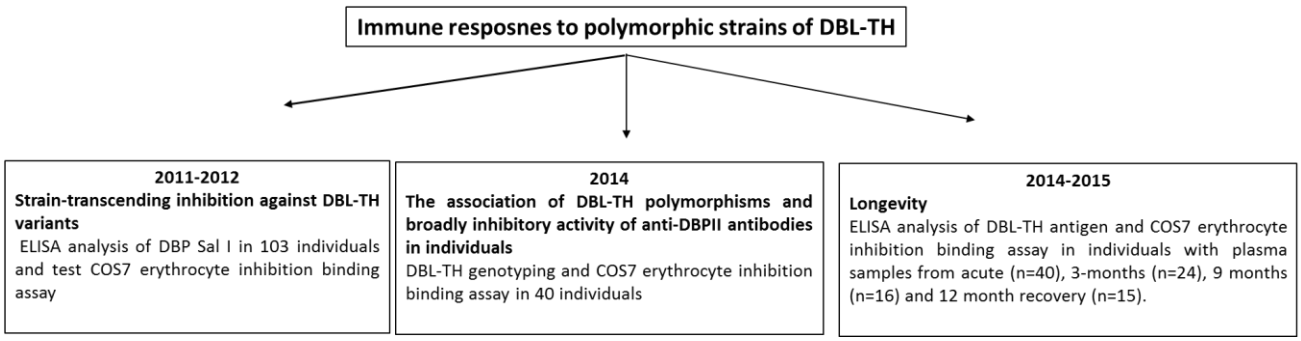


Figure 1: Study design. Shown is flowchart depicting the three subcohort used in the study.

2. Measurement of antibody response to DBPII antigen by ELISA

Serological response against recombinant DBP reference Sal I was quantified by ELISA. Recombinant DBP region II (rDBPII) was expressed as a glutathione S-transferase (GST) fusion protein in E. coli. It was then affinity purified on glutathione and cleaved from GST with thrombin using standard methods. Purified rDBP region II was added to 96-well plates at 2 ug/mL and incubated overnight at 4oC. Wells were incubated with blocking buffer (2% skim milk in PBS) for 2 hr and washed three times with wash buffer. The diluted plasma (n=103) at 1:200 was added to allow binding to rDBP antigen and incubated for 1 hr at 37°C. Bound rDBP and human plasma were detected with goat anti-human IgG-alkaline phosphatase (1:1,000 dilution; KPL, Maryland, USA). The anti-DBPII antibody activity was detected by recording the absorbance (OD) at 405 nm. The overage absorbance and standard deviation were calculated for each plasma sample. A baseline OD was established using plasma from 40 samples of non-malaria exposed Thai individuals and this control value was subtracted from test OD values to standardize the assay. The samples were considered positive when the OD value was greater than or equal to the mean plus 2 standard deviations of negative controls. The antibody reactivity in human plasma was classified into three groups: high responder, low responders and non-responders.

3. Identification of DBL-TH haplotypes in *P. vivax* patients

To identify the DBP region II genotype of the causative agent in acutely infected P. vivax patients, 40 blood spot samples were taken for DNA isolation and amplification. DBPII genes were PCR amplified as described in detail previously [Chootong et al 2012]. In brief, PCR cycling conditions for each primer pair were 90 sec initial denaturation at 94°C, followed by 30 cycles of 15 sec denaturation at 94°C, 35 sec annealing at 60°C, and 60 sec extension at 68°C, and a final extension step of 2 min at 68°C. The PCR products were subsequently sequenced using the dideoxynucleotide chain termination method (Applied Biosystems, Foster City, CA). The alignment of complete sequences of PvDBPII genes from 40 isolates were analyzed by CLUSTAL and percent similarity was assessed using BioEdit software.

4. COS7 culture and transfection

COS7 cell erythrocyte binding assays were carried out to evaluate the ability of neutralizing antibodies in P. vivax patient to inhibit binding of DBL-TH variant and reference Sal I haplotypes to human erythrocytes. Expression plasmid constructs were engineered to express DBL-TH or reference Sal I alleles on the surface of transiently transfected COS-7 cells as fusion proteins to the N-terminus of enhanced green fluorescent protein (EGFP) [Michon et al, 2012]. Recombinant plasmids were transfected into green monkey kidney cells (COS-7, American Type Culture Collection, and Manassas, VA, USA) by the use of Lipofectamine 2000 reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). COS-7 cells were seeded in 24-well culture plates (4.5 × 104 cells/well) in Dulbecco's Modified Eagle Medium (DMEM, Sigma, USA) with 10% fetal bovine serum (Gibco BRL, Life Technologies, Rockville, MS, USA). Recombinant plasmid DNA (100 ng/well) was mixed with Lipofectamine (2% Lipofectamine 2000/well) in DMEM without serum and

then added into transfected wells (100 ul/well) and incubated in a humidified incubator with 5 % CO2 at 37 °C for 42-44 hr. The detection of the C-terminal green fluorescent protein (GFP)-expressing vector was used as positive control for checking transfection efficiency.

5. Measurement of the inhibitory efficiency of anti-DBPII antibodies against DBL-TH binding by COS7 cell inhibition binding assay

The inhibition of anti-DBPII neutralizing antibodies against DBPII-erythrocyte binding was performed as previously reported [Michon et al, 2000]. Briefly, 42-44 hr after transfection, diluted human plasma from high responders was pre-incubated with the transfected COS-7 cells expressing DBL-TH or reference Sal I haplotypes for 1 hr at 37°C before addition of a 10% suspension of Duffy positive human erythrocytes in each well followed by 2 hr incubation. Unbound erythrocytes were removed by washing the well with PBS. DBP-erythrocyte binding was quantified by counting rosettes observed over 30 fields of view (magnification, x200). The binding-inhibition was determined by assessing the number of rosettes in wells of transfected COS7 cells in the presence of plasma relative to rosettes in wells of transfected cells in presence of medium control. The positive and negative inhibition binding controls were 3C9 mouse monoclonal antibodies against DBPII.7.18 haplotype and medium control, respectively. Experiments for each human plasma sample were done in triplicate wells and were repeated two times.

$$\% \text{ inhibition} = \frac{[1-(\text{number of rosette in the presence of vivax plasma})] \times 100}{\text{number of rosette in the presence of healthy control plasma}}$$

6. Recombinant protein DBL-TH production

DNA coding for DBP was amplified by PCR from 3 different alleles of *Plasmodium vivax* DBP (DBP-SalI, DBL-TH2, -TH4, -TH5, -TH6 and -TH9) (Table 1) present in Thai malaria endemic area. The amplified products were be cloned into an expression vector (pET21a+) with a C-terminal histidine tag. The resulting plasmid (pET21a^{+}-DBPII) was transformed into BL21 (DE3) LysE Escherichia coli (Invitrogen). Cells were grown in LB medium in, induced with 1 mM IPTG (isopropyl-**β**-D-thiogalactopyranoside), collected by centrifugation, and stored at -80°C until needed. Recombinant DBP-SalI, DBL-TH2, -TH4, -TH5, -TH6 and -TH9 protein was purified from inclusion bodies and the recombinant proteins were checked for purity by visualizing with SDS-PAGE. Eluted fractions containing enriched protein will be then refolded by rapid dilution as previously described. The final product will be concentrated to 1 mg/ml using the Amicon ultra centrifugal filter units (Millipore) and then stored at -80°C until needed.

Table1: The Polymorphic residues of DBL-TH haplotypes

DBL Alleles	Amino acid position														
	308	313	333	371	375	384	385	386	390	417	424	433	437	475	503
DBL-Sal I	R	.	L	K	.	D	E	K	R	N	L	.	W	P	I
DBL-TH2	.	.	F	E	.	G	K	Q	.	K	I	.	R	.	.
DBL-TH4	.	.	F	.	.	G	K	Q	H	K	I	.	R	.	K
DBL-TH5	.	.	.	E	.	G	.	N	.	K	I	.	R	.	K
DBL-TH6	.	.	.	.	.	G	.	H	.	.	.	.	.	.	.
DBL-TH9	.	.	F	.	.	.	.	.	.	.	.	I	R	.	K

7. Antisera against DBL-TH4 and DBL-TH5 haplotypes

To produce antisera against recombinant DBL-TH2, -Th4, -TH5, -TH6, -TH9 protein antigens for study of cross-reactivity among polymorphic strain of DBL-TH, female BALB/c mice will be used at 5 to 7 weeks of age. Groups of three mice will be injected intraperitoneally with 30 μg of DBL-TH2, -TH6, -TH9 and phosphate-buffered saline (PBS) with Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MO). Booster injections will be given 2 and 4 weeks after the priming using the same amount of antigen with Freund's incomplete adjuvant (Sigma-Aldrich), and mouse sera will be collected 2 weeks after the final boost. All animal experimental protocols will be approved by the Institutional Animal Care and Use Committee of Kangwon National University, and the experiments were conducted according to the Ethical Guidelines for Animal Experiments University.

8. Strain-transcending immunity against a panel DBL-TH antigens

The cross reactivity and/or variant specific antibodies against different variant haplotypes of PvDBL-TH were evaluated by immunodepletion assay. Antigens concentration at 2 μg/mL of each variant were overnight coated on 96-well plate in 4 ^{o}C. The wells were blocked with blocking buffer (2.5% BSA in PBS-T) for 1 hour at room temperature and well-washed by PBS-T before adding mice sera. An antibody from each group of immunized mice, PvDBL-TH2, -TH4, -TH5, -TH6 and TH9, were prepared at a dilution of 1:4,000 in blocking buffer. The prepared antisera were depleted by its specific immunized-variant in first two well for 30 min. After that, depleted sera were transferred to next two well and incubated another 30 min. These serial incubations were repeated until antibodies with a particular variant antigen completely depleted as determined by the development of color of standard ELISA method. Subsequently, those antibody-depleted sera were allowed to react with the remaining heterologous variant for evaluation of cross-reactivity among difference haplotype of PvDBL-TH by ELISA.

For inhibitory function of strain-transcending neutralizing antibody against a panel of PvDBL-TH variant strains, antisera depleted with its homologous strain were used to evaluate the potential of cross-inhibition efficiency against a panel PvDBL-TH binding to erythrocytes, PvDBL-TH1, TH2, -TH4, -TH5, -TH6, -

TH7, -TH8, -TH9 and PvDBPII reference Sal I, compared to undepleted sera by perform COS7 erythrocyte inhibition binding assay.

Results (ผลงานวิจัยที่ได้รับ)

1. Inhibition activity of anti-DBPII antibodies against PvDBL-TH binding

To evaluate inhibitory response against PvDBL-TH haplotypes in high anti-DBPII responders, the reactivity of naturally acquired antibodies in vivax patients (n=103) was tested against recombinant DBPII protein by ELISA. The anti-DBPII antibody levels in acute human plasma were significantly higher than naïve controls (*P. vivax* patient, overage optical density [OD] = 0.25 ± 0.08, naive controls, OD = 0.13 ± 0.030, $P < 0.05$, Figure 2A). The serological responses to DBP were used to classify patients into three groups; high responders (HR) (OD = 0.41 to 0.69), low responders (LR) (OD = 0.20 to 0.40) and non-responders (NR) (OD < 0.20) (Figure. 2A-2B). The samples were considered positive when OD value was greater than or equal to the mean plus 2 standard deviations of naive controls. There were 7, 49 and 47 patients in the high responder, low responder and non-responder categories, respectively (Figure2B).

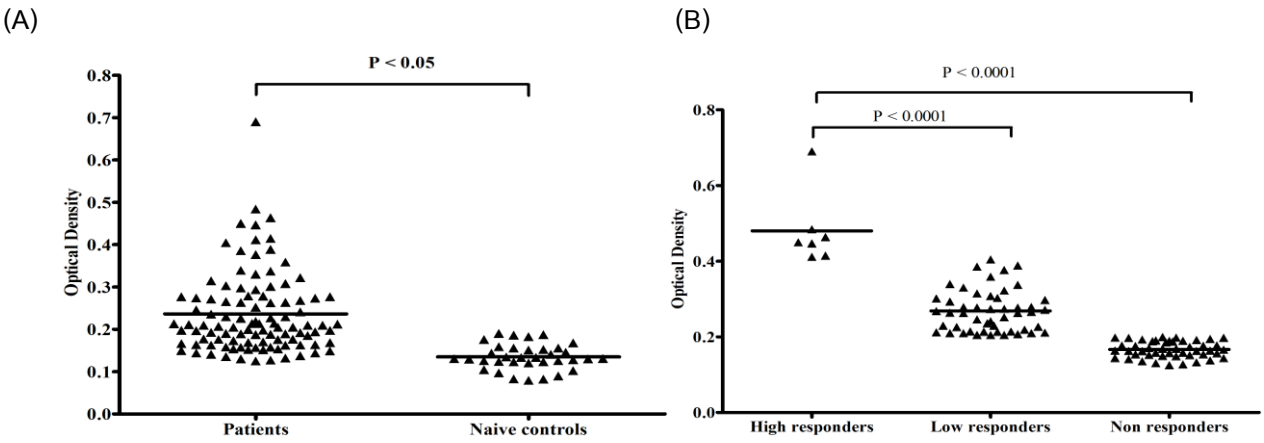


Figure2. Antibody recognition of recombinant PvDBPII. The scatter plot graph shows the anti-DBPII antibody levels in Thai patients compare to naive control as measured by ELISA. **(A)** Anti-DBPII levels were significantly higher in patients with acute *P. vivax* than in naive controls, **(B)** ELISA data classified patients into 3 groups: high responder (HR), low responders (LR) and non-responders (NR). Each dot represents the mean of optical density values in double wells for each sample. The line represents the mean value. Significance was determined by non-parametric analysis using the Mann-Whitney U test. The level of significant was set at $p < 0.05$

The previous study identified the common DBL-TH haplotypes among P. vivax isolates [Chootong et al, 2012]. Therefore, in this study, the individual plasma samples of high responders (HR) (n=7) were used to evaluate inhibitory function of neutralizing antibody against a panel of PvDBL-TH haplotypes by COS7 cell binding-inhibition assay. All HR patients strongly inhibited PvDBL-TH2 and -TH7 binding to human erythrocytes, >80% inhibition activity (Figure3). Most high responders had no inhibitory activity against DBL-

TH5 variant binding with only HR3 and HR6 inhibiting this variant. There was no HR patient that inhibited reference DBPII Sal I binding to erythrocyte at >80%. Interestingly, two high responders, HR3 and HR6, inhibited binding of all DBL-TH variants by >80% inhibition activity (Figure3).

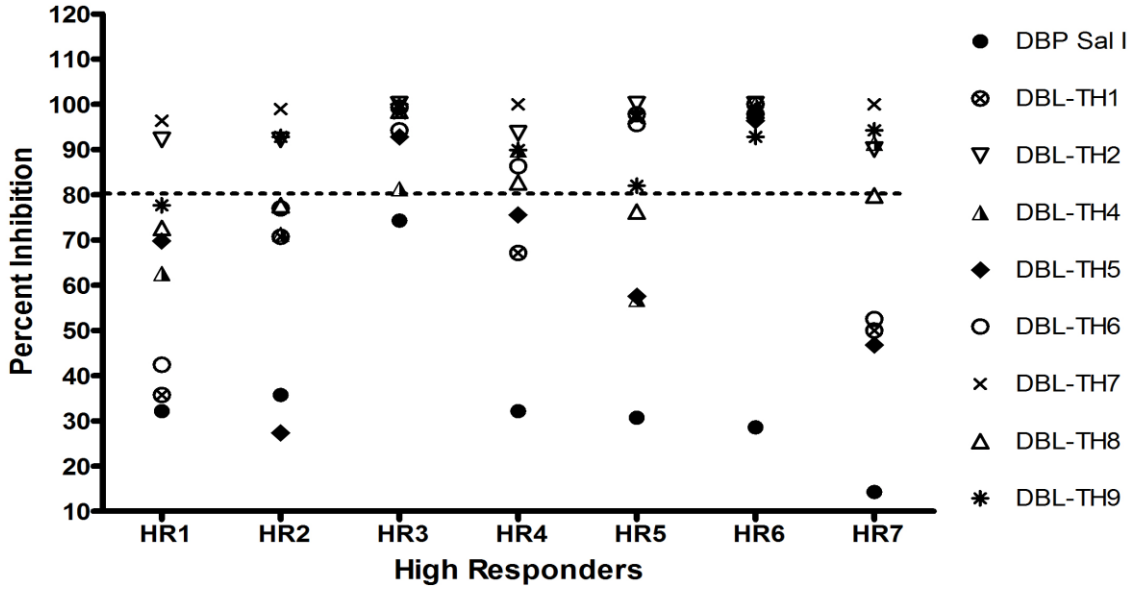


Figure 3. Functional inhibition of anti-DBPII antibodies in high responder samples against the panel of DBL-TH variants. Transfected COS7 cells expressing DBL-TH alleles, DBL-TH1, -TH2, -TH3, -TH4, -TH5, -TH6, -TH7, -TH8, -TH9 and reference Sal I were incubated with 1:100 plasma dilution for 1 hr at 37ºC followed by incubation with a 10% suspension of human erythrocytes for 2 hrs. The number of rosettes was compared between wells of transfected cells incubated with plasma relative to wells without plasma (30 fields of view, magnification x200). The symbols represent mean percent inhibition of two experiments tested in duplicate wells.

2. Broad inhibition of anti-DBPII antibodies against DBL-TH haplotypes

To further analyze the ability of plasma samples from HR3 and HR6 (Figure3) to inhibit both homologous and heterologous DBL-TH haplotypes, plasma from HR3 and HR6 were serially diluted from 1:500 to 1:2000 and tested for inhibitory function of anti-DBPII antibodies by COS7 cell binding-inhibition assay. The result showed that antibodies in HR3 (Figure 4A) and HR6 (Figure 3B) strongly inhibited DBL-TH1, -TH2, -TH6, -TH7, -TH8, and -TH9, with >80% inhibition at 1:500, 1:1,000 and 1:2,000 plasma dilutions whereas only low inhibitory efficiency was shown towards DBL-TH4 and -TH5 haplotypes (at 1:500 dilution, HR3; -TH4 = 59.10%, -TH5 = 47.74%, HR6; -TH4 = 72.23%, -TH5 = 61.68%) (Figure 4A-4B). These data suggest that PvDBL-TH4 and -TH5 variants are composed of critical polymorphic residues which may change antigenic character and alter the potency of neutralizing antibody. The cross inhibition of antibody against DBL-TH1, -TH2, -TH6, -TH7, -TH8, and -TH9 may indicate the sharing of conserved epitopes among PvDBL-TH variants.

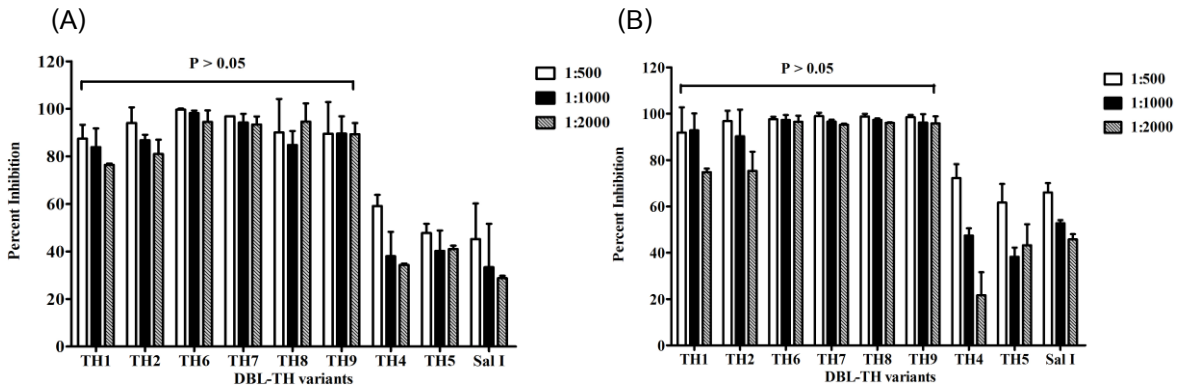


Figure 4. Broad inhibition by high responder samples of erythrocyte binding to PvDBL-TH haplotypes. The transfected COS7 cell expressing DBPII reference Sal I or PvDBL-TH variants were pre-incubated with plasma at dilution 1:500, 1:1000 and 1:2000 for inhibition of DBPII-erythrocyte binding. The charts show the mean inhibition of each DBL-TH variant. Inhibitory function against the panel of DBL-TH variants by HR3 **(A)** and HR6 samples **(B)**. Each chart represents the mean of two independent experiments with each dilution tested in triplicate. Error bars represent ± standard deviation. Statistical significance was determined using one-way analysis of variance (ANOVA) and multiple comparison analysis by Bonferroni test.

3. The association between DBPII polymorphisms and inhibition of anti-DBPII antibodies

To further evaluate the association of DBPII polymorphisms and inhibition activity of anti-DBPII antibodies in P. vivax exposed individuals, 40 additional blood samples from acute *P. vivax* patients were collected in 2014. DBPII polymorphism analysis was used to identify DBL-TH haplotypes in acute P. vivax patients and their plasma was used for evaluation of inhibitory activity against DBL-TH haplotypes by COS7 cell binding-inhibition assay. The patterns of DBPII polymorphisms in individuals of acutely P. vivax patients were classified into 2 groups, those infected with Sal I strain and those infected with DBL-TH haplotypes. There were 22 (55%) and 18 (45%) patients infected with DBP Sal I strain and DBL-TH haplotypes, respectively. For patients infected with DBL-TH haplotypes, 12 haplotypes were defined among P. vivax isolates. Nine haplotypes (DBL-TH1, -TH2, -TH3, TH4, -TH6, -TH7, -TH8 and -TH9) were similar to our previously reported haplotypes (Chootong et al 2012). Three new DBL-TH haplotypes, DBL-TH10, DBL-TH11 and DBL-TH12 were first identified in this Thai endemic area. The highest frequency was DBL-TH1 haplotype (33.33%).

Since the mutation of amino acids has been shown to change antigenic character of DBPII [8], in this study, DBL-TH4 and DBL-TH5 haplotypes which contain multiple polymorphic residues and reference Sal I strain were used for characterization of broadly anti-DBPII neutralizing antibodies. The result showed that nine (22.50%) patients developed anti-DBPII neutralizing antibody against both DBL-TH4 and -TH5

binding to erythrocytes, > 80% inhibition activity. One (31.82%) patients broadly inhibited both DBL-TH4 and DBL-TH5 and reference Sal I (Table 1). In contrast, thirteen (32.50%) patients highly inhibited only one strain, DBL-TH4 or DBL-TH5 or reference Sal I (Table 2). Seventeen patients did not develop antibody against DBL-TH4 or TH5 or Sal I binding.

Table 2. Inhibitory function of anti-DBPII antibodies in *P. vivax* individuals against heterologous PvDBL-TH4, PvDBL-TH5 and reference Sal I binding to human erythrocytes measured by COS7 cell binding-inhibition assay

Sample ID	DBPII haplotypes	Functional Assay		
		DBL-TH4	DBL-TH5	Reference Sal I
2	DBP-Sal I	HI	HI	NI
3	DBP-Sal I	HI	HI	NI
9	DBP-Sal I	HI	HI	HI
15	DBP-Sal I	HI	HI	NI
16	DBL-TH1	HI	HI	NI
24	DBP-Sal I	HI	HI	NI
29	DBP-Sal I	HI	HI	NI
31	DBP-Sal I	HI	HI	NI
33	DBL-TH1	HI	HI	NI
36	DBP-Sal I	NI	HI	HI

Table 3. Inhibitory function of anti-DBPII antibodies in *P. vivax* individuals against heterologous DBL-TH4 or DBL-TH5 and homologous reference Sal I binding to human erythrocytes measured by COS7 cell binding-inhibition assay

Sample ID	DBPII haplotypes	Functional Assay		
		DBL-TH4	DBL-TH5	Reference Sal I
5	DBL-TH3	NI	HI	NI
11	DBP-Sal I	HI	NI	NI
17	DBL-TH11	HI	NI	NI
18	DBP-Sal I	HI	NI	NI
19	DBP-Sal I	HI	NI	NI
21	DBL-TH2	NI	HI	NI
22	DBP-Sal I	NI	HI	NI
25	DBL-TH2	HI	NI	NI
28	DBL-TH8	HI	NI	NI
30	DBL-TH12	NI	HI	NI
32	DBP-SalI	HI	NI	NI
35	DBP-Sal I	NI	NI	HI
37	DBL-TH4	HI	NI	NI

4. Recombinant DBL protein expression and purification

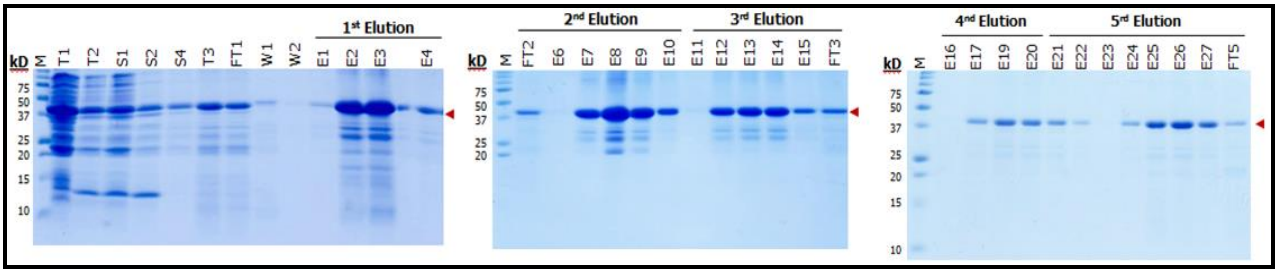
4.1 Cloning and expression of gene encoding PvDBL-TH2, -TH4, -TH5, -TH6 and -TH9

A codon synthetic gene encoding PvDBPII region (GenBank Accession No. AAA63423: amino acids from 194 to 521) with C-terminal 6-His tag was previously cloned in pET21a+ expression vector (Novagen Inc. USA). Primer pair: 5'-GGT GGT GGT GCT CGA GTG TCA CAA CTT CCT GAG TAT T-3' and 5'-TAA TAC GAC TCA CTA TAG GG-3' was used to amplify DNA encoding PvDBPII by polymerase chain reaction (PCR). The amplified product was cloned at *BamHI* and *Xhol* restriction sites of pET23a+. The resultant expression plasmid pET23a(+)-PvDBPII was transformed into *E.coli* Origami2 (DE3) pLysS competent cells (Novagen Inc., USA). The transformant colonies were screened for expression of PvDBL-TH4/5. The colonies were grown in shake-flasks using 2000 ml Luria Broth (LB) supplemented with Ampicillin (100μg/ml) at 37°C. PvDBL-TH expression was induced by addition of 1 mM IPTG when culture reached an OD600 of 1.0. Cells were harvested six hours post induction. Cell lysates were analyzed by SDS-PAGE.

4.2 Cell lysis, isolation, washing and solubilisation of Inclusion bodies

Cell pellet was harvested for two times by centrifugation and resuspended in wash cell buffer (100 mM Tris pH 8.0, 1 M NaCl) then centrifuged at 10,000 rpm for 30 min at 4 °C. Pellet was resuspended two times in lysis buffer (1 M NaCl, 3% Sucrose, 0.2 mM EDTA, 50 mM Tris pH 8, 1% Triton X-100, 1 mM DDT, 1 mM PMSF) containing lysozyme 10 mg/ml and DNase 2 mg/ml. Sonicated by using a sonicator until a homogenous slurry formed. Lysed cells were centrifuged at 10,000 rpm for 30 min at 4 °C to pellet the inclusion bodies (IB). IBs were washing for two times with wash cell buffer (1 M NaCl, 3% Sucrose, 0.2 mM EDTA, 50 mM Tris pH 8, 1% Triton X-100, 3 M Urea, 1 mM DDT, 1 mM PMSF) then sonicated and centrifuged at 10,000 rpm for 30 min at 4 °C. IBs were solubilized with buffer containing 8 M Urea, 0.5 M NaCl, 50 mM Phosphate buffer. Solubilized IB was shaking overnight and centrifuged at 10,000 rpm for 15 min, supernatant was collected and add 15 mM of beta-mercaptoethanol before binding with 1 mL Ni-NTA. The IB solution was rotate at room temperature for 2 hrs then pass through the poly-pep chromatography column. The unbound proteins in column were wash off with IB washing buffer 1 (20 mM Phosphate buffer, 0.5 M NaCl, 10 mM Immidazole, 8 M Urea, pH 7.8) and 2 (20 mM Phosphate buffer, 0.5 M NaCl, 10 mM Immidazole, 8 M Urea, pH 6.3), respectively. Elute bound protein with elution buffer (20 mM Phosphate buffer, 0.5 M NaCl, 250 mM Immidazole, 8 M Urea, 15 mM beta-mercaptoethanol). Check protein concentration by nano drop and or protein colorimetric assay as per manufacturer's instructions. Check purity of protein by SDS-PAGE. Pool the fraction with clean protein and proceed to refold.

(A)



(B)

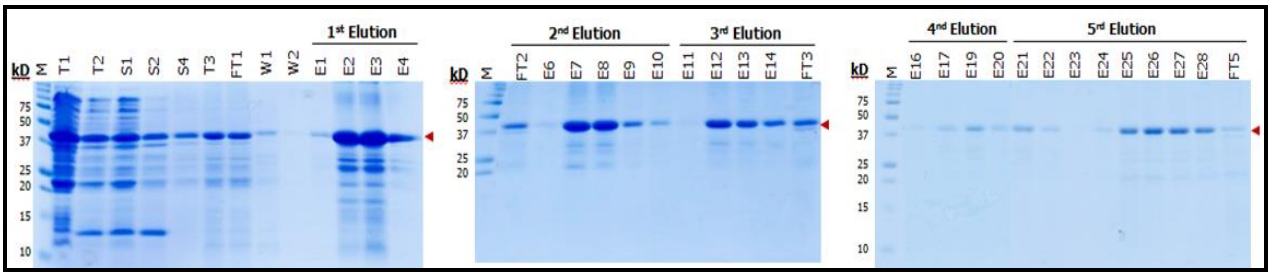
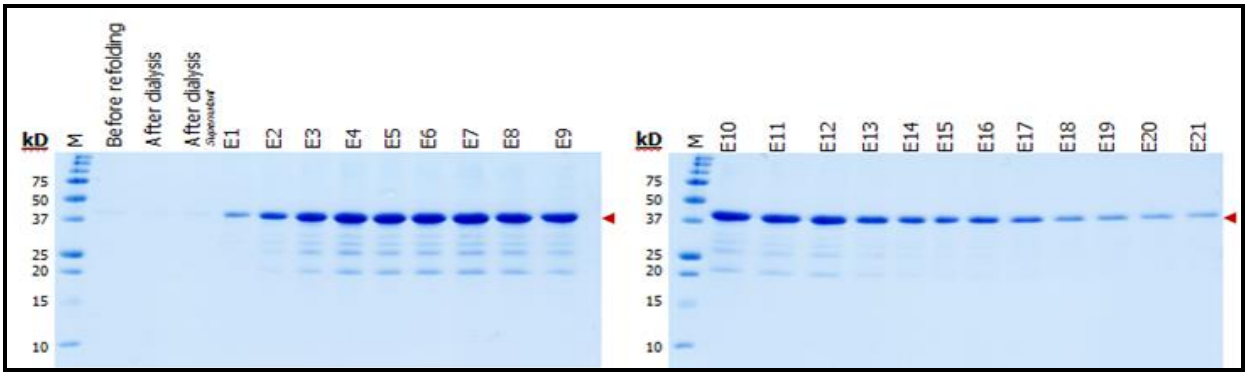


Figure 5: SDS-PAGE show unfold protein of **(A)** DBL-TH4 haplotype and **(B)** DBL-TH5 haplotype.

4.3 Refolding by rapid dilution method and purification by ion exchange chromatography

Unfold form of PvDBPII protein 20 mg was added to refolding buffer A containing 50 mM Tris pH 8, 1 M Urea, 1 mM EDTA, 0.5 M Arginine, 2 mM Cysteine, 0.67 mM Cystamine dihydrochloride, 1 mM Reduced glutathione and 0.25 mM Oxidized glutathione. Refolding buffer B containing β-cyclodextrin (18 g/liter) was slowly added. Refolding was carried out for 36 h at 10 °C with constant stirring. Transfer the refolded protein into 12 kDa dialysis tubing membrane (Sigma) and sealed tubes tightly then dialyze in 5 volumes of dialysis buffer (50 mM Phosphate buffer, 1 M Urea, pH 6.3) while stirring at 4 °C. Change buffers every 12 hr, 3 times within 36 hrs. Pool the dialyzed protein and filter through 0.45 µM to remove particulate material. The refolded protein was purified by Ion exchange chromatography on HiTrap Columns Prepacked with Sepharose: HiTrap SP Sepharose FF (GE Healthcare). Recombinant PvDBPII was eluted using a step gradient of 0.2, 0.5, 1 and 1.5 M NaCl. Elutes were checked for purity by SDS-PAGE on a 12% acrylamide gel.

(A)



(B)

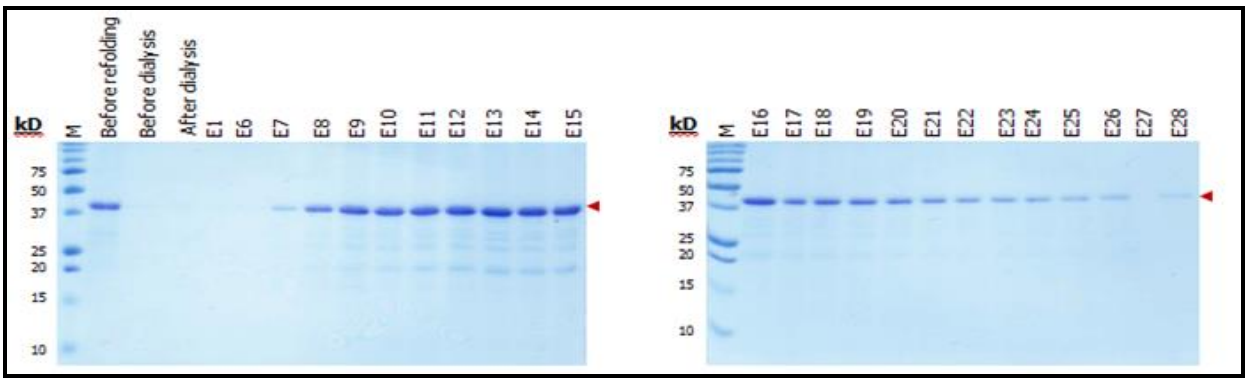
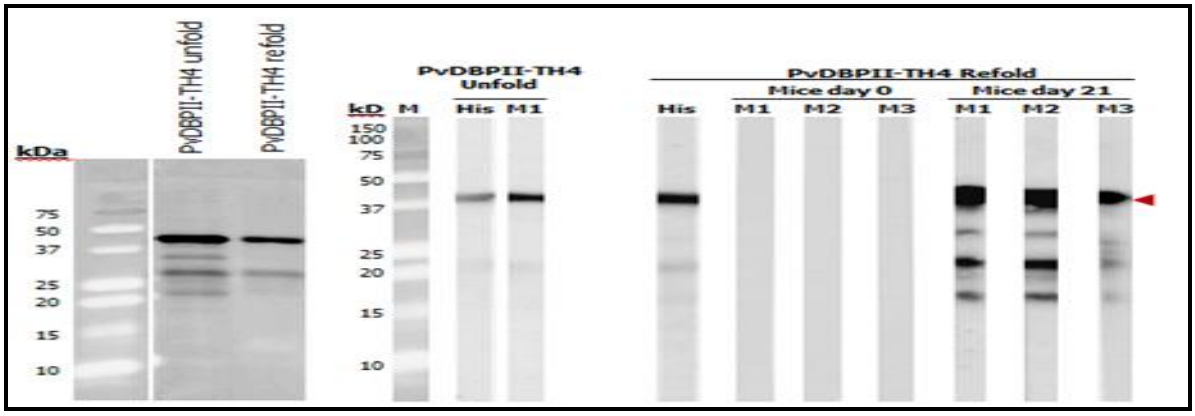


Figure 6: SDS-PAGE show refold protein **(A)** DBL-TH4 **(B)** DBL-TH5

5. The immunogecity of DBL-TH polymorphism strain in immunized mice

PvDBL-TH4 and DBL-TH5 recombinant proteins were used for raising antibodies. Female BALB/c mice (DaehanBiolink Co., Eumsung, ROK) were used at 5 to 7 weeks of age. Groups of three mice were injected intraperitoneally with 30 μg of PvDBL-TH4, PvDBL-TH5, and phosphate-buffered saline (PBS) with Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MO). Booster injections were given 2 and 4 weeks after the priming using the same amount of antigen with Freund's incomplete adjuvant (Sigma-Aldrich), and mouse sera were collected 2 weeks after the final boost. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Kangwon National University, and the experiments were conducted according to the Ethical Guidelines for Animal Experiments University.

(A)



(B)

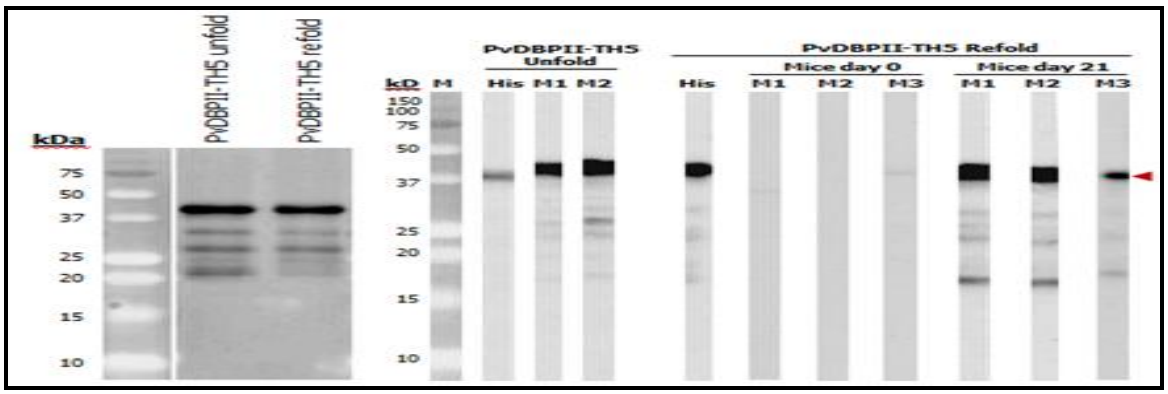


Figure 7: Western blot analysis of DBL-TH4/DBL-TH5 to check specificity of protein with anti-protein (Mouse serum, 1000:1 dilution) and anti-His (2000:1 dilution).

6. Antibody responses against polymorphic haplotypes of DBL-TH during *P. vivax* infection

Serological response against rPvDBL-TH antigens were examined by indirect ELISA. The result shown *P. vivax* patients produced antibody against all five PvDBL-TH variants, which the levels is significantly higher than those in endemic villagers and healthy controls. Based on OD value, DBL-TH5 antigen showed the highest reactivity to P. vivax human plasma (PvDBL-TH5, P. vivax patient, OD=0.354 ± 0.165, endemic villager, OD=0.223 ± 0.188, healthy controls, OD= 0.123 ± 0.024) whereas DBL-TH9 had a low reactivity, (*P. vivax* patient, OD = 0.256 ± 0.218, endemic villagers, OD = 0.117 ± 0.017, healthy controls, OD = 0.094 ± 0.027 (Figure 8).

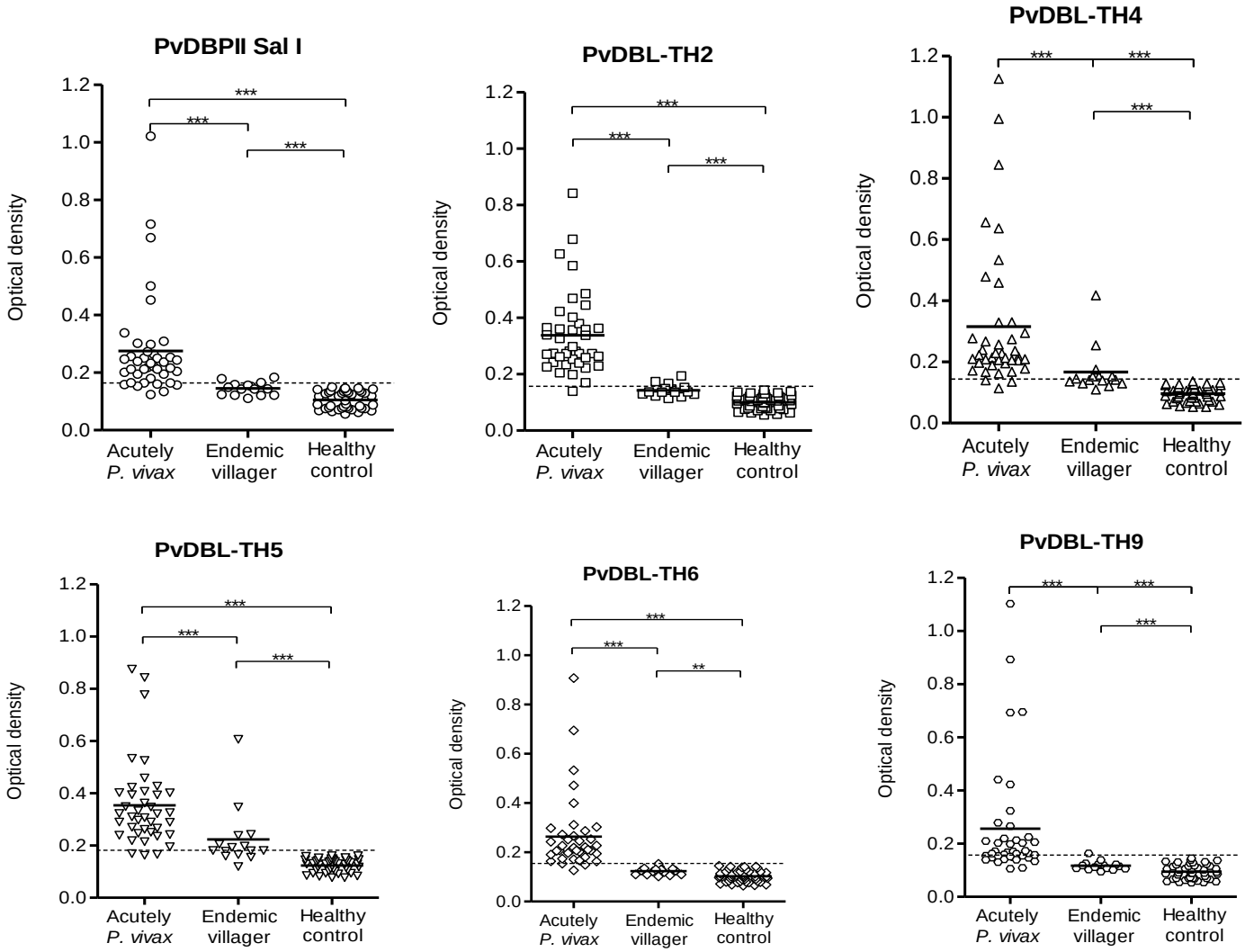


Figure 8: A significant antibody responses against polymorphic DBL-TH antigen in P. vivax patients

Table 4: Prevalence and average of anti-PvDBL-TH antibody responses in acute P. vivax subjects, endemic villager and healthy control.

DBPII alleles	Acute *P. vivax* subjects (AC)				Endemic villagers (EV)			Healthy controls (HC)		
	Percent positive[a] (%)	Mean[b]	*P*-value[c]		Percent positive[a] (%)	Mean[b]	*P*-value[c] compare with HC	Percent positive[a] (%)	OD values	
			compare with EV	compare with HC					Mean[b]	Cut-off[d]
DBPII-Sal I	85.0	0.275	< 0.0001	< 0.0001	20.0	0.145	0.0001	0	0.104	0.164
DBL-TH2	97.5	0.338	< 0.0001	< 0.0001	40.0	0.143	< 0.0001	0	0.100	0.148
DBL-TH4	92.5	0.315	< 0.0001	< 0.0001	46.7	0.166	< 0.0001	0	0.096	0.143
DBL-TH5	92.5	0.354	0.0002	< 0.0001	73.3	0.223	< 0.0001	0	0.123	0.172
DBL-TH6	97.5	0.263	< 0.0001	< 0.0001	6.7	0.123	0.0025	0	0.102	0.149
DBL-TH9	77.5	0.256	< 0.0001	< 0.0001	6.7	0.117	0.0074	0	0.094	0.149

[a]Percent positive: the percentage of seropositive individuals who had OD values greater than the cutoff value.

[b] Mean: the average of antibody level against each antigen presented as mean OD value.

[c] P value of the difference between the mean antibody levels of acute P. vivax subjects with endemic villagers or healthy controls compared using the Mann–Whitney U test. NS: not significant.

[d] Cutoff: the cutoff value calculated by using mean ± 2SD of the OD value of healthy control.

Table 5: Pattern of antibody response to DBP antigen in individuals of acute P. vivax subjects

Sample ID	PvDBPII antigens					
	DBPII Sal I	DBL-TH2	DBL-TH4	DBL-TH5	DBL-TH6	DBL-TH9
PV01	N/R	x	x	N/R	x	N/R
PV02	x	x	x	x	x	x
PV03	x	x	x	x	x	x
PV04	x	x	x	x	x	x
PV05	x	x	x	x	x	x
PV06	x	x	x	x	x	x
PV07	N/R	x	x	N/R	x	N/R
PV08	x	x	x	x	x	x
PV09	x	x	x	x	x	x
PV10	x	x	x	x	x	x
PV11	N/R	x	x	x	x	x
PV12	x	x	x	x	x	x
PV13	x	x	x	x	x	x
PV14	N/R	x	x	x	x	x
PV15	x	x	x	x	x	x
PV16	x	x	x	x	x	x
PV17	x	x	x	x	x	x
PV18	N/R	x	x	x	x	N/R
PV19	x	x	x	x	x	x
PV20	x	x	x	x	x	x
PV21	x	x	x	x	x	x
PV22	x	x	x	x	x	x
PV23	x	x	x	x	x	x

Sample ID	PvDBPII antigens					
	DBPII Sal I	DBL-TH2	DBL-TH4	DBL-TH5	DBL-TH6	DBL-TH9
PV24	x	x	x	x	x	x
PV25	x	x	x	x	x	x
PV26	x	x	x	x	x	x
PV27	x	x	x	x	x	x
PV28	x	x	x	x	x	N/R
PV29	x	x	x	x	x	x
PV30	x	x	x	x	x	x
PV31	x	x	x	x	x	x
PV32	x	x	x	x	x	x
PV33	x	x	x	x	x	N/R
PV34	x	x	N/R	x	x	N/R
PV35	N/R	N/R	N/R	N/R	N/R	N/R
PV36	x	x	x	x	x	x
PV37	x	x	x	x	x	x
PV38	x	x	N/R	x	x	N/R
PV39	x	x	x	x	x	N/R
PV40	x	x	x	x	x	x

***** Positive response to each antigen is indicated as X whereas negative response to each antigen is indicated as N/R**.**

7. The prevalence of antibody responses to polymorphic strain of PvDBL-TH

The seroprevalence study of anti-DBP antibody responses in symptomatic of *P. vivax* infection showed approximately 85%, 97.5%, 92.5%, 92.5%, 97.5% and 77.5% positive antibody titer to PvDBPII Sal I, PvDBL-TH2, -TH4, -TH5, -TH6, and -TH9 antigens, respectively (Table 4). A high seroprevalence of antibody responses to polymorphic strain of DBL-TH antigens suggested that theses polymorphic strain has a good immunogenicity, it could induce antibody resposnes during P. vivax infection. Furthermore, a study of anti-PvDBL-TH resposnes in endemic villagers showed 20%, 40%, 46.7%, 73.3%, 6.7% and 6.7% of individuals were seropositive for PvDBPII reference Sal I, PvDBL-TH2, -TH4, -TH5, -TH6 and -TH9 antigens, respectively, supporting the exposure to *P. vivax* parasites in malaria-endemic area could induce immunity to PvDBL-TH antigens. There was no significant correlation between the level of anti-PvDBL-TH antibodies with gender and age were observed in this study.

The data analysis of seropositive prevalence against PvDBL-TH antigens in individuals of *P. vivax* patients showed 72.5% of patients had broadly reactive antibodies specific to all polymorphic strain of PvDBL-TH antigenic and PvDBPII reference Sal I. All patients were seropositive to polymorphic PvDBP-TH antigens, > 3 strains (Table 5). There was correlation between antibody responses to polymorphic PvDBL-TH2, -TH4, -TH5, -TH6, -TH9 and reference Sal I in individual of *P. vivax* patients (Spearman's correlation coefficients, $P < 0.0001$). Interestingly, 5 patients (12.5%) were seropositive against only polymorphic PvDBL-TH strains but they did not produce antibody responses to PvDBPII reference Sal I strain. There was no patients producing antibody specific to only PvDBPII reference Sal I. Together, our data indicated that although PvDBP antigen has a high polymorphism, it has immunogenicity by triggering antibody responses. The immune system of *P. vivax* patients have ability to produce antibody against polymorphic strain of PvDBP. Therefore, a protective PvDBP vaccine development should base on the common DBP variant strains in malaria-endemic areas.

8. Longevity of antibody responses to polymorphic strain of DBL-TH antigen in *P. vivax* exposure.

In this study, the cross sectional study was carried out to demonstrate the longevity of anti-DBP antibody responses. The result showed a maintainnance of antibdoy respsones after recover from *P. vivax* infection. At 3-month recovery, antibody levels were still higher detected but it was obviously reduced to the theshold levels at 9-month recovery (Figure 9). Among seropositive of DBP antigens, 8 patients was folowed for antibody responses at acute phase, 3 months, 9 months and 12 months of infection. The result showed that most seropositive patients (PV02, PV03, PV05 and PV08) lower produced antibodies specific to reference DBP Sal I and polymorphic strain DBL-TH2, -TH4, -TH5, -TH6 and TH9 at 3 month recovery and the antibodies were not detected at 9 month recovery. There were 2 patients (PV03 and PV06) producing higher anti-DBP antibody specific to DBL-TH2, -TH4, -TH5, -TH6 and TH9 and PvDBP II reference Sal I antigen at 3 month recovery but it was substantially lower at 9 motnh recovery. Only anti-

DBL-TH antibody responses of PV02 still seropositive at 9 month and 12 recovery of *P. vivax* infection (Figure 10).

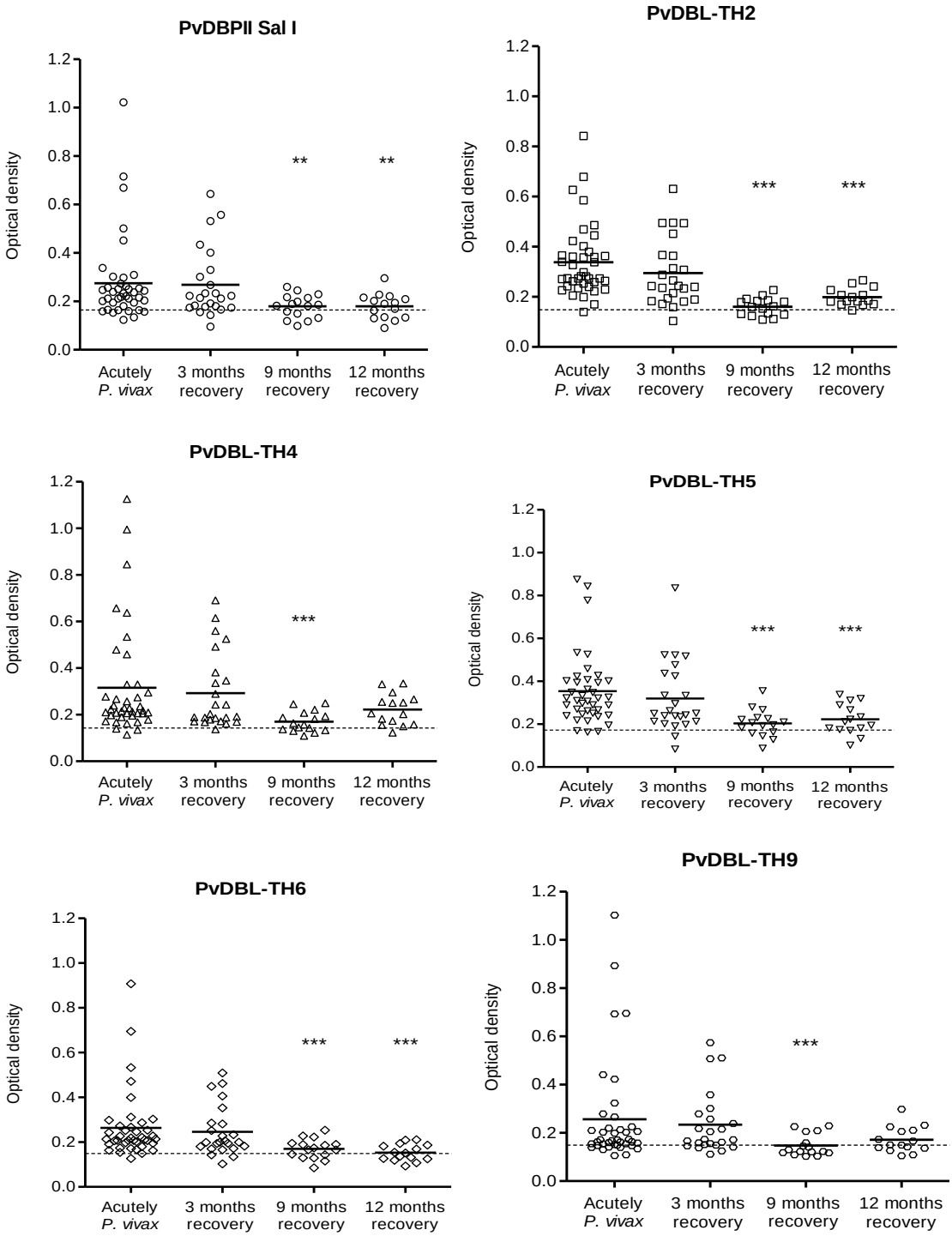


Figure 9: Cross-sectional study show the presence of antibody response to polymorphic strain of DBL-TH in symptomatic and recovery phase of *P. vivax* infection .

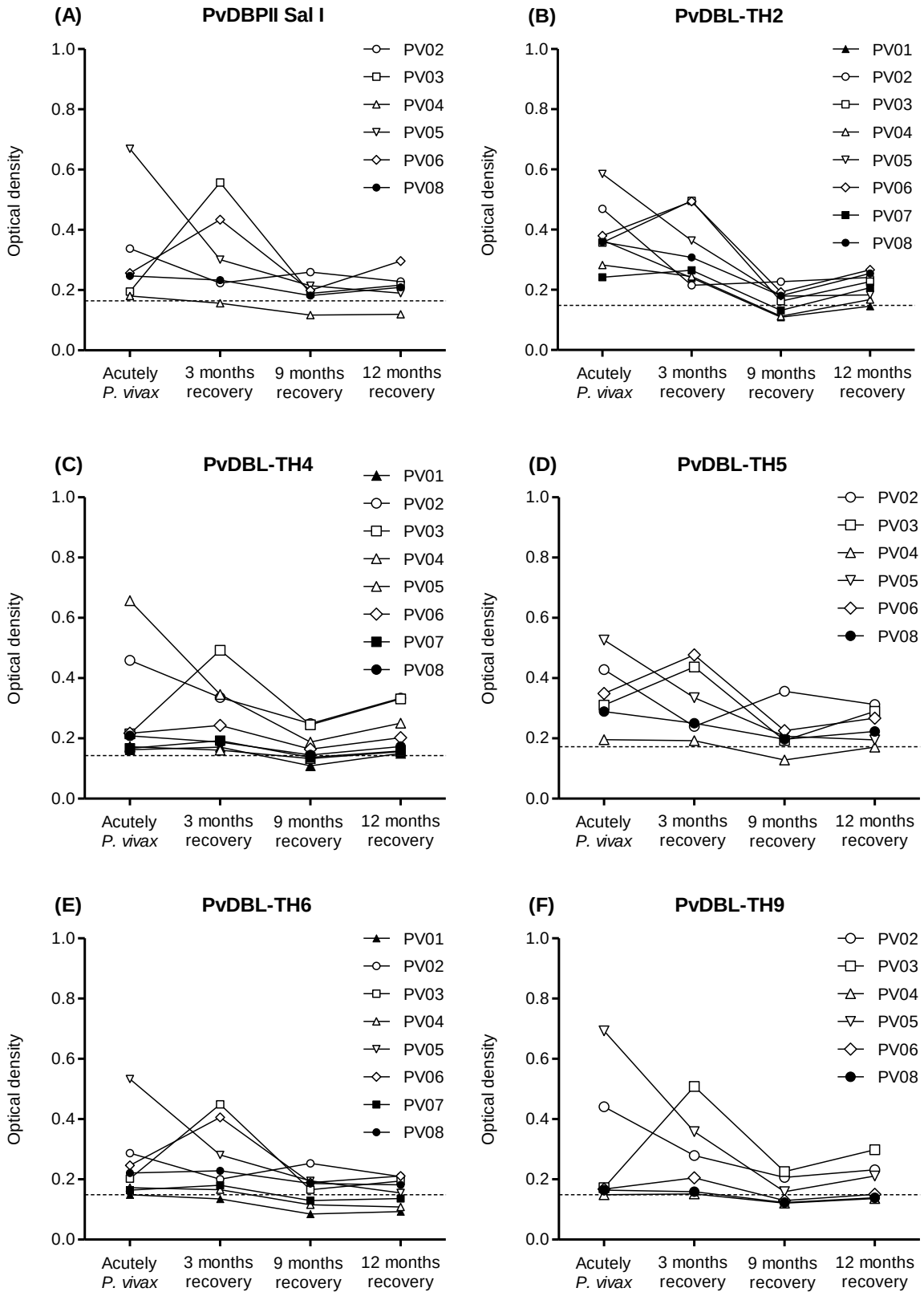


Figure 10: The longevity of anti-DBP antibody responses in indivudals of *P. vivax* patients

9. Inhibition activity of antibodies against polymorphic PvDBL-TH binding to erythrocytes

To evaluate prevaluence of inhibition efficiency of anti-DBPII antibodies in seropositive patients, COS7 assay expressed DBL-TH or reference Sal I on cell surface was pre-incubated with human plasma from seropositive patients before adding human erythrocytes. The result showed that in individuals of seropositive pateints had broadly inhibited DBL-TH-erythrocyte binding. All seropositive patients highly inhibited DBL-TH2, -TH4 and -TH9 binding. Most PvDBL-TH5 seropositive individuals had highly inhibitory activity against PvDBL-TH5-expressing COS7 cell (DBL-TH5, PV02=90.48%, PV03=87.60%, PV04=87.65%, and PV08=89.14%) exception with PV05 and PV06, only 72.73% and 72.18% of inhibition activity was observed. All patients did not produce neutralizing antibodies against PvDBL-TH6 and PvDBPII reference Sal I, <80% percent inhibition (Figure 11). These data indicated that in individual patients could produce broadly neutralizing antibody against polymorphic strain of DBL-TH binding to human erythrocytes. The further study for identification of conserved epitopes among DBL-TH antigen will be useful for conserved epitope-based DBP vaccine development.

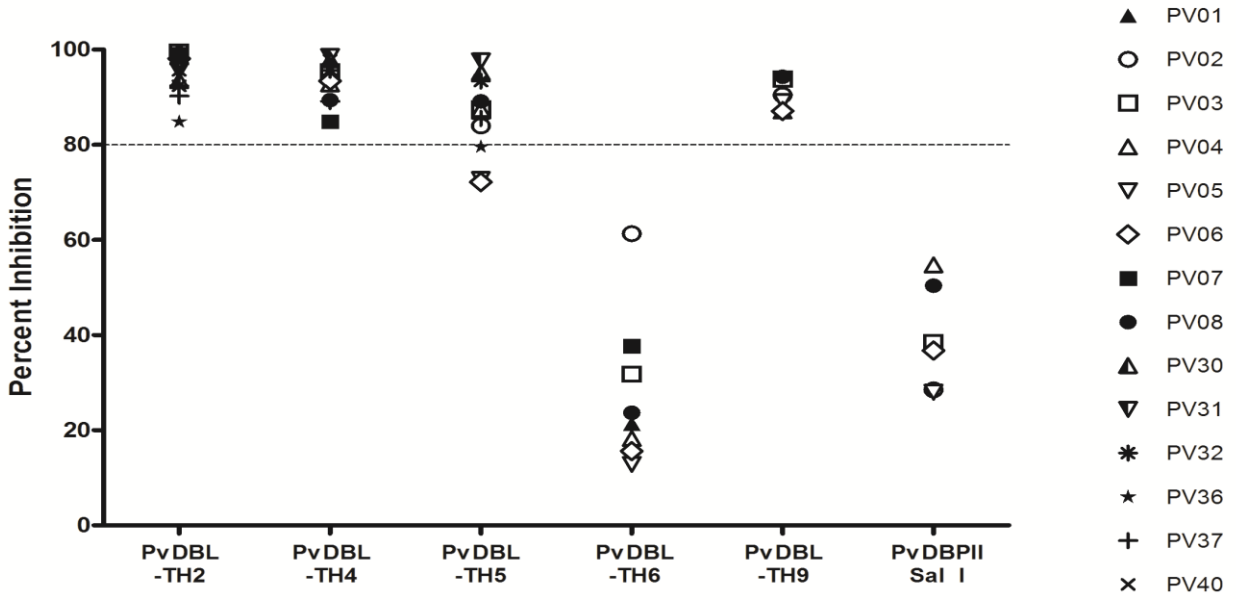


Figure 11: The inhibitory activity of seropositive PvDBL-TH patients

10. Longevity of neutralizing antibodiea against DBL-TH binding to human erythrocyte

As protective PvDBP vaccine should trigger memory B cell responses and elicit stability of neutralizing antibodies, in this study, the seropositive patients who had strong inhibitory activity in acute phase was followed after anti-malaria treatment at 3 months, 9 months and 12 months. Therefore, only neutralizing antibodies against, PvDBL-TH2, -TH4, -TH5, and -TH9 were followed in recovery phase. All seropositive patients who had neutralizing antibodies against PvDBL-TH-2 and -TH4 binding to human erythrocytes maintained their inhibitory efficiency, more than 80% inhibition, in all followed up period during 12-month of observation (Figure 12A-12B). Three of PvDBL-TH5 seropositive patients were stably inhibited the binding of PvDBL-TH5 to human erythrocyte although a transient decreased at 9-month

recovery in PV03 was observed. Only neutralizing antibodies from PV08 patient was reduced activity <80% at 3-month recovery phase and continuous declined overtime (Figure 12C). Similarly, inhibition activity of neutralizing antibodies against PvDBL-TH9 from PV02 and PV03 patients were decreased after recovery from *P. vivax* infection at 3 months whereas another 4 patients of PvDBL-TH9-seropositive individuals kept >80% inhibition activity over 12-month of study (Figure 12D).

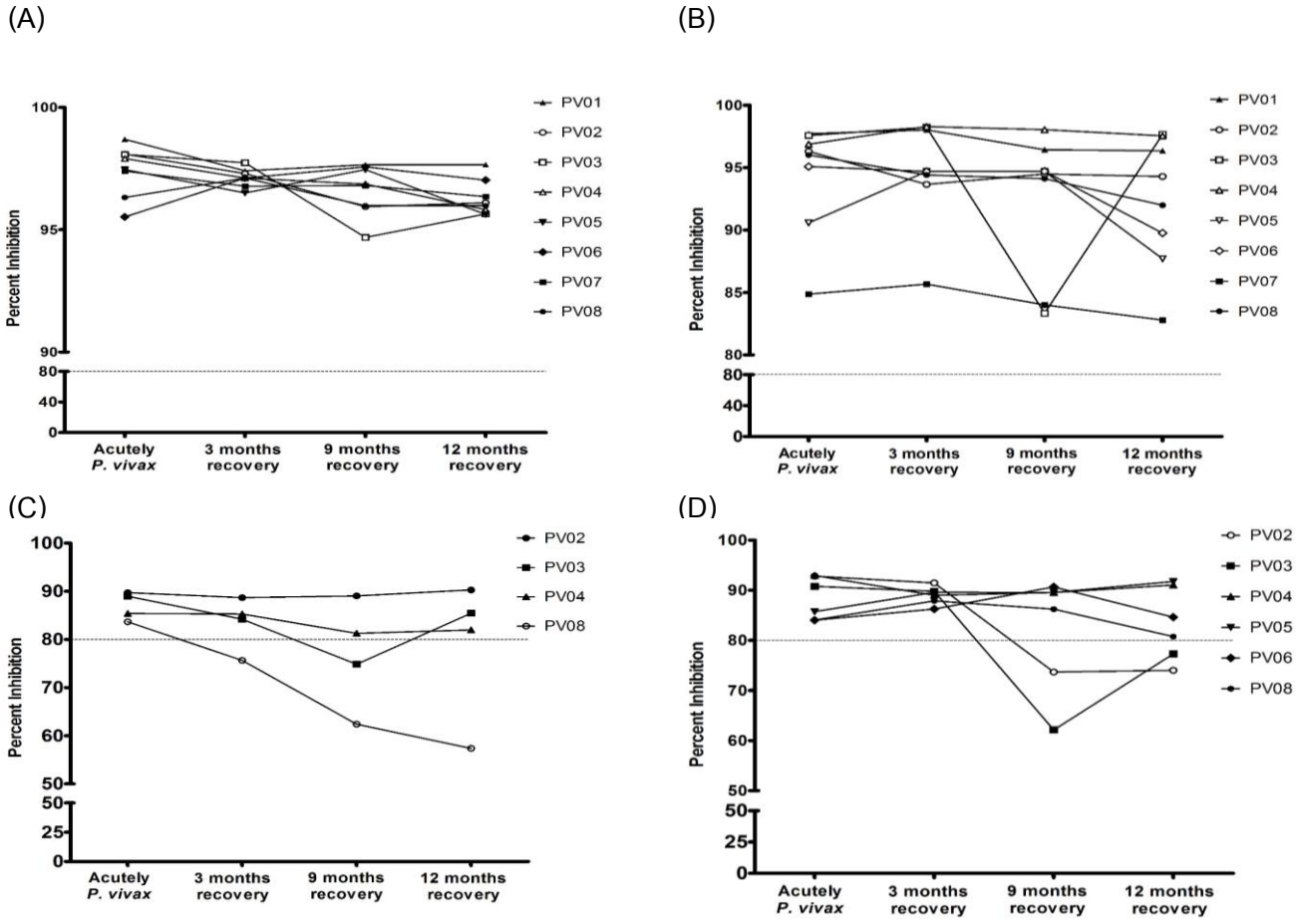


Figure 12: Stability of neutralizing antibgodies against PvDBL-TH binding to human erythrocytes.

11. Characterization of broadly neutralizng antibodies against a panel of polymorphic DBL-TH strain

11.1. Cross reactivity of anti-DBP antibody responses

The antisera from PvDBL-TH2, -TH4, -TH5, -TH6 and -TH9 immunization was used for cross-relativity with heterologous DBL-TH strain as well as rMSP1-19 antigen. The reactivity before and after depletion with homologous strain was measured by ELISA depletion assay. In this assay, the antisera against was depleted with each homologous rDBL-TH antigen and only antibodies to different site of antigen remained to bind to secondary plate. PvDBL-TH2 antisera was depleted by homologous DBL-TH2 variants and there was no positive antibody response to TH4, -TH5, -TH6 and -TH9 variants and reference Sal I strain (Figure 13). For PvDBL-TH4 antisera, the antisera depleted with homologous PvDBL-TH4 strain significantly recognized heterologous PvDBL-TH2 variant whereas no reactivity against heterologous PvDBL-TH5, -TH6, -TH9 and

reference Sal I (Figure13). Contrastly, anti-PvDBL-TH5 antibody depletion strongly bind to heterologous PvDBL-TH2, -TH4, -TH6, -TH9 variants and reference Sal I (Figure 12C). Anti-PvDBL-TH6 depletion had ability to recognize heterologous PvDBL-TH2, -TH4, -TH9 variants and reference Sal I (Figure13), also anti-PvDBL-TH9 depletion recognized heterologous PvDBL-TH2, -TH4, -TH6 variants and reference Sal I (Figure13).

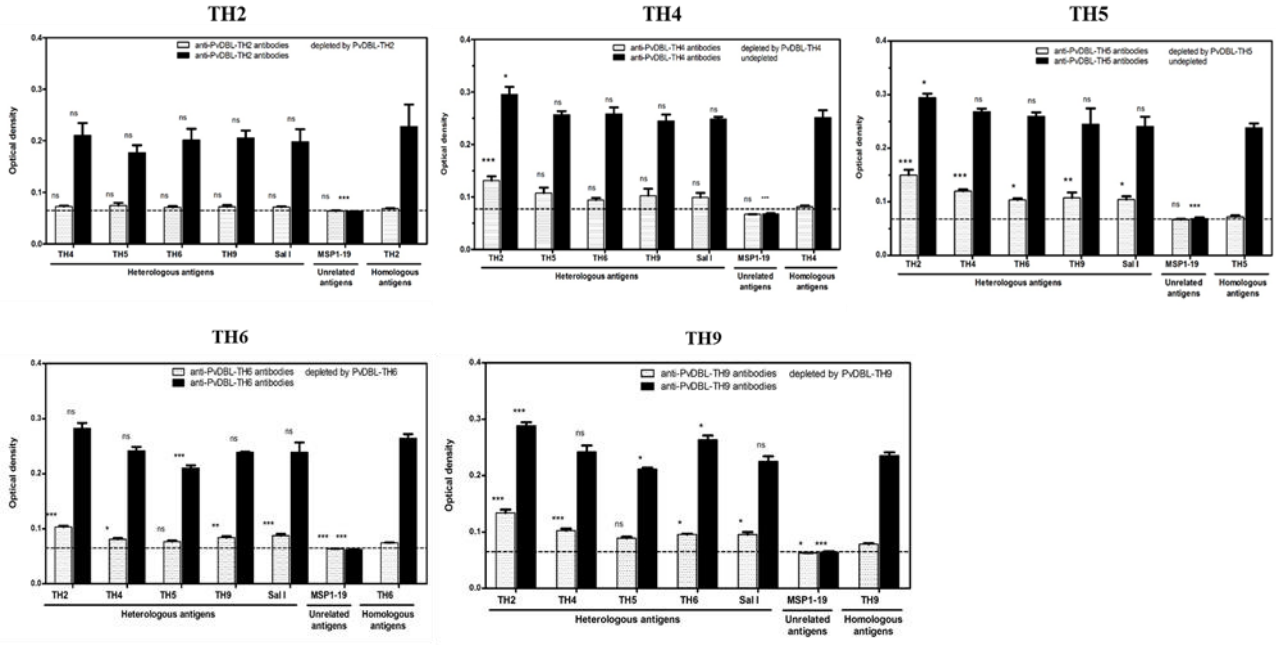


Figure 13: Cross reactivity of anti-DBP antibodies in antisera. Antisera against DBL-TH2 , -TH4, -TH5, -TH6 and -TH9 was tested the binding activity against a panel DBL-TH variants and reference Sal I by ELISA depletion assay. PvMSP1-19 protein was used as unrelated antigen to confirm specificity of the depletion reaction. The mean OD values from duplicate wells are shown in the figure. The horizontal line was shown the cut off value.

11.2. Strain-transcending inhibition of anti-PvDBL-TH antibodies.

The antisera against was evaluated inhibition activity against COS7 cell surface-expressed DBL-TH variants binding to human erythrocytes. Inhibition of DBL-TH-erythrocyte binding was determined by assessment number of rosette in the cultures transfected COS7 cells incubated with difference concentration of PvDBL-TH antisera. The result showed a dose-dependent inhibitory responses of antisera to PvDBL-TH-2, -TH4, -TH5, -TH6, -TH9 (Figure14). The determination of 50% inhibition concentration of antisera against DBL-TH2, -TH4, -TH5, -TH6 and -TH9 was 10 ug/ml, 6.56 ug/ml, 11.49 ug/ml, 9.96 ug/ml and 11.27 ug/ml, respectively (Figure 14). Then, we used the IC50 of each antisera to evaluate the functional activity of antibody against the panel of DBL-TH variants expressed in the COS7 cell assay. The results revealed no difference in the inhibitory activity of PvDBL-TH2 and -PvDBL-TH4 antisera against DBL-TH variant binding whereas it showed significant variation against reference Sal I binding (Figure14), suggesting the epitope target of this neutralizing antibody was conserved among DBL-TH variants. Contrastly, there was a significant difference of inhibition activity of anti-PvDBL-TH6 against DBL-TH5 binding (Figure15). Also,

the inhibition of anti-PvDBL-TH9 against DBL-TH4 and reference Sal I binding was greatly variation form PvDBL-TH2, -TH5 and -TH6 binding to erythrocytes (Figure15). Interestingly, the inhibition of DBL-TH5 antisera against a panel DBL-TH was significantly difference but it was not difference in reference Sal I binding indicating variant residues of DBL-TH were the target epitopes of anti-DBP neutralizing antibodies (Figure15).

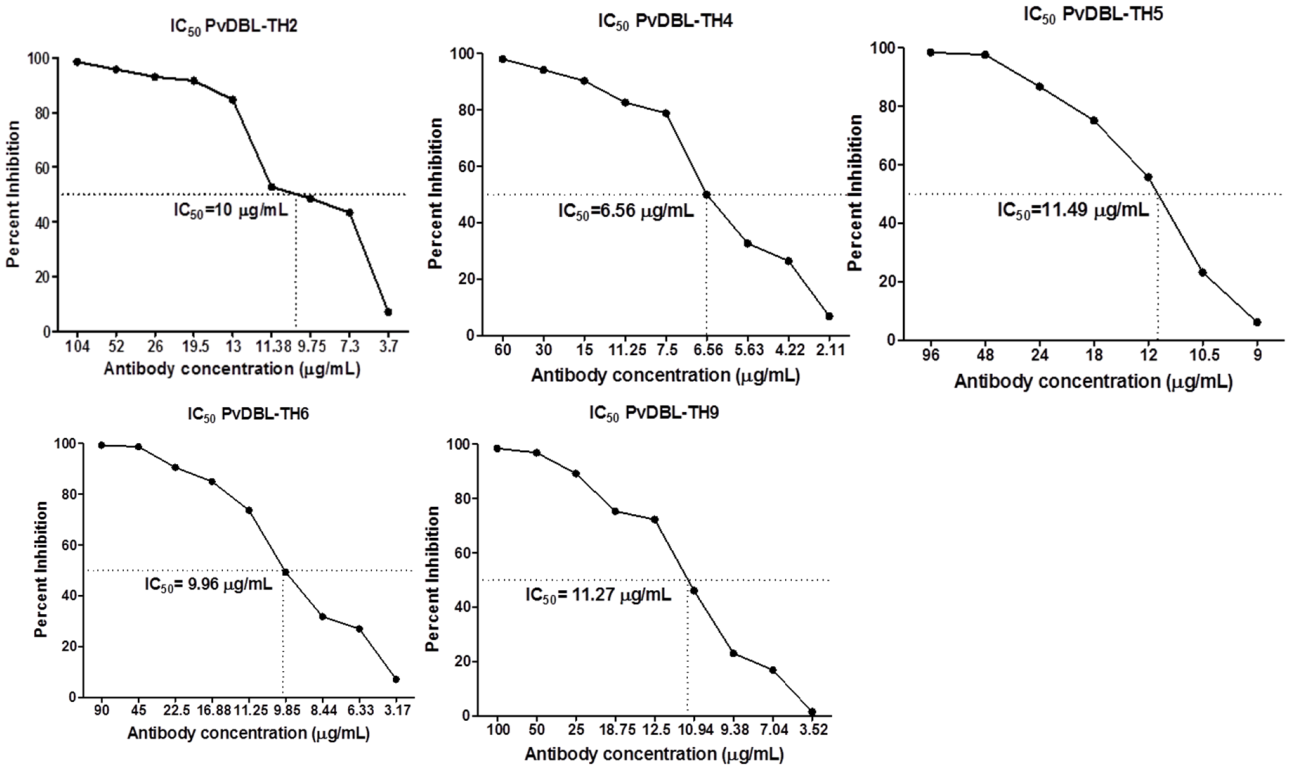


Figure 14: Determination of 50% inhibition concentration of antisera. Antisera against DBL-TH2, -TH4, -TH5, -TH6 and –TH9 were tested for their inhibitory function against the panel DBL-TH variants and reference Sal I. The transfected COS7 cells expressing DBL-TH or DBPII-Sal allele were incubated with antisera at different concentrations and with human erythrocytes.

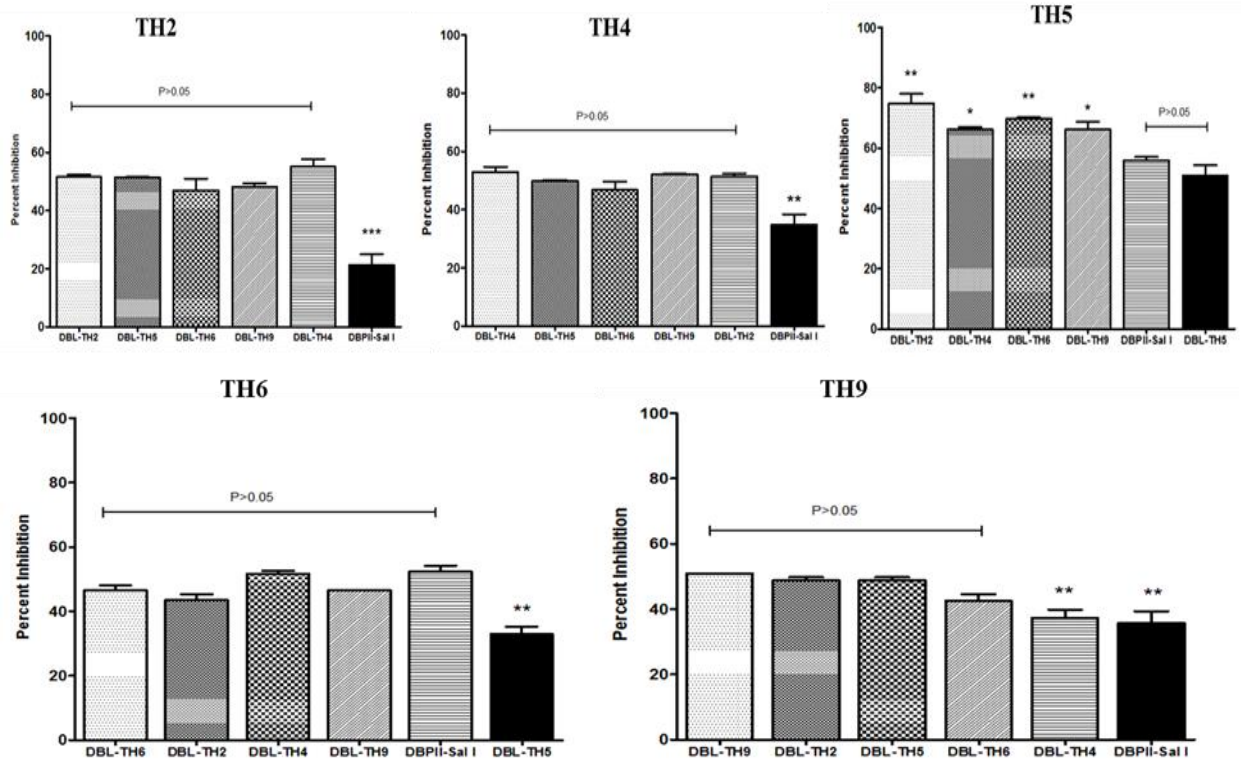


Figure 15: Inhibition of erythrocyte binding to DBL-TH expressed on COS-7 cells. The transfected COS7 cell expressing DBPII reference Sal I or DBL-TH variants were pre-incubated with antisera at concentrations set to the IC50 of the homologous DBL-TH strain for inhibition of DBPII-erythrocyte binding. The charts show the mean inhibition of each DBL-TH variant. COS7 experiments for each monoclonal antibody were done in triplicate wells of each DBL variant and were repeated two times. Statistical significance was determined using one-way analysis of variance (ANOVA)

ผลงานวิจัยที่ได้จากโครงการ

1. ผลงานวิจัยตีพิมพ์ในวรสารนานาชาติ

1.1 Sudarat Wongkidakarn, Amy M. McHenry, Jetsumon Sattabongkot, John H Adams, **Patchanee Chootong***. Strain-Transcending Inhibitory Antibodies against Homologous and Heterologous Strains of Duffy Binding Protein region II. Plos One (2016);11(5):e0154577. Impact factor 3.23

1.2 Siriruk Changrob, Amy H Mchenry, Francis N tumbgiab, Myat Htut Nyunt, Muh Fauzi, Jin Hee Han, Eun-Taek Han, John H Adams and **Patchanee Chootong***. The immunogenicity of polymorphic haplotypes of *Plasmodium vivax* Duffy Binding Protein II in induction of strain-transcending immune response. **(Submitted)**

2. ผลงานวิจัยเสนอในงานประชุมวิชาการนานาชาติ

2.1 เสนอแบบโปสเตอร์ในงานประชุมวิชาการนานาชาติในหัวข้อเรื่อง” The strain-transcending inhibition response against Duffy binding protein II Thai haplotypes in *Plasmodium vivax* malaria-exposed individuals” ณ งานประชุม the international conference “The 14th Awaji International Forum on Infection and Immunity” ประเทศญี่ปุ่น วันที่ 6 เดือน กันยายน พ.ศ. 2558 (บดคัดย่อแสดงในเอกสารภาคผนวก)

2.2 เสนอผลงานวิจัยแบบโปสเตอร์ในงานประชุมนานาชาติในหัวข้อเรื่อง ” The presence of immune resposnes against polymorphic haplotypes of *Plasmodium vivax* Duffy binding protein II vaccine candidate in natural exposure “ณ งานประชุม the international conference Awaji 15th International Forum on Infection and Immunity ณ ประเทศญี่ปุ่น วันที่ 9 เดือน กันยายน พ.ศ. 2559 (บดคัดย่อแสดงในเอกสารภาคผนวก)

2.3 เสนอผลงานวิจัยแบบโปสเตอร์ในงานประชุมวิชาการนานาชาติในหัวข้อเรื่อง” Antibody resposnes against polymorphic haplotypes of Plasmodium vivax Duffy binding protein II: approach to antibody based vaccine design“ณ งานประชุม 3th Congress of MU-ASEAN Association of School of Medical Technology Conference 2016 ณ ประเทศไทย วันที่ 3 เดือน พฤศจิกายน พ.ศ. 2559 (บดคัดย่อแสดงในเอกสารภาคผนวก)

PLOS ONE

RESEARCH ARTICLE

Strain-Transcending Inhibitory Antibodies against Homologous and Heterologous Strains of Duffy Binding Protein region II

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Abbreviations: DBPII, Duffy binding protein region II; DBL-TH, Duffy binding ligand Thai haplotypes.

Abstract

Duffy binding protein region II (DBPII) is a promising vaccine candidate against vivax malaria. However, polymorphisms of DBPII are the major obstacle to designing a successful vaccine. Here, we examined whether anti-DBPII antibodies from individual *P*. *vivax* exposures provide strain-transcending immunity and whether their presence is associated with DBPII haplotypes found in patients with acute *P*. *vivax*. The ability of antibodies to inhibit DBL-TH-erythrocyte binding was tested by COS7 erythrocyte binding inhibition assay. Seven samples of high responders (HR) were identified from screening anti-DBPII levels. HR no.3 and HR no.6 highly inhibited all DBL-TH binding to erythrocytes, by >80%. Antibodies from these two patients' plasma had the potential to be broadly inhibitory against DBL-TH1, -TH2, -TH6, -TH7, -TH8 and -TH9 haplotypes when plasma was serially diluted from 1:500 to 1:2000. To further examine the association of DBPII haplotypes and the ability of antibodies to broadly inhibit DBL-TH variants, the individual samples underwent sequencing analysis and the inhibitory function of the anti-DBPII antibodies was tested. The patterns of DBPII polymorphisms in acute patients were classified into two groups, DBPII Sal I (55%) and DBL-TH variants (45%). Plasma from Sal I and DBPII-TH patients who had the highest inhibition against Sal I or DBL-TH4 and -TH5 was serially diluted from 1:500 to 1:2000 and their inhibitory capacity was tested against a panel of DBL-TH haplotypes. Results provided evidence of both strain-transcending inhibition as well as strain-specific inhibition by antibodies that blocked erythrocyte binding against some DBL-TH variants and against homologous alleles. This study demonstrated broad inhibition by anti-DBPII antibodies against DBL-TH haplotypes in natural *P*. *vivax* exposed individuals. The identification of conserved epitopes among DBL-TH may have implications for vaccine development of a DBPII-based vaccine against diverse *P*. *vivax* infections.

Introduction

The malaria asexual cycle involves repeated cycles of parasite growth and subsequent destruction of host erythrocytes. Each cycle is characterized by production of merozoites which recognize and invade new erythrocytes for continuation of the parasite cycle. The clinical manifestations of malaria are associated with asexual erythrocytic stages of the parasites [1]. Therefore, targeting these stages may help reduce clinical symptoms during malaria. The merozoite proteins, which play a role in parasite invasion, are important candidates for vaccine development to block parasite invasion and limit blood- stage growth.

The *Plasmodium vivax* Duffy Binding Protein (PvDBP) is released from micronemes during initial attachment of merozoites to the erythrocytes and for junction formation to complete the invasion process. The critical binding motif of PvDBP is referred to as DBP region II (DBPII) or the DBL domain. The interaction between DBPII and its cognate receptor, the Duffy antigen/receptor for chemokine (DARC) on the reticulocyte surface is required for parasite invasion [2, 3]. Naturally acquired anti-DBP antibody are widespread in people living in malaria endemic area and these antibodies can block DBP-erythrocyte binding and inhibit parasite invasion in short-term culture [4–7]. These data support the potential of DBP as a key vaccine development against blood-stage *P. vivax* malaria. However, analysis of genetic diversity of *dbpII* alleles among *P. vivax* isolates from different geographical regions showed high rates of polymorphisms. Therefore, it may hamper vaccine development as some variant residues alter immune recognition of the DBP antigen.

DBPII polymorphisms significantly change antigenic character and sensitivity to neutralizing antibodies [8, 9]. It has been shown that dominant B-cell epitopes in DBPII are polymorphic surface exposed motifs. These dominant polymorphic epitopes tend to create an inherent bias toward a strain specific immune response [6, 10]. However, this parasite immune evasion mechanism can be overcome since some individuals exposed to *P. vivax* in endemic areas are capable of producing broadly inhibitory anti-DBPII antibodies [5, 11]. Therefore, an alternative approach to design DBPII vaccine is to focus the immune response toward conserved epitopes targeting strain-transcending immunity. A recent study indicated cross immunity of anti-DBPII neutralizing antibodies. The DEKnull vaccine candidate had the potential to induce broadly neutralizing antibodies capable of inhibiting heterologous *dbpII* alleles. The removal of dominant polymorphic DEK variant epitopes tended to focus development of immune responses towards the more conserved neutralizing epitopes in the native Sal I strain [12]. Therefore, DBPII based immunogens that target the immune response to conserved functional epitopes may be necessary to avoid induction of strain-specific responses to dominant variant epitopes.

To approach DBPII vaccine development in Thailand, serological responses and inhibitory function of anti-DBPII antibodies were characterized in natural *P. vivax* exposures. Individuals produced anti-DBPII antibodies that significantly increased in titer during acute infection but there was no correlation between antibody titer and inhibitory function [13]. Polymorphic patterns of Thai isolates were defined into 9 haplotypes (DBL-TH1, -TH2, -TH3 . . .etc). The polymorphisms of DBL-TH variants changed the specificity of naturally acquired antibodies. A monoclonal antibody against the DBP7.18 variant (accession no. AAL79051.1) inhibited heterologous DBL-TH haplotypes, indicating anti-DBPII antibodies recognize conserved epitopes that are shared between DBPII Thai variants [14]. Therefore, optimization of immunological responses to conserved DBL-TH epitopes in order to induce broadly inhibitory anti-PvDBPII neutralizing antibody is necessary for effective vaccine development against diverse *P. vivax* in Thai endemic areas. The present study was designed to evaluate whether sequence polymorphisms in *dbpII* genes among *P. vivax* Thai isolates affect the recognition and specificity of

naturally occurring antibody against DBPII Thai variant antigens. An association between DBPII polymorphisms and anti-DBPII inhibitory response was observed in acute *P. vivax* patients infected with Thai isolates.

Results

Inhibition activity of anti-DBPII antibodies against DBL-TH binding

To evaluate inhibitory response against DBL-TH haplotypes in high anti-DBPII responders, the reactivity of naturally acquired antibodies in vivax patients (n = 103) was tested against recombinant DBPII protein by ELISA. The anti-DBPII antibody levels in acute human plasma were significantly higher than naïve controls (*P. vivax* patient, overage optical density [OD] = 0.25 ± 0.08, naive controls, OD = 0.13 ± 0.030, $P < 0.05$, Fig 1A). The serological responses to DBP were used to classify patients into three groups; high responders (HR) (OD = 0.41 to 0.69), low responders (LR) (OD = 0.20 to 0.40) and non-responders (NR) (OD < 0.20) (Fig 1A and 1B). The samples were considered positive when OD value was greater than or equal to the mean plus 2 standard deviations of naive controls. There were 7, 49 and 47 patients in the high responder, low responder and non-responder categories, respectively (Fig 1B).

Our previous study identified the common DBL-TH haplotypes among *P. vivax* isolates [14]. Therefore, in this study, the individual plasma samples of high responders (HR) (n = 7) were used to evaluate inhibitory function of neutralizing antibody against a panel of DBL-TH haplotypes by COS7 cell binding-inhibition assay. All HR patients strongly inhibited DBL-TH2 and -TH7 binding to human erythrocytes, >80% inhibition activity (Fig 2). Most high responders had no inhibitory activity against DBL-TH5 variant binding with only HR3 and HR6 inhibiting this variant. There was no HR patient that inhibited reference DBPII Sal I binding to erythrocyte at >80%. Interestingly, two high responders, HR3 and HR6, inhibited binding of all DBL-TH variants by >80% inhibition activity (Fig 2).

Broad inhibition of anti-DBPII antibodies against DBL-TH haplotypes

To further analyze the ability of plasma samples from HR3 and HR6 (Fig 2) to inhibit both homologous and heterologous DBL-TH haplotypes, plasma from HR3 and HR6 were serially diluted from 1:500 to 1:2000 and tested for inhibitory function of anti-DBPII antibodies by COS7 cell binding-inhibition assay. The result showed that antibodies in HR3 (Fig 3A) and HR6 (Fig 3B) strongly inhibited DBL-TH1, -TH2, -TH6, -TH7, -TH8, and -TH9, with >80% inhibition at 1:500, 1:1000 and 1:2000 plasma dilutions whereas only low inhibitory efficiency was shown towards DBL-TH4 and -TH5 haplotypes (at 1:500 dilution, HR3; -TH4 = 59.10%, -TH5 = 47.74%, HR6; -TH4 = 72.23%, -TH5 = 61.68%) (Fig 3A and 3B). These data suggest that DBL-TH4 and -TH5 variants are composed of critical polymorphic residues which may change antigenic character and alter the potency of neutralizing antibody. The cross inhibition of antibody against DBL-TH1, -TH2, -TH6, -TH7, -TH8, and -TH9 may indicate the sharing of conserved epitopes among DBL-TH variants.

The association between DBPII polymorphisms and inhibition of anti-DBPII antibodies

To further evaluate the association of DBPII polymorphisms and inhibition activity of anti-DBPII antibodies in *P. vivax* exposed individuals, 40 additional blood samples from acute *P. vivax* patients were collected in 2014. DBPII polymorphism analysis was used to identify DBL-TH haplotypes in acute *P. vivax* patients and their plasma was used for evaluation of inhibitory activity against DBL-TH haplotypes by COS7 cell binding-inhibition assay. The

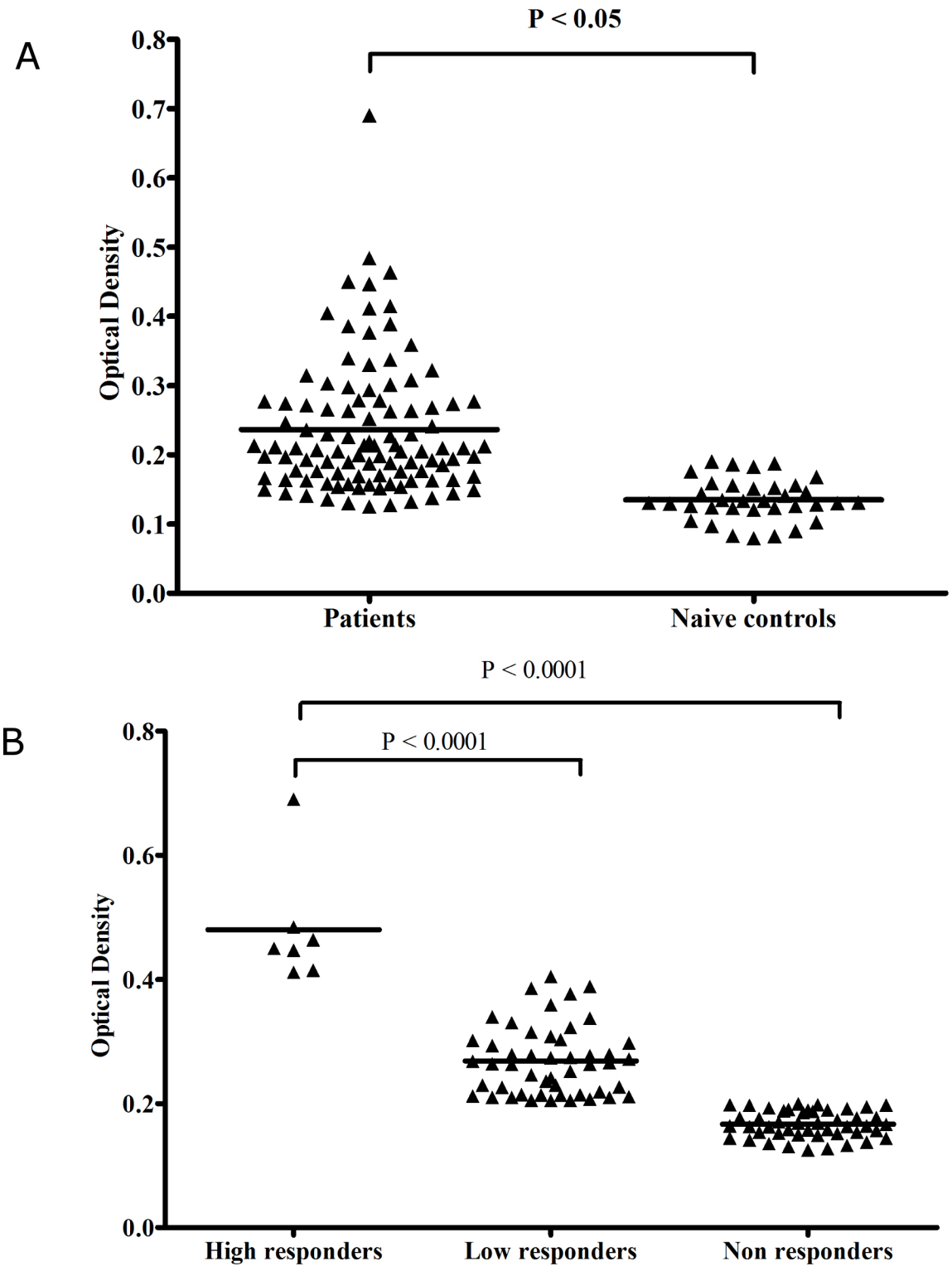

Fig 1. Antibody recognition of recombinant PvDBPII. The scatter plot graph shows the anti-DBPII antibody levels in Thai patients compare to naive control as measured by ELISA. **(A)** Anti-DBPII levels were significantly higher in patients with acute *P*. *vivax* than in naive controls, **(B)** ELISA data classified patients into 3 groups: high responder (HR), low responders (LR) and non-responders (NR). Each dot represents the mean of optical density values in double wells for each sample. The line represents the mean value. Significance was determined by non-parametric analysis using the Mann-Whitney U test. The level of significant was set at $P < 0.05$.

doi:10.1371/journal.pone.0154577.g001

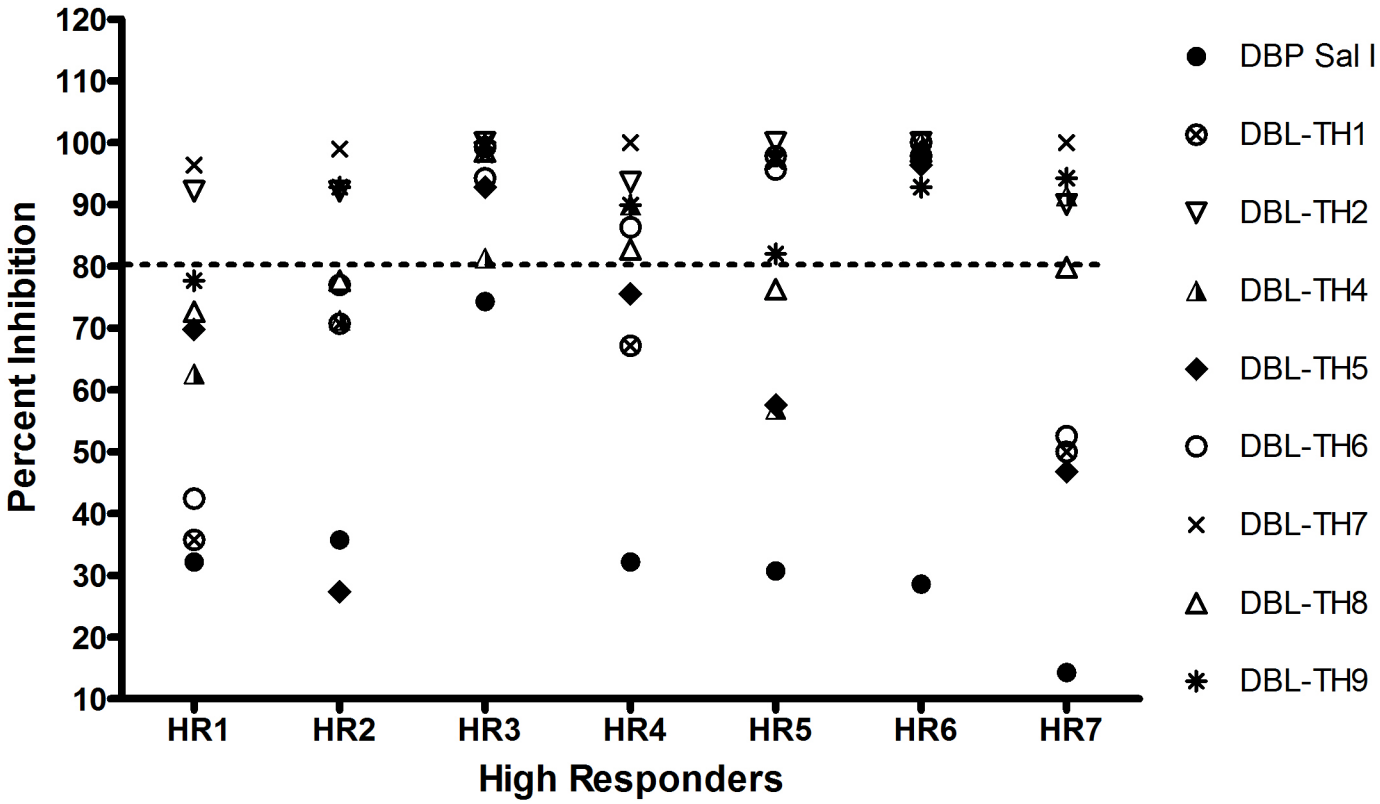

Fig 2. Functional inhibition of anti-DBPII antibodies in high responder samples against the panel of DBL-TH variants. Transfected COS7 cells expressing DBL-TH alleles, DBL-TH1, -TH2, -TH3, -TH4, -TH5, -TH6, -TH7, -TH8, -TH9 and reference Sal I were incubated with 1:100 plasma dilution for 1 hr at 37°C followed by incubation with a 10% suspension of human erythrocytes for 2 hrs. The number of rosettes was compared between wells of transfected cells incubated with plasma relative to wells without plasma (30 fields of view, magnification ×200). The symbols represent mean percent inhibition of two experiments tested in duplicate wells.

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patterns of DBPII polymorphisms in individuals of acutely *P. vivax* patients were classified into 2 groups, those infected with Sal I strain and those infected with DBL-TH haplotypes. There were 22 (55%) and 18 (45%) patients infected with DBP Sal I strain and DBL-TH haplotypes, respectively. For patients infected with DBL-TH haplotypes, 12 haplotypes were defined among *P. vivax* isolates. Nine haplotypes (DBL-TH1, -TH2, -TH3, TH4, -TH6, -TH7, -TH8 and -TH9) were similar to our previously reported haplotypes [14]. Three new DBL-TH haplotypes, DBL-TH10, DBL-TH11 and DBL-TH12 were first identified in this Thai endemic area. The highest frequency was DBL-TH1 haplotype (33.33%) (Text in S1 Table).

Since the mutation of amino acids has been shown to change antigenic character of DBPII [8], in this study, DBL-TH4 and DBL-TH5 haplotypes which contain multiple polymorphic residues and reference Sal I strain were used for characterization of broadly anti-DBPII neutralizing antibodies. The result showed that nine (22.50%) patients developed anti-DBPII neutralizing antibody against heterologous DBL-TH4 and -TH5 binding to erythrocytes, >80% inhibition activity. Interestingly, one (2.5%) patients broadly inhibited both heterologous DBL-TH4, DBL-TH5 and holomologous reference Sal I strain (Table 1). In contrast, twelve (30.00%) patients had inhibitory antibody against only heterologous DBL-TH4 or DBL-TH5 strain. One patient (2.5%) developed anti-DBPII neutralizing antibody response against only homologous reference Sal I strain whereas no inhibitory response to heterologous DBL-TH4 or

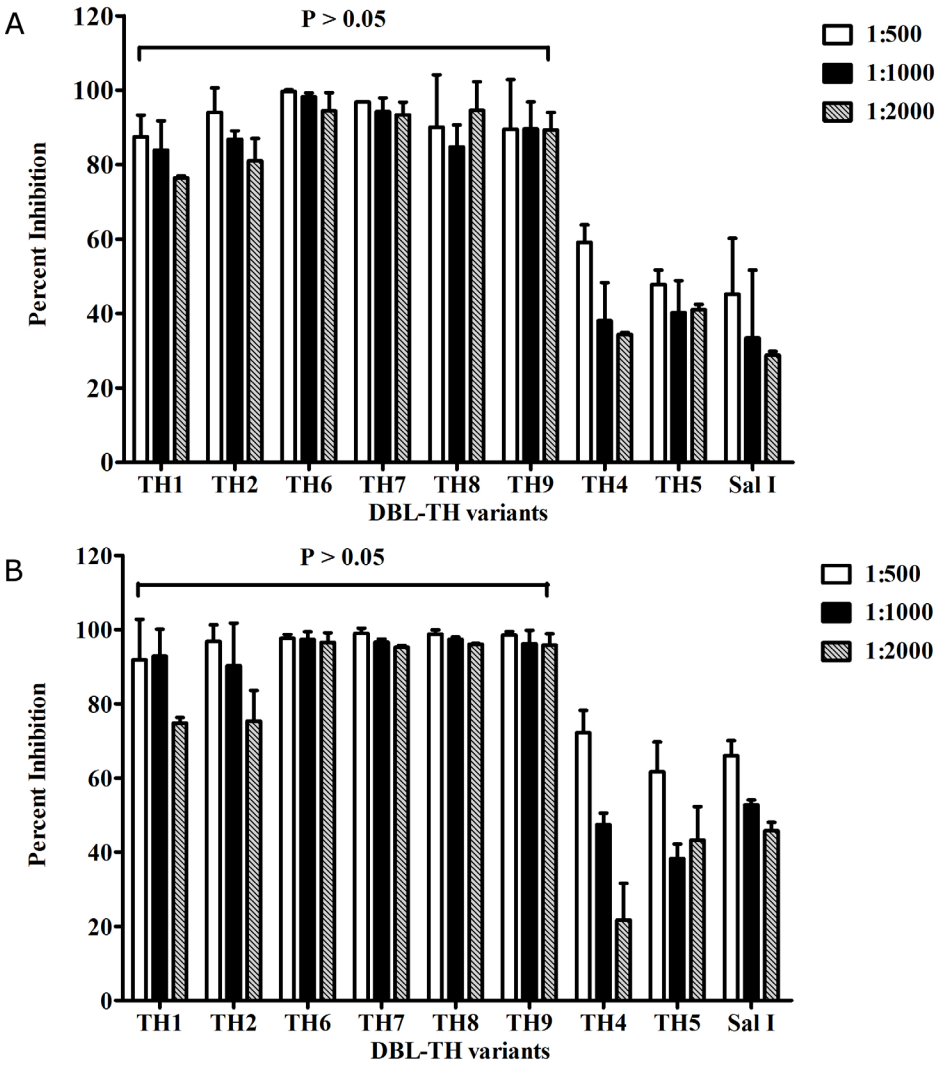

Fig 3. Broad inhibition by high responder samples of erythrocyte binding to DBL-TH haplotypes. The transfected COS7 cell expressing DBPII reference Sal I or DBL-TH variants were pre-incubated with plasma at dilution 1:500, 1:1000 and 1:2000 for inhibition of DBPII-erythrocyte binding. The charts show the mean inhibition of each DBL-TH variant. Inhibitory function against the panel of DBL-TH variants by (A) HR3 and (B) HR6 samples. Each chart represents the mean of two independent experiments with each dilution tested in triplicate. Error bars represent ± standard deviation. Statistical significance was determined using one-way analysis of variance (ANOVA) and multiple comparison analysis by Bonferroni test.

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DBL-TH5 (Table 2). Seventeen patients (42.50%) did not develop antibody against DBL-TH4 or TH5 or Sal I binding.

Anti-DBPII antibodies block both homologous and heterologous DBL-TH haplotypes binding to erythrocytes

To support the ability of anti-DBPII antibodies in inhibitory response against both homologous and heterologous during *P. vivax* infection, plasma samples from acute *P.vivax* patients no.9 and no.15 who had the highest broadly inhibition activity against heterologous DBL-TH4, DBL-TH5 and reference Sal I binding were used to evaluate inhibition against a panel of

Table 1. Inhibitory function of anti-DBPII antibodies in *P. vivax* individuals against heterologous DBL-TH4 and DBL-TH5 or heterologous DBL-TH4, DBL-TH5 and homologous reference Sal I binding to human erythrocytes measured by COS7 cell binding-inhibition assay

Sample ID	DBPII haplotypes	Functional Assay		
		DBL-TH4	DBL-TH5	Reference Sal I
2	DBP-Sal I	HI*	HI	NI*
3	DBP-Sal I	HI	HI	NI
9	DBP-Sal I	HI	HI	HI
15	DBP-Sal I	HI	HI	NI
16	DBL-TH1	HI	HI	NI
24	DBP-Sal I	HI	HI	NI
29	DBP-Sal I	HI	HI	NI
31	DBP-Sal I	HI	HI	NI
33	DBL-TH1	HI	HI	NI
36	DBP-Sal I	NI	HI	HI

* NI = Non inhibition (<80% inhibition), HI = High inhibition (>80% inhibition)

doi:10.1371/journal.pone.0154577.t001

DBL-TH haplotypes by COS7 cell binding-inhibition assay after serial dilution from 1:500 to 1:2000.

For patient no.9, the inhibition capacity typified strain-transcending inhibition showing broad inhibition against heterologous DBL-TH2, -TH4, -TH5, -TH6, -TH7, -TH8, -TH9 and homologous Sal I strain (Fig 4A). Percent inhibition was not significantly different among these DBL-TH haplotypes at 1:500, 1:1000 and 1:2000 dilution (Fig 4A, $P > 0.05$). However, statistical analysis demonstrated significantly different inhibitory activity against DBL-TH1 when plasma was diluted at 1:1000 or 1:2000 (Fig 4A, $P < 0.05$). In contrast, inhibition activity against DBL-TH binding of patient no.15 was not strain-transcending. There was significantly different inhibition against 8 DBL-TH haplotypes and the Sal I haplotype binding to human erythrocyte when plasma was serially diluted from 1:500 to 1:2000 (Fig 4B, $P < 0.05$).

Table 2. Inhibitory function of anti-DBPII antibodies in *P. vivax* individuals against heterologous DBL-TH4 or DBL-TH5 or homologous reference Sal I binding to human erythrocytes measured by COS7 cell binding-inhibition assay

Sample ID	DBPII haplotypes	Functional Assay		
		DBL-TH4	DBL-TH5	Reference Sal I
5	DBL-TH3	NI*	HI*	NI
11	DBP-Sal I	HI	NI	NI
17	DBL-TH11	HI	NI	NI
18	DBP-Sal I	HI	NI	NI
19	DBP-Sal I	HI	NI	NI
21	DBL-TH2	NI	HI	NI
22	DBP-Sal I	NI	HI	NI
25	DBL-TH2	HI	NI	NI
28	DBL-TH8	HI	NI	NI
30	DBL-TH12	NI	HI	NI
32	DBP-Sal I	HI	NI	NI
35	DBP-Sal I	NI	NI	HI
37	DBL-TH4	HI	NI	NI

* NI = Non inhibition (<80% inhibition), HI = High inhibition (>80% inhibition)

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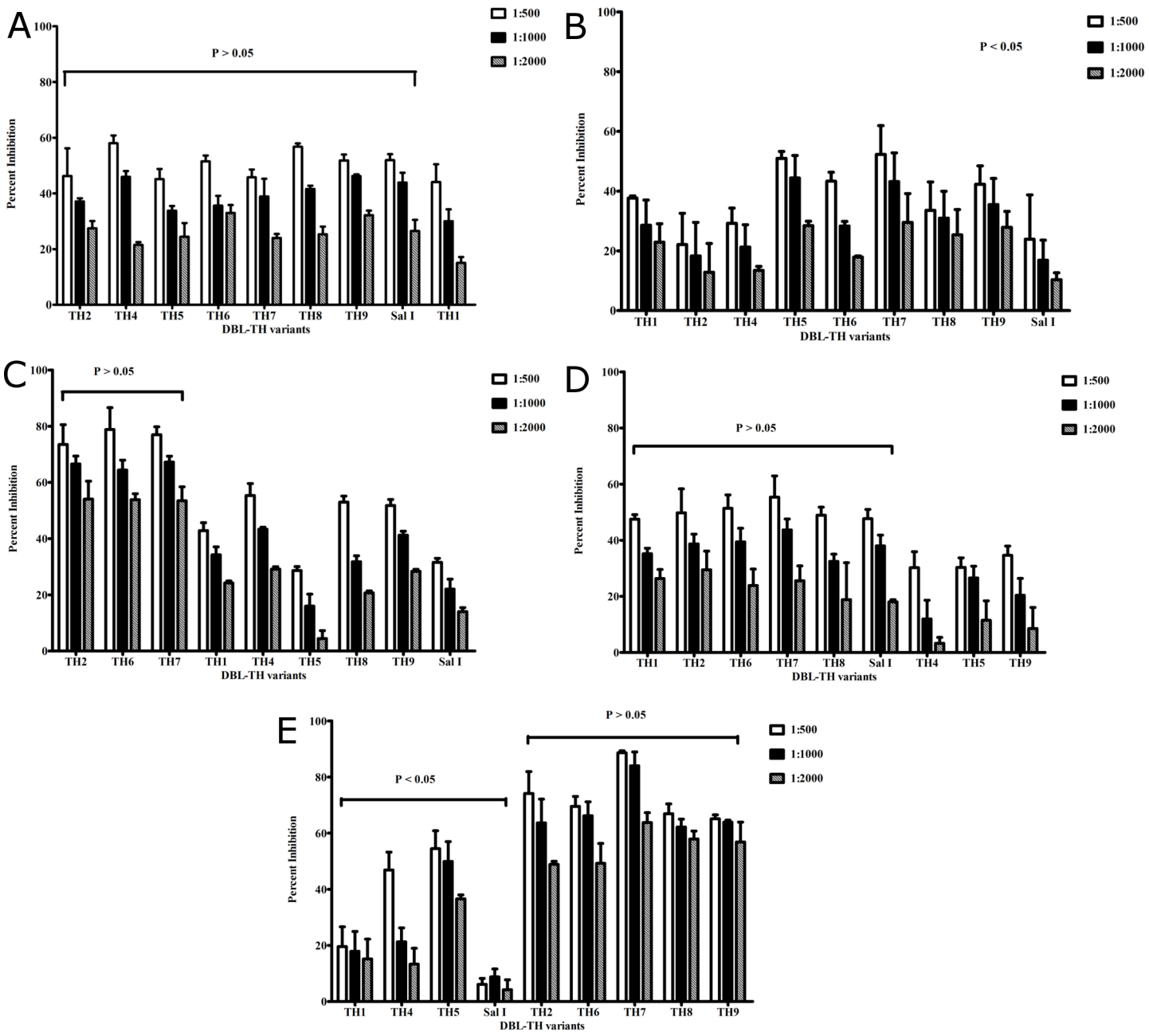

Fig 4. Inhibition efficiency of anti-DBPII antibodies in *P. vivax* exposed individuals. A and B: Inhibitory function against a panel DBL-TH haplotypes of vivax patients infected with Sal I strain and had the strongest inhibitory immunity against both heterologous DBL-TH4 and DBL-TH5 strain **(A)** patient no.9, **(B)** patient no.15. C, D and E:. Inhibitory function against a panel DBL-TH of vivax patients infected with high polymorphism DBL-TH strain and had the strongest inhibitory immunity against heterologous DBL-TH4 or DBL-TH5 or Sal I strain, **(C)** patient no.25 infected with DBL-TH2 strain, **(D)** patient no.37 infected with DBL-TH4 strainand **(E)** infected with new DBL-TH strain, patient no.30. The transfected COS7 cells expressing Thai DBPII alleles were incubated with plasma and with human erythrocytes. The number of rosettes was compared between wells of transfected cells incubated with antibodies relative to wells without antibodies. Each chart represents the mean of two independent experiments with each dilution tested in triplicate. Error bars represent ± standard deviation. Statistical significance was determined using one-way analysis of variance (ANOVA) and multiple comparison analysis by Bonferroni test.

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To study broadly inhibition activity of anti-DBPII antibodies in patients who infected with high polymorphism DBL-TH variant strain, patient plasma no.25 who infected with DBL-TH2

Table 3. Polymorphic residues of DBL-TH haplotypes infected in *P. vivax* patients at the time of enrollment.

DBPII alleles	Amino acid position														
	308	313	333	371	375	384	385	386	390	417	424	433	437	475	503
DBPII-Sal I	R	.	L	K	.	D	E	K	R	N	L	.	W	P	I
DBL-TH1	.	.	F	.	.	.	.	.	.	.	I	.	R	A	K
DBL-TH2	.	.	F	E	.	G	K	Q	.	K	I	.	R	.	.
DBL-TH3	.	.	F	.	.	G	.	.	H	.	.	.	.	.	K
DBL-TH4	.	.	F	.	.	G	K	Q	H	K	I	.	R	.	K
DBL-TH5	.	.	.	E	.	G	.	N	.	K	I	.	R	.	K
DBL-TH6	.	.	.	.	.	G	.	H	.	.	.	.	.	.	.
DBL-TH7	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.
DBL-TH8	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.
DBL-TH9	.	.	F	.	.	.	.	.	.	.	I	.	R	.	K
DBL-TH10	.	P	.	.	.	.	.	.	.	.	.	H	.	.	.
DBL-TH11	.	.	F	.	.	.	K	.	.	.	.	.	.	.	.
DBL-TH12	S	.	F	.	D	G	K	N	H	K	I	.	R	.	.

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and patient plasma no.37 who infected with DBL-TH4 were diluted and tested inhibition capacity against a panel DBL-TH binding. The result showed that patient no.25 who infected with DBL-TH2, showed high inhibition against homologous DBL-TH2 and heterologous DBL -TH6 and -TH7 haplotypes at 1:500, 1:1000 and 1:2000 plasma dilutions (Fig 4C, $P > 0.05$). However, this patient had a low inhibitory immune response to heterologous DBL-TH1, -TH4, -TH5, TH-8, -TH9 and Sal I strain at 1:500, 1:1000 and 1:2000 plasma dilutions (Fig 4C). For patient no.37 who was infected with DBL-TH4 showed broad inhibition against heterologous DBL-TH1, -TH2, -TH6, -TH7, -TH8 and Sal I strain when plasma was diluted at 1:500, 1:1000 and 1:2000 (Fig 4D, $P > 0.05$) whereas inhibition activity against homologous DBL-TH4 and heterologous -TH5, -TH9-erythrocyte binding was very low at 1:500, 1:1000 and 1:2000 plasma dilutions (Fig 4D).

In this study, three novel DBPII Thai haplotypes, DBL-TH10, -TH11 and -TH12 were identified (Table 3). To analyze the association of DBPII Thai polymorphisms and broad inhibition by neutralizing antibodies, plasma from patient no.30, infected with DBL-TH12 haplotype, was examined. Anti-DBPII antibodies in this patient highly inhibited heterologous DBL-TH2, -TH6, -TH7, -TH8 and -TH9 haplotypes at 1:500, 1:1000 and 1:2000 plasma dilutions (Fig 4E, $P > 0.05$). However, the inhibition activity against heterologous DBL-TH1, -TH4, -TH5 and Sal I was significantly different among DBL-TH haplotypes at 1:500, 1:1000 and 1:2000. These data suggest that patients infected with the new DBL-TH variant produced neutralizing antibody specific to conserved epitopes of DBL-TH2, -TH6, -TH7, -TH8 and -TH9 (Fig 4E, $P < 0.05$), but strain-specific antibodies towards DBL-TH1, -TH4, -TH5 and Sal I (Fig 4E).

Discussion

Developing a vaccine for *P. vivax* represents a major challenge especially considering the limitation of *in vitro* cultures. To date, a completely effective vaccine that can be introduced to clinical practice for malaria is not available. Most vivax vaccine candidate antigens such as DBP, Merozoite Surface Protein 1 (MSP1) and Apical Membrane Antigen 1 (AMA-1) are highly polymorphic surface proteins that stimulated strain-specific immunity [6, 8, 15, 16]. This antigenic diversity is one of the biggest challenges in vaccine development since it enables the parasite to evade the host immune responses [17, 18]. Therefore, to design an efficient anti-malarial

vaccine, worldwide information of the circulating antigenic variants is necessary for the formulation of a polyvalent vaccine, which would be effective in different malaria-endemic areas [19, 20]. Recently, an alternative approach is the design of a vaccine focusing the immune response toward conserved epitopes that are the target of neutralizing inhibitory antibodies [12]. Understanding mechanisms of sequence variation in *dbpII* alleles and the immunological response to DBPII variant antigens may help in designing vaccines. Therefore, the purpose of this study was to evaluate whether sequence polymorphisms in *dbpII* genes affect the inhibitory function of naturally occurring antibody to eight sequence variants of DBL-TH antigens (DBL-TH1, -TH2, -TH4, -TH5, -TH6, -TH7, -TH8 and -TH9), which may have implications for development of DBPII-based vaccine.

The analysis of genetic diversity of *dbpII* alleles among *P. vivax* isolates has been reported from different geographical regions, including Brazil [21], Colombia [22], South Korea [23], Thailand [24], Sri Lanka [25], Iran [26], Myanmar [27] and Papua New Guinea [28, 29]. Among the common polymorphic residues were K371E, D384G, E385K, K386N, N417K, L424I, W437R and I503K [21–30]. Although these polymorphisms do not appear to interfere with receptor recognition, some of them (R308S, D384K and K386N) affect the ability of acquired neutralizing antibodies to inhibit DBP function [8, 10, 13]. In our current study, inhibitory responses against DBL-TH4 and -TH5 haplotypes in high responders showed strain specificity whereas broadly neutralizing inhibition was shown in their response against DBL-TH1, -TH2, -TH6, -TH7, -TH8 and -TH9. This suggests that mutant residues (D384K, K386Q/N, N417K, L424I, W437R, and I503K), which are present in both DBL-TH4 and -TH5, may alter antibody recognition. Additionally, sequence alignment of DBL-TH2 and -TH4 indicated shared common polymorphic residues (L333F, D384G, E385K, K386Q, N417K, L424I, W437R) and only residues, K371E, R390H and I503K were different between DBL-TH2 and -TH4 [14]. I503K has previously been shown to be part of a haplotype that alters sensitivity to inhibition [8]. Therefore, these data suggest that these polymorphic residues contained in the DBL-TH4 haplotype may interfere with strain-transcending immunity of anti-DBPII antibodies.

To understand protective immunity against DBL-TH haplotypes in individuals exposed to *P. vivax* infection, the inhibitory function of naturally acquired anti-DBPII antibodies against a panel DBL-TH variants was evaluated in acutely infected *P. vivax* patients infected with Sal I or DBL-TH haplotypes. The result demonstrated that patients who were infected with *P. vivax* expressing the DBP Sal I allele showed a high prevalence of strain-transcending inhibition towards DBL-TH strains with 50.0% and 40.1% of Sal I patients having broad inhibition against DBL-TH4 and DBL-TH5 haplotypes, respectively, whereas only 13.82% of patients produced antibodies to inhibit homologous Sal I DBP. Additionally, most vivax patients infected with DBL-TH variants had inhibitory antibodies against heterologous DBL-TH4 (33.33%) or DBL-TH5 (27.78%) whereas patients had no inhibitory immune response against heterologous Sal I stain. These data suggested that vivax patients infected with Sal I strain could produce broadly neutralizing antibodies to heterologous *dbpII* Thai variant alleles. However, the patients infected with DBL-TH variants rarely produced broadly neutralizing antibodies against reference Sal I stain. Interestingly, some vivax patients infected with DBL-TH variants had anti-DBPII antibodies to inhibit both homologous and heterologous DBL-TH variants. Together, this study support that DBPII variation is an evasion mechanism responsible for strain-specific immunity and that stable broadly neutralizing antibodies are achieved when antibodies target functionally conserved epitopes.

Strain-transcending immunity of anti-DBPII immune response has been observed in previous studies. Children who had long-term exposure to malaria showed high levels of anti-DBP inhibitory antibody and had strain-transcending protection against *P. vivax* infection [5]. This

finding is in line with other studies that demonstrated that long-term residents living in vivax malaria endemic areas were able to inhibit erythrocyte binding to two common variants (Sal I and Acre-1) [31]. The cross reactivity of anti-DBPII antibody to heterologous variants (DBPI, DBPV, DBPVI, DBPIX, DBPX) was also demonstrated in Iranian vivax infected individuals [11]. Additionally, a study by *Ntumngia et al* [32] revealed that monoclonal antibodies specific to DBP7.18 variants (accession no. AAL79051.1) recognize conserved epitopes of heterologous *dbpII* alleles including DBPII Thai haplotypes [14]. Therefore, for DBPII vaccine design, it should be possible to produce broadly neutralizing immunity to all the DBPII variants represented in *P. vivax* endemic areas. Here, the association of DBPII Thai polymorphisms with broad inhibition of anti-DBPII antibodies was examined in acutely infected *P. vivax* patients. Among vivax patients infected with reference Sal I strain, patient no.9 produced broadly neutralizing antibody blocking heterologous DBL-TH and Sal I-erythrocyte binding (Fig 4A) whereas patient no.15 produced allele-specific neutralizing antibodies towards a panel of DBL-TH haplotypes (Fig 4B, $P < 0.05$). Among vivax patients infected with DBPII Thai variants, patient no.25, infected with DBL-TH2, produced neutralizing antibody against homologous DBL-TH2 and heterologous DBL-TH6 and -TH7 (Fig 4C), while patient no.37, infected with DBL-TH4, produced a broader range of neutralizing antibody against heterologous DBL-TH1, -TH5, -TH6, -TH9 and reference Sal I strain but showed no inhibitory capacity against homologous DBL-TH4 variant (Fig 4D). Moreover, vivax patient no.30, infected with DBL-TH12, showed strain-transcending immunity against heterologous DBL-TH2, -TH6, -TH7, -TH8 and -TH9 (Fig 4E). Together, these data suggests that vivax patients infected with DBPII Thai variants are able to produce anti-DBPII neutralizing antibody against some heterologous DBPII Thai variants. These neutralizing antibodies appear to share recognition of conserved B-cell epitopes to overcome the strain specific immunity.

In summary, we have demonstrated that *P. vivax* patients in Thai endemic areas are able to produce neutralizing antibodies that can block DBL-TH variants-erythrocyte binding. Moreover, this study further demonstrates that strain-transcending anti-DBPII immunity against heterologous *dbpII* Thai alleles occurs in some vivax infected individuals. Further study is necessary to identify conserved B-epitopes from *P. vivax* field isolates that can overcome DBPII variation as one of the biggest challenge of DBPII-based vaccine development.

Materials and Methods

Ethics Statement

This study was approved by the Committee on Human Rights Related to Human Experimentation, Mahidol University, and the Ministry of Health, Thailand (MUIRB2012/079.2408). The participant information sheet was written and approved by Committee on Human Rights Related to Human Experimentation, Mahidol University, and the Ministry of Health, Thailand. The informed consent was signed by each participant before the blood sample was collected. The selecting criteria of the patients were as followings: (1) systolic blood pressure not less than 90 mm, (2) body temperature not higher than 40°C, (3) hematocrit not less than 25% and (4) age of 18 or above. Those who did not fit the criteria were excluded. The minority participants were not involved in the study.

Blood sample preparation

We collected blood samples from acutely infected *P. vivax* patients at malaria clinics in Chumphon province, which is in the Southern part of Thailand. The areas are malaria endemic with a high prevalence of *P. vivax* infections. One hundred three sera samples were collected in 2011–2012 from *P. vivax* patients for screening for anti-DBPII responses and evaluation of

strain-transcending inhibition against DBL-TH variants. Forty blood samples were collected in 2014 to study the association of DBL-TH polymorphisms and broadly inhibitory activity of anti-DBPII antibodies in individuals with *P. vivax* exposure. Individuals were assigned for DBPII sequence analysis and testing of inhibitory function of antibodies against Thai DBPII haplotypes. Three blood spots were collected on filter paper from each consenting *P. vivax* patient for preparation of parasite isolates. Parasite genomic DNA was extracted with a QIAamp DNA mini kit (Qiagen, Valencia, CA, USA). The confirmation of *P. vivax* infection was performed by microscopic examination of thin and thick Giemsa-stained blood smears. Blood samples for malaria-naive controls were obtained from 35 healthy volunteers who live in Bangkok and had no history of exposure to *Plasmodium* parasites. Acute *P. vivax*-infected volunteers who registered at Malarial Clinics and naïve control subjects were asked for informed consent under the protocol approved by the Ethic Committee on Human Rights Related to Human Experimentation, Mahidol University (MUIRB2012/079.2408).

Measurement of antibody response to DBPII antigen by ELISA

Serological response against recombinant DBP reference Sal I was quantified by ELISA. Recombinant DBP region II (rDBPII) was expressed as a glutathione S-transferase (GST) fusion protein in E. coli. It was then affinity purified on glutathione and cleaved from GST with thrombin using standard methods [33]. Purified rDBP region II was added to 96-well plates at 2 μg/mL and incubated overnight at 4°C. Wells were incubated with blocking buffer (2% skim milk in PBS) for 2 hr and washed three times with wash buffer. The diluted plasma (n = 103) at 1:200 was added to allow binding to rDBP antigen and incubated for 1 hr at 37°C. Bound rDBP and human plasma were detected with goat anti-human IgG-alkaline phosphatase (1:1000 dilution; KPL, Maryland, USA). The anti-DBPII antibody activity was detected by recording the absorbance (OD) at 405 nm. The overage absorbance and standard deviation were calculated for each plasma sample. A baseline OD was established using plasma from 40 samples of non-malaria exposed Thai individuals and this control value was subtracted from test OD values to standardize the assay. The samples were considered positive when the OD value was greater than or equal to the mean plus 2 standard deviations of negative controls. The antibody reactivity in human plasma was classified into three groups: high responder (OD = 0.41 to 0.69), low responders (OD = 0.20 to 0.40) and non-responders (NR) (OD $<$ 0.20) [10, 13].

Identification of DBL-TH haplotypes in *P. vivax* patients

To identify the DBP region II genotype of the causative agent in acutely infected *P. vivax* patients, 40 blood spot samples were taken for DNA isolation and amplification. DBPII genes were PCR amplified as described in detail previously [14]. In brief, PCR cycling conditions for each primer pair were 90 sec initial denaturation at 94°C, followed by 30 cycles of 15 sec denaturation at 94°C, 35 sec annealing at 60°C, and 60 sec extension at 68°C, and a final extension step of 2 min at 68°C. The PCR products were subsequently sequenced using the dideoxynucleotide chain termination method (Applied Biosystems, Foster City, CA). The alignment of complete sequences of PvDBPII genes from 40 isolates were analyzed by CLUSTAL and percent similarity was assessed using BioEdit software.

COS7 culture and transfection

COS7 cell erythrocyte binding assays were carried out to evaluate the ability of neutralizing antibodies in *P. vivax* patient to inhibit binding of DBL-TH variant and reference Sal I haplotypes to human erythrocytes. Expression plasmid constructs were engineered to express

DBL-TH or reference Sal I alleles on the surface of transiently transfected COS-7 cells as fusion proteins to the N-terminus of enhanced green fluorescent protein (EGFP) [7]. Recombinant plasmids were transfected into green monkey kidney cells (COS-7, American Type Culture Collection, and Manassas, VA, USA) by the use of Lipofectamine 2000 reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). COS-7 cells were seeded in 24-well culture plates (4.5×10^4 cells/well) in Dulbecco's Modified Eagle Medium (DMEM, Sigma, USA) with 10% fetal bovine serum (Gibco BRL, Life Technologies, Rockville, MS, USA). Recombinant plasmid DNA (100 ng/well) was mixed with Lipofectamine (2% Lipofectamine 2000/well) in DMEM without serum and then added into transfected wells (100 μl/well) and incubated in a humidified incubator with 5% CO_2 at 37°C for 42–44 hr. The detection of the C-terminal green fluorescent protein (GFP)-expressing vector was used as positive control for checking transfection efficiency.

Measurement of the inhibitory efficiency of anti-DBPII antibodies against DBL-TH binding by COS7 cell binding-inhibition assay

The inhibition of anti-DBPII neutralizing antibodies against DBPII-erythrocyte binding was performed as previously reported [7, 34]. Briefly, 42–44 hr after transfection, diluted human plasma from high responders was pre-incubated with the transfected COS-7 cells expressing DBL-TH or reference Sal I haplotypes for 1 hr at 37°C before addition of a 10% suspension of Duffy positive human erythrocytes in each well followed by a 2 hr incubation. Unbound erythrocytes were removed by washing the well with PBS. DBP-erythrocyte binding was quantified by counting rosettes observed over 30 fields of view (magnification, ×200). The binding-inhibition was determined by assessing the number of rosettes in wells of transfected COS7 cells in the presence of plasma relative to rosettes in wells of transfected cells in presence of medium control. The positive and negative inhibition binding controls were 3C9 mouse monoclonal antibodies against DBPII.7.18 haplotype [14] and medium control, respectively. Experiments for each human plasma sample were done in triplicate wells and were repeated two times.

Statistical analysis

Comparison of anti-DBPII antibody levels between unpaired groups (patients compared to naive controls) was performed using the Mann-Whitney U test. The inhibition activity for each plasma sample was compared between all the DBL-TH alleles and tested for any statistically significant differences in antibody reactivity and inhibitory responses by one-way analysis of variance and multiple comparison analysis by Bonferroni test. P-values < 0.05 were considered significant. The statistical analysis was performed and graphs prepared using GraphPad Prism (v. 5; GraphPad Software, San Diego, CA, USA).

Supporting Information

S1 Table. DBPII haplotypes as causative agent of *P. vivax* infection in individuals at time of enrollment. Data are presented DBL-TH haplotypes in acutely infected *P. vivax* patients (n = 40) Blood spot samples were taken for DNA isolation and amplification. DBPII genes were PCR amplified and PCR products were subsequently sequenced. The alignment of complete sequences of PvDBPII genes from 40 isolates were analyzed by CLUSTAL and percent similarity was assessed using BioEdit software.
(DOCX)

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Author Contributions

Conceived and designed the experiments: PC. Performed the experiments: SW. Analyzed the data: PC SW. Contributed reagents/materials/analysis tools: JS JHA. Wrote the paper: SW PC AMM JHA.

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