VARIATIONS IN ANTIBODY RESPONSE TO *PLASMODIUM VIVAX* DUFFY BINDING PROTEIN REGION II OF INFECTED INDIVIDUALS IN THAILAND

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Abstract. Duffy binding protein region II of Plasmodium vivax (PvDBPII) is a key target for vaccine-mediated immunity despite its highly polymorphic nature. Genetic diversity of PvDBPII within and between P. vivax isolates vary according to geographic regions, which might affect host immune response. This study evaluated DBPII allelic variations and naturally acquired immunity in P. vivax-infected individuals from malaria endemic areas of Thailand. Twenty-three 15-mer DBP peptides (DBPps) covering a critical binding motif in PvDBPII of P. vivax Thai isolates were evaluated for their immunogenicity in P. vivax-infected individuals (n = 82) compared to healthy non-parasite exposed controls (n = 39). Eighty-seven percent P. vivax-infected individuals had significantly higher anti-PvDBPII total IgG against six DBPps compared to controls. Anti-PvDBPII IgG1 was the most prominent subclass. Low IgG3 levels against Thai P. vivax-specific DBPp4 and DBPp5 and non-polymorphic DBPp22 were also detected. High IgG1 and low IgG3 response to DBPp4 and DBPp5 were associated with DBPp4 L333F (CTT>TTT) and DBPp5 S351C (AGT>TGT) mutations, in which the point mutation was C/A>T, whereas T/C>A/C mutation was present in other DBPps. These preliminary data revealed variations in levels of anti-PvDBPII IgG subclasses in P. vivax-infected individuals, which might impact vaccine development strategies against vivax malaria.

Keywords: *Plasmodium vivax*, Duffy binding protein, IgG subclass, malaria infection, polymorphism

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INTRODUCTION

Plasmodium vivax infection is a significant cause of morbidity and mortality in malaria and poses challenges for control and elimination of this debilitating disease, with over one-third of world's population (3.3 billion people) estimated to be at risk in 2017: 1.6 billion in Southeast Asia, 661 million in Western Pacific region and 629 million in Africa (Battle and Baird, 2021). *P. vivax* invades reticulocytes, a process requiring interaction between parasite surface Duffy binding protein (DBP) and cognate human Duffy antigen on reticulocyte/erythrocyte surface as an essential initial step (Souza-Silva *et al*, 2010; Golassa *et al*, 2020). Duffy-negative individuals are typically resistant to *P. vivax* infection (Miller *et al*, 1976); however, transmission of *P. vivax* among Duffy antigen receptor for chemokine (DARC)-negative individuals has been described in Africa (Ryan *et al*, 2006; Wurtz *et al*, 2011; Mendes *et al*, 2011; Ngassa Mbenda and Das, 2014; Lo *et al*, 2015; Abdelraheem *et al*, 2016) and in South America (Cavasini *et al*, 2007; Carvalho *et al*, 2012). *Pv*DBP is a prime target of protective immune response and promising candidate for vaccine development (Chen *et al*, 2016).

*Pv*DBP is a 140-kDa type 1 integral membrane protein consisting of seven regions: an N-terminal leader peptide sequence (region I), two cysteine rich regions (region II and VI), a hydrophobic region (region III), and a C-terminal transmembrane domain (region VII). The receptor-binding domain lies within 330 amino acids (aa) of the cysteine-rich region characterized by 12 conserved cysteine residues, known as DBP region II (*Pv*DBPII), the minimal binding domain to DARC on Duffy-positive red blood cells (RBCs) (Sim *et al*, 1994). The critical erythrocyte binding motifs of *Pv*DBPII lies between cysteines 4-7 (aa 291-460) within a region spanning 170 aa (Chitnis and Miller, 1994) including cysteines 5-8 (Chitnis *et al*, 1996). Within the identified binding motif, cysteine residues are conserved while other amino acids are highly polymorphic (Ampudia *et al*, 1996; Xainli *et al*, 2000).

Seven regions of the hypervariable portion of *Pv*DBPII are critical targets for host protective immunity (Tsuboi *et al*, 1994) and contain potential cross-reactive epitopes to elicit strain-transcending inhibitory antibodies (Abs) (Singh *et al*, 2018). Individuals residing in malaria endemic areas develop high titers of naturally acquired *Pv*DBPII-binding inhibitory Abs that can block *Pv*DBPII-DARC interaction in vitro by divergent *Pv*DBPII alleles with similar capabilities (King *et al*, 2008). Properties of such inhibitory antibodies correlate with protection against *P. vivax* infection (Singh *et*

al, 2018). Naturally acquired Abs to *Pv*DBPII were detected in people residing in a village of Colombia where *P. vivax* malaria is not endemic (Michon *et al*, 1998), and in areas with unstable and hypo-endemic *P. vivax* malaria transmission in Iran (Zakeri *et al*, 2011). The presence of Abs to recombinant *Pv*DBPII with an additive effect of Abs against *P. vivax* Erythrocyte Binding Protein II (*Pv*EBPII) shows a strong correlation with protection against *P. vivax* infection in a high endemic area of malaria transmission in Papua New Guinea (He *et al*, 2019). In an animal model, Abs against recombinant *Pv*DBP either from immunized rabbit or affinity-purified human serum can inhibit *P. vivax* reticulocyte invasion (Grimberg *et al*, 2007). Thus, the invasion inhibitory activity of anti-*Pv*DBP antibodies, which prevents the *Pv*DBP-DARC interaction, provides a key proof-of-concept that these antibodies inhibit erythrocyte invasion by *P. vivax*.

Analyses of genetic diversity of DBPII alleles among geographically distinct *P. vivax* isolates indicate that the polymorphic residues are mostly in the binding ligand domain and varies according to geographic areas (Tsuboi *et al*, 1994; Ampudia *et al*, 1996; VanBuskirk *et al*, 2004; Sousa *et al*, 2006; Grimberg *et al*, 2007; Babaeekho *et al*, 2009; Hwang *et al*, 2009; Premaratne *et al*, 2011; Ju *et al*, 2012). *Pv*DBPII of Thai isolates has been detected containing five synonymous and twenty-five nonsynonymous (D384G, R390H, L424I, W437R, and I503K) polymorphisms among 30-point mutations compared to the reference Sal I strain, with L424I having the highest frequency (Gosi *et al*, 2008). Using Nei and Gojobori's method (Nei and Gojobori, 1986) the different rates of synonymous and nonsynonymous mutations are likely due to differences in selection pressure. Phylogenetic analysis of DBPII among Thai *P. vivax* isolates indicates the existence of six discrete allele groups, with groups 4 and 6 typically unique to Thai isolates compared to other studies (Gosi *et al*, 2008), which could dramatically alter both parasite binding ability to human red blood cell receptors and its antigenic characteristics (Fraser *et al*, 1997; Wongkidakarn *et al*, 2016). Continuous nonsynonymous polymorphisms generated within *Pv*DBPII ligands may help the parasite to evade from host's immune system (Xainli *et al*, 2002) or to avert the mechanisms of host protective immunity against DBP (Cole-Tobian *et al*, 2002). Understanding the polymorphic nature of specific parasite ligands in DBPII especially in the N-terminal cysteine-rich region among geographically distinct *P. vivax* isolates and naturally acquired immunity among populations in different

malaria endemic areas are the key focuses of future vaccine development (Longley *et al*, 2016).

Studies on the potential antigenic characteristics of DBPII allelic variation among *P. vivax* isolates in Thailand and their effects on averting naturally acquired immunity are limited (Chootong *et al*, 2014). Here, we evaluated whether DBPII allelic variations could foil the naturally acquired immunity in *P. vivax*-infected individuals in the country. The results might impact rational design of a broadly protective vaccine against *P. vivax* malaria.

MATERIALS AND METHODS

Collection of plasma samples

Stored plasma samples (n = 82) from symptomatic *P. vivax*-infected individuals living in malaria endemic areas of Thailand and healthy individuals (n = 39) residing in Bangkok, a non-malaria region, who had no known previous exposure to malaria and no travel history to malaria-endemic regions in the prior two years were from a collection of 2012-2013 (Maneerattanasak *et al*, 2017).

Research protocols were approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University (approval no. MUTM 2015-038-01). No prior written consent from each sample donor in this study was required because this is the retrospective study using the leftover stored specimens.

Collection of *P. vivax* blood stage antigens

Antigens were prepared from pooled enriched *P. vivax*-infected blood using sulfoethyl cellulose and Sephadex G-25 chromatography followed by Percoll step-gradient centrifugation as previously described (Khusmith *et al*, 1998), and stored at -80°C until used.

Selection of *Pv*DBPII potential peptide antigens

Twenty-three 15-mer linear peptides (with overlapping 4-aa sequence) were designed corresponding to sequences of the cysteine-rich region of *Pv*DBPII suggested as being targets of host protective immunity (Tsuboi *et al*, 1994). The 15-mer linear peptides were derived from the aa sequence at the critical binding motif mapped to a DBP portion of *P. vivax* Thai isolates (Gosi *et al*, 2008), including the 170-aa spanning region between cysteine 4-7 that represents the critical binding motifs of *Pv*DBPII shared among geographically distinct *P. vivax* isolates (Chitnis and Miller, 1994; Chitnis *et al*, 1996). The 15-mer linear peptides with 95% purity (Bio Basic Canada Inc, Ontario, Canada) are designated DBPp1 - DBPp23 (Fig 1).

Peptide location	Polymorphic position	Peptide sequence
291-305		DBPp1 CMKELTNLVNNTDTN
306-316	306, 308	DBPp2 TDTN**L**LH**S**DITFRKLY
317-327		DBPp3 RKLYLKRKLIYDAAV
328-338	333	DBPp4 DAAVEGDLL**F**KLNNY
345-355	351	DBPp5 LNNYRYNKDF**C**KDIR
356-366		DBPp6 KDIRWSLGDFGDIIM
367-371	367, 371	DBPp7 DIIM**T**GYS**E**
372-382	375, 378	DBPp8 GYS**E**VVE**N**NLRSIFG
383-393	384, 385, 386, 390	DBPp9 SIFGT**GKN**AQQ**H**RKQ
394-404	390, 398, 404	DBPp10 **H**RKQWWNE**T**KAQIW**R**
405-415	404	DBPp11 QIW**R**AMMYSVKKRLK
416-426	417, 419, 424	DBPp12 KRLKG**K**F**M**WICK**I**NV
427-437	424, 433, 436, 437	DBPp13 K**I**NVAVNIEP**K**IY**TR**
438-448	436, 437	DBPp14 IY**TR**IREWGRDYVSE
449-459		DBPp15 YVSELPTEVQKLKEK
460-469		DBPp16 KLKEKCDGKINYTDK
470-480	475	DBPp17 YTDKKVCKV**A**PCQNA
481-491	486	DBPp18 CQNACKSYD**E**WITRK
492-502		DBPp19 ITRKKNQWDVLSNKF
503-513	503, 507, 513	DBPp20 SNKF**K**SVK**H**AEKVQ**K**
514-524	513	DBPp21 **K**VQTAGIVTPYDILK
525-535		DBPp22 DILKQELDEFNEVAF
536-546		DBPp23 EVAFENEINKRDGAY

Fig 1 - Amino acid sequences of 23 synthesized 15-mer linear peptides corresponding to a polymorphic domain of *Plasmodium vivax* Duffy binding protein (DBP) region II of parasite isolates in Thailand

Note: Names of DBP peptides, DBPp1-DBPp23, are shown in blue and variant amino acids in underlined bold red compared to those of *Plasmodium vivax* Sal I strain (Gosi et al, 2008).

Detection of antibodies against *P. vivax* asexual blood stage antigens

Plasma samples were initially confirmed to contain antibodies against *P. vivax* asexual blood stage lysate antigens using ELISA previously described (Pitabut *et al*, 2007). Controls were plasma from healthy non-infected individuals. Tests were performed in duplicate.

Total IgG and IgG subclasses against DBPp1-DBPp23 were evaluated using ELISA. In brief, 96-well microtiter plates (Costar, Broadway/ Cambridge, MA) were coated overnight at 4°C with 100-µl aliquot of 5 µg/ml each DBPp in 0.05 M carbonate-bicarbonate buffer pH 9.6. Wells were washed with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T), incubated for one hour

at 37°C with 2.5% (w/v) powdered milk in PBS-T, washed as described, and incubated with 100-µl aliquot of 1:5 diluted plasma in PBS-T containing 1.25% powdered milk for one hour at 37°C. Wells were washed as described and incubated for one hour at 37°C with horse radish peroxidase (HRP)-conjugated mouse anti-human IgG (Invitrogen, Waltham, MA) (1:1,000 dilution). HRP reaction was performed using 3,3',5,5′-tetramethylbenzidine (Invitrogen, Waltham, MA) for 30 minutes in the dark, reaction terminated by addition of 2 M H_2SO_4 solution and $A_{450\ nm}$ measured using an ELISA reader (Sunrise™, Tecan, Austria). Control samples were plasma from healthy non-infected individuals. Tests were carried out in duplicate.

Anti-*Pv*DBPII-IgG subclasses were identified using ELISA as described above using 1:5 diluted plasma samples and mouse HRP-conjugated anti-human IgG1, IgG2, IgG3, and IgG4 monoclonal antibodies (clones Fc, Fd, Hinge, and Fc; Invitrogen) (1:1,000 dilution in PBS-T containing 1.25% powdered milk).

Statistical analysis

$A_{450\ nm}$ values were expressed as median plus an interquartile range (IQR) and independent data between groups compared using a Mann-Whitney U test. Antibody association with age, parasitemia and malaria exposure were evaluated using a Spearman's rank correlation test. A p-value <0.05 is considered statistically significant. Calculations were performed using a Statistical Package for the Social Sciences (SPSS) version 18.0 (SPSS Inc, Chicago, IL) and Prism version 7.05 (GraphPad Software Inc, San Diego, CA).

RESULTS

Characteristics of *P. vivax*-infected individuals

P. vivax-infected individuals were male, median age of 24 years (range 15-52 years, IQR = 20-31). Based on clinical records, 24/82 (29%) *P. vivax*-infected individuals presented first malaria infection while fifty-eight (71%) had previous malaria experiences varying from 1-3 episodes. Duration of malaria infection upon admission and previous malaria episodes among *P. vivax*-infected individuals varied from one month to two years. Median parasite count from microscopic examination was 13,155 parasite/µl (IQR = 6,204 - 21,843 parasite/µl).

Peptide sequences and polymorphic positions of 23 linear peptides

The synthesized peptide sequences with overlapping 4-aa residues corresponded to the sequence at the critical binding motif mapped to a DBP portion, aa 291-546, of

P. vivax Thai isolates (Fig 1) (Gosi *et al*, 2008). The sequence included the 170-aa spanning region between cysteine 4-7 (aa 291-460) that represented the critical binding motifs of *Pv*DBPII discrete alleles shared among *P. vivax* isolates from distinct geographic regions (Chitnis and Miller, 1994; Chitnis *et al*, 1996).

Total IgG response to *Pv*DBPII linear peptides

All plasma samples of *P. vivax*-infected individuals were confirmed to contain total IgG against *P. vivax* blood stage lysate antigens (median $A_{450\ nm}$ = 0.410, IQR = 0.240 -0.611) significantly higher than median $A_{450\ nm}$ (0.160, IQR = 0.133 - 0.391) of non-infected individuals (*p*-value <0.001) (Fig 2).

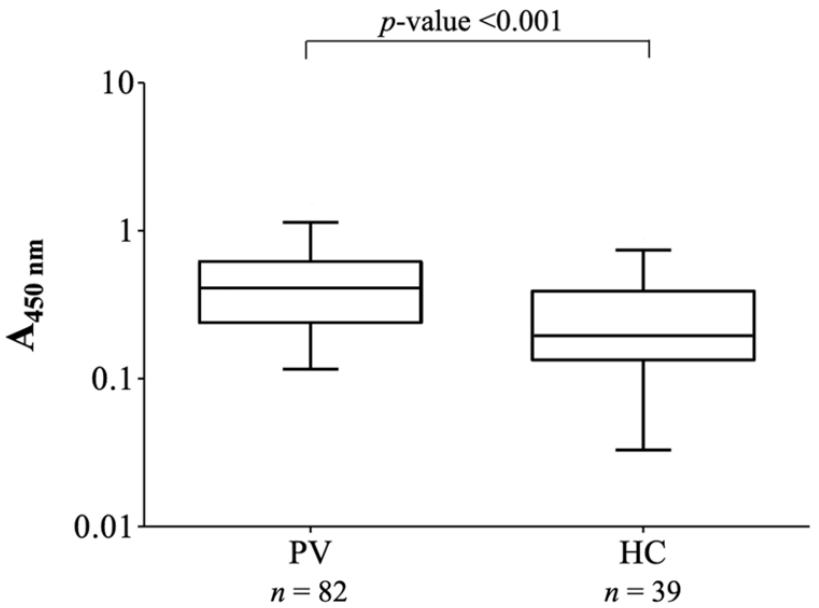

Fig 2 - Levels of anti-*Plasmodium vivax* Duffy binding protein (DBP) region II total IgG in plasma of *P. vivax*-infected (PV) and healthy non-infected (HC) individuals in Thailand (2012-2013)

Plasma total IgG levels were determined using ELISA, employing horse radish peroxidase (HRP)-conjugated mouse anti-human IgG (Invitrogen, Waltham, MA) (1:1,000 dilution) and 3,3′,5,5′-tetramethylbenzidine substrate (Invitrogen).

A_{450nm}: Absorbance reading at 450 nm which was measured using a plate reader

Horizontal line indicates median value, vertical line indicates A450 nm and box indicates the median and interquartile range.

Anti-*Pv*DBPII total IgG against the 15-mer linear peptides (DBPp1-DBPp23) demonstrated 71/82 (86%) *P. vivax*-infected individuals have significantly higher specific anti-*Pv*DBPII total IgG against one or more of six linear peptides, namely, DBPp4, DBPp5, DBPp14, DBPp16, DBPp21, and DBPp22 (Table 1) compared to controls (p-value = 0.020, <0.001, 0.002, <0.001, 0.004, and 0.002, respectively using a Mann Whitney U test) (Fig 3A). While the numbers of specific anti-*Pv*DBPII total IgG positive responders to these six DBPp varied from 70-78%, other DBPp exhibited very low or undetectable anti-*Pv*DBPII IgG responses in tested individuals (data not shown). Thus, only *P. vivax* plasma samples having specific total IgG response to the six DBPp were further evaluated for presence of anti-*Pv*DBPII IgG subclasses.

Anti-*Pv*DBPII IgG subclasses and percent reactive samples responders

A positive responder is defined as a *P. vivax*-infected individual who has a level of plasma anti-*Pv*DBPII-IgG subclass greater than the median of normal healthy control for each DBPp. IgG1 responders were detected against all six test DBPps (Fig 3B), followed by IgG2 (against DBPp5, -16 and -22) (Fig 3C) and IgG3 (against DBPp4, -5 and -22)

Table 1

Sequence and variant position of six antigenic 15-mer linear peptides of *Plasmodium vivax* Duffy binding protein (DBP) region II in blood plasma of individuals with vivax malaria in Thailand (2012-2013)

DBP linear peptide	Peptide sequence	Mutation position	Mutation*	SNV
DBPp4	DAAVEGDLLFKLNNY	333	L > F	CTT>TTT
DBPp5	LNNYRYNKDFCKDIR	351	S > C	AGT>TGT
DBPp14	IYRRQIEWGRDYVSE	437	W > R	TGG>CGG
DBPp16	KLKEKCDGKINYTDK	-		
DBPp21	KVQTAGIVTPYDILK	513	T > K	ACG >AAG
DBPp22	DILKQELDEFNEVAF	-		

**Plasmodium vivax* Sal I reference strain (Gosi *et al*, 2008)

SNV: single nucleotide variation

Variant amino acid is indicated in bold italic.

(Fig 3D), then IgG4 (against DBPp5) (Fig 3E). When the cumulative percent of *P. vivax* IgG subclass responders to the six DBPps were compared normalized to IgG1, consistently high percent IgG2, -3 and -4 responders to DBPp5 were observed, followed by IgG2 and -3 responders to DBPp22, and lowest against DBPp14 with IgG2, -3 and -4 responders (Fig 4).

Association of anti-*Pv*DBPII antibody levels with age, cumulative exposure and parasitemia

Data on age, cumulative and episodes of malaria exposure of subjects were from clinical records and parasitemias determined from microscope examination of slides. Anti-*Pv*DBPII total IgG and IgG subclass levels were not associated with age (15-52 years of age)

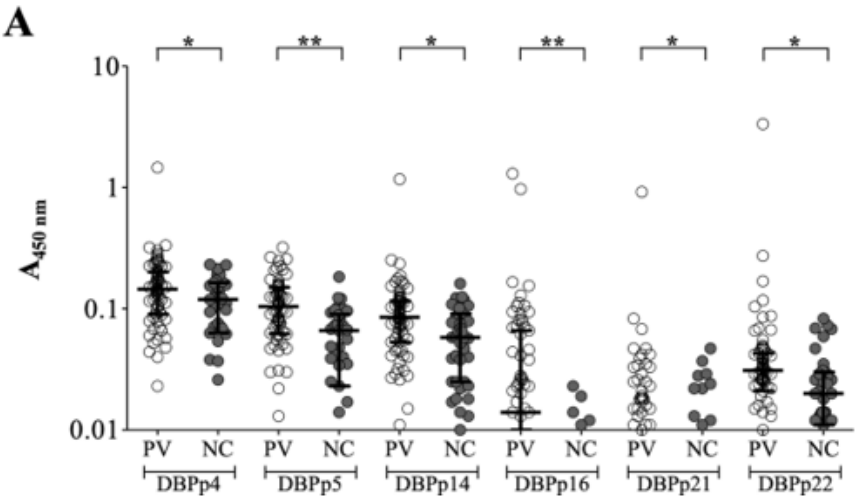

Fig 3 - Levels of anti-*Plasmodium vivax* Duffy binding protein region II peptides (DBPp4, -5, -14, -16, -21, and -22) total IgG (A), IgG1 (B), IgG2 (C), IgG3 (D), and IgG4 (E) in plasma of *P. vivax*-infected (PV) (n = 82) and healthy non-infected (HC) (n = 39) individuals in Thailand (2012-2013)

ELISA experiments were performed as described in legend to Fig 2. Horizontal line indicates median value and vertical line interquartile range. Only samples with $A_{450\ nm} \geq 0.01$ are included.

*p-value <0.05; **p-value <0.001 respectively using Mann Whitney U test

A_{450nm}: Absorbance reading at 450 nm; IgG: Immunoglobulin G

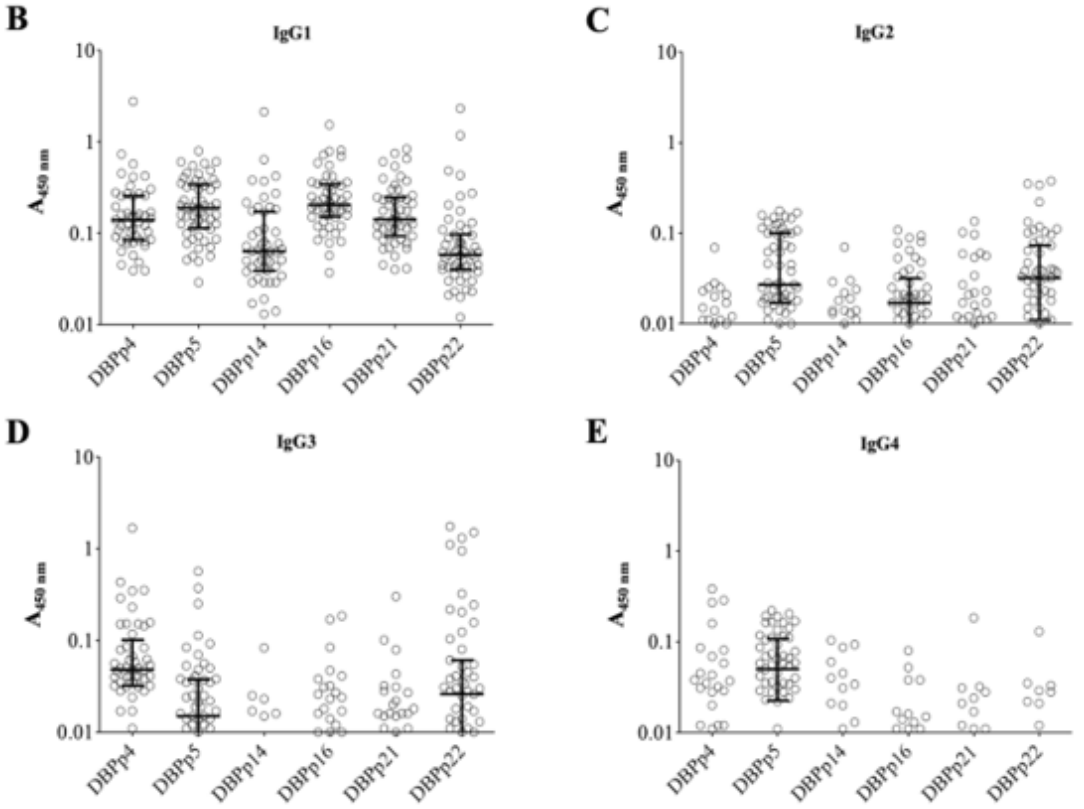

Fig 3 - Levels of anti-*Plasmodium vivax* Duffy binding protein region II peptides (DBPp4, -5, -14, -16, -21, and -22) total IgG (A), IgG1 (B), IgG2 (C), IgG3 (D), and IgG4 (E) in plasma of *P. vivax*-infected (PV) (n = 82) and healthy non-infected (HC) (n = 39) individuals in Thailand (2012-2013) (cont)

ELISA experiments were performed as described in legend to Fig 2. Horizontal line indicates median value and vertical line interquartile range. Only samples with $A_{450\ nm}$ ≥0.01 are included.

*p-value <0.05; **p-value <0.001 respectively using Mann Whitney U test

A_{450nm}: Absorbance reading at 450 nm; IgG: Immunoglobulin G

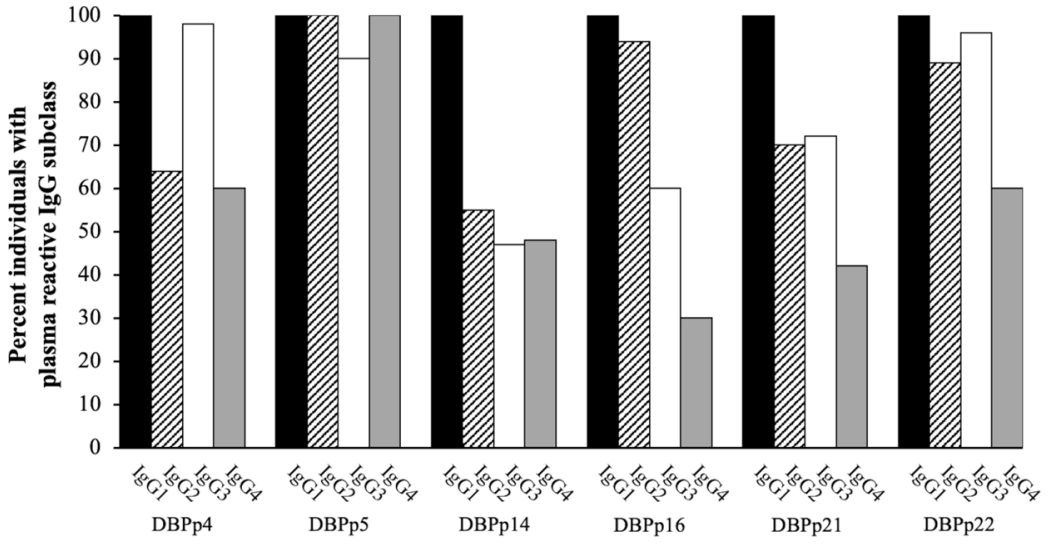

Fig 4 - Percent *Plasmodium vivax*-infected individuals with plasma IgG subclasses reactive to *P. vivax* Duffy binding protein region II peptides DBPp4, -5, -14, -16, -21, and -22

Note: Data are from Fig 3.

IgG: Immunoglobulin G

or with parasitemia (from low of <500 parasite/μl to high >500 parasite/μl) (data not shown). Comparisons of anti-*Pv*DBPII total IgG levels against the six test DBPps with numbers of malaria exposure demonstrated a slight decrease in total IgG levels for all DBPps, with the highest correlation observed for DBPp5 (r = -0.262; p-value = 0.043, using Spearman's rank correlation test) (Fig 5).

DISCUSSION

The study conducted in Thailand during 2012-2013 of subjects exposed to *P. vivax* in areas where malaria was endemic showed development naturally acquired antibodies to B-cell linear epitopes of the cysteine-rich ligand domain in the critical binding motif of DBPII of local *P. vivax* isolates, as demonstrated by measurement of levels total IgG and four IgG subclasses against 23 15-mer peptides with overlapping 4 amino acids.

Immune responses to the critical binding motif of *Pv*DBP region II inhibiting parasite binding to reticulocyte receptors are believed to play a crucial role in protection

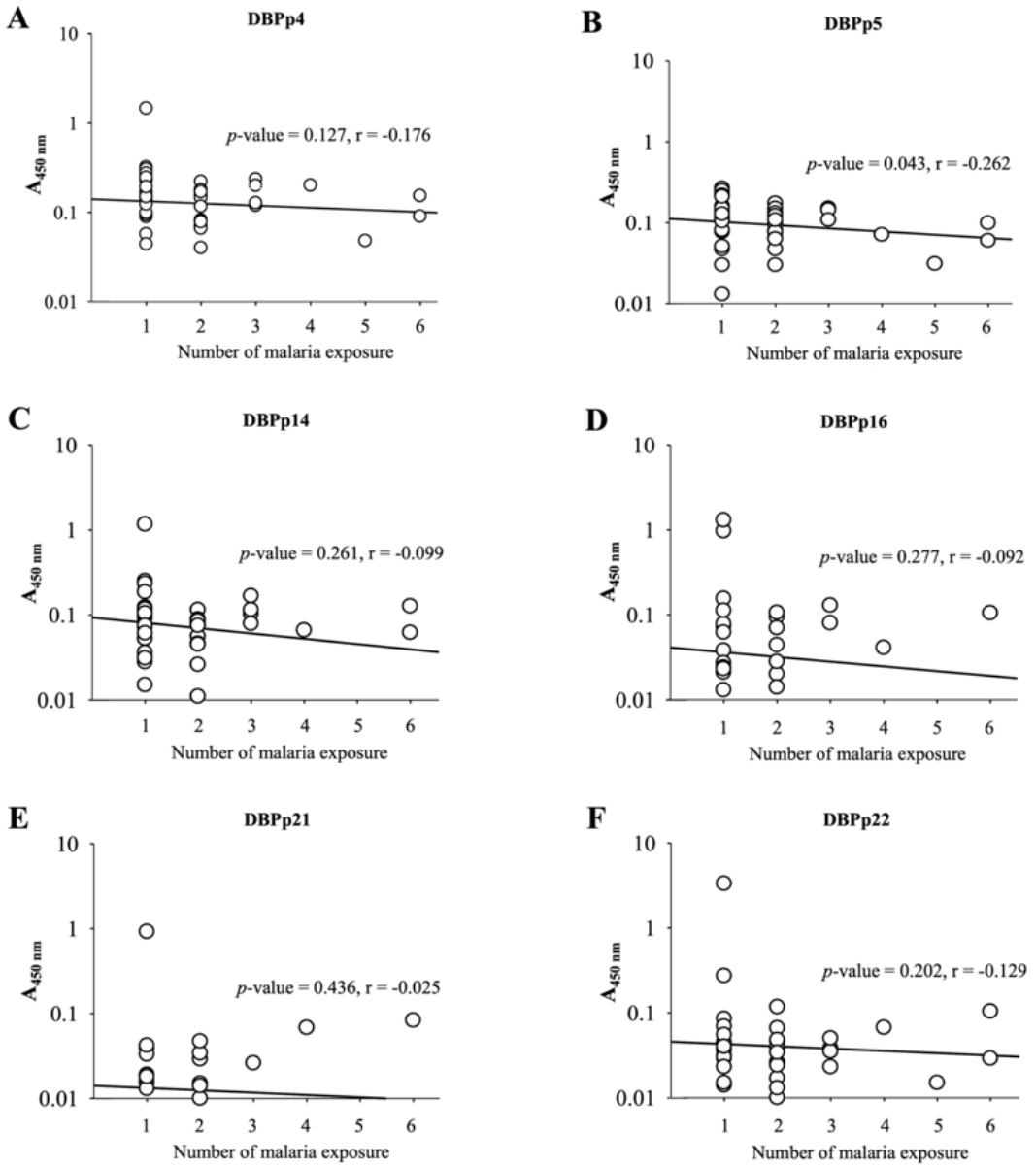

Fig 5 - Association between anti-PvDBPII IgG reactivity to PvDBPII peptides DBPp4, DBPp5, DBPp14, DBPp16, DBPp21 and DBPp22 with numbers of malaria exposure of *P. vivax*-infected individuals

Note: IgG levels were measured as described in legend to Fig 2. Only samples with A_{450n}m ≥0.01 are included.

A_{450nm}: Absorbance reading at 450 nm; r: correlation coefficient

against *P. vivax* infection (Fraser *et al*, 1997; Michon *et al*, 1998; Xainli *et al*, 2002). Although 23 15-mer peptides covering *Pv*DBP region II aa sequence 291-546 were tested, plasma IgG levels are significantly elevated compared to non-exposed controls against only 6 peptides, four of which contained variant amino acids compared to reference *P. vivax* Sal I strain (Fig 1) (Gosi *et al*, 2008).

Variable serological reactivity profiles to *Pv*DBPII were previously reported in high malaria-endemic areas of Papua New Guinea (PNG) (He *et al*, 2019). High IgG reactivity to linear epitopes in reduced or denatured recombinant *Pv*DBP was observed in PNG individuals who have with varying serological reactivity profile, from high titer (in a few subjects) to poor and negligible anti-*Pv*DBP reactivity (in the majority of subjects) (Fraser *et al*, 1997). In young PNG children, specific IgG responses to four alleles of *Pv*DBPII (AH, O, P and Sal1), *Pv*DBPIII-V and *Pv*EBPII were detected, among which response to *Pv*EBPII is more prevalent and associated strongly with protection against clinical vivax malaria (Nicolete *et al*, 2016). Several factors that might have influenced the apparent non-respondence against the majority of *Pv*DBPII peptides in our study include: i) poor immunogenicity of these DBPps (George *et al*, 2019), ii) variable antibody response to diverse *Pv*DBPII peptides in *P. vivax*-infected individuals, iii) number of malaria exposures, and iv) limited sample size. Further studies will be needed to clarify the relevance and importance of these factors employing larger sample sizes from different geographic regions of the country.

Determination of responses of IgG subclasses to *Pv*DBPII antigens allows evaluation of protective activity as IgG subclasses mediate distinct immune effector functions against *Pv*DBPII AH and *Pv*EBPII (He *et al*, 2019), as well as *P. vivax* rhoptry proteins *Pv*RALP1-Ecto and *Pv*RhopH2 (Kochayoo *et al*, 2019). In our study, among the four IgG subclasses tested, anti-*Pv*DBPII peptides specific IgG1 was predominant, with relative high numbers responder against both variant and reference *Pv*DBPII peptides, indicating that anti-*Pv*DBPII antibodies were biased towards cytophilic antibody subclasses as previously reported in Brazilian (Tran *et al*, 2005; de Sousa *et al*, 2014) and Colombian (Maestre *et al*, 2010) populations.

Antigenic stimulation may first trigger B-cells to produce IgG1 and IgG3 that are crucial for parasite clearance and then subsequently stimulate B cells to produce IgG2 and IgG4 (Wang *et al*, 2016). However, weak stimulation of IgG2 and IgG4 to particular DBPps in our study might be due to short-term antibody response

to *Pv*DBPII or to weak IgG response to these particular *P. vivax* antigens (Xainli *et al*, 2003; Liu *et al*, 2022). Furthermore, antigenic properties of antigens impact the body's ability to generate IgG isotypes (Stanisic *et al*, 2015). These preliminary results provide evidence of the potential critical *Pv*DBPII binding motifs that are involved in or represent sequences associated with natural acquired immunity in malaria-endemic region population (Wang *et al*, 2016). Again, further studies as described above are required to confirm these observations.

SNV polymorphisms in DBP gene at domain II were suggested to involve natural acquired immunity (Xainli *et al*, 2003; Souza-Silva *et al*, 2010) and to be associated with immune evasion (Batchelor *et al*, 2011). High anti-*Pv*DBPII total IgG level against polymorphic *Pv*DBPII linear epitopes indicates the possibility of a role polymorphism in altering antibody reactivity (Xainli *et al*, 2003). Acquired antibody is only partially directed against polymorphic motifs within *Pv*DBPII domain that can affect antibody binding by induction of high levels of specific total IgG and IgG1 by polymorphic variants and diminishing reactivity in non-polymorphic sequences, suggesting that DBPII genetic polymorphisms may have arisen from host immune pressure.

It is worth noting that IgG subclass response profiles induced during infection differed depending on antigen types and polymorphisms, which might affect their binding properties (Anchang-Kimbi *et al*, 2016). The highest frequency of polymorphisms common among Thai *Pv*DBPII was observed in five amino acids [D384G (DBPp9), R390H (DBPp9), L424I DBPp12, W437R (DBPp13), and I503K (DBPp20)] with L424I having the highest frequency (Gosi *et al*, 2008). A combination of L424I, W437R and I503K variants might alter antibody production (Chootong *et al*, 2014).

It is of interest that SNV associated with high IgG1 and low IgG3 levels reactive to the polymorphic peptides, DBPp4 and DBPp5, involves a change of C/A to T, whereas a change of T/C to A/C occurs in other DBPps. This is the first observation of a T point mutation associated with increased IgG1 and low IgG3 levels. Poor immunogenicity of some cysteine-rich DBPps have been reported in other molecules important for red blood cell invasion by malaria parasites, such as MSP119 and MSP1 domain of *P. vivax* and *P. falciparum* respectively (Egan *et al*, 1997; Quin *et al*, 2001). These findings highlight the importance of the association of SNVs in *Pv*DBPII with naturally acquired antibody response to *P. vivax*. Future studies should focus on the comparison of immunity against *Pv*DBPII of Thai isolates with other

isolates from different geographical regions, which should provide a better understanding of the relationship between polymorphisms in *Pv*DBPII among *P. vivax* isolates and natural immunity.

Our study demonstrates that cumulative exposure to parasites, age and parasitemia were not associated with levels of anti-*Pv*DBPII total IgG and IgG subclasses. Previously Michon *et al* (2000) and Xainli *et al* (2003) observed significant increase in levels of anti-DBP antibodies (average or maximum response) with age, suggesting a boosting of DBP antibody response with accumulated age-related exposure. McCallum *et al* (2017) noted increases in age and parasite density give rise to an increase of IgG subclass polarization in response to malarial antigens. On the other hand, limited immunity to *Pv*DBPII in young PNG children cohort was reported (Cole-Tobian *et al*, 2009). The lack of association of anti-*Pv*DBPII antibody level with age or parasite density in our study could be due to (i) narrow age ranges of the study adult cohort and (ii) majority of infected adults presenting mild vivax malaria with similar parasitemia.

Acquisition and maintenance of anti-malarial antibodies depend on parasite exposure (Folegatti *et al*, 2017). In the Brazilian Amazon malaria-endemic regions, individuals experiencing a number of previous clinical malaria episodes exhibit higher antibody levels against *Pv*DBPII compared to those having only one episode (Ceravolo *et al*, 2005; de Sousa *et al*, 2014). Thus, the lack of association of malaria exposure with anti-*Pv*DBP antibody level against DBPps among *P. vivax*-infected individuals in our study might be due to duration of malaria infection and number of previous malaria episodes prior to admission. Future studies should include *P. vivax*-infected individuals of all age groups, both mild and severe cases and with low and high parasitemia.

In summary, the results demonstrate variations in naturally acquired antibody to *Pv*DBPII in *P. vivax* infected individuals living in malaria endemic regions of Thailand, with IgG1 subclass being predominant. SNVs (from C/A to T) in *Pv*DBPII suggested that SNVs affected IgG and IgG subclasses response. In order to gain a better understanding of the protective nature of these antibodies to different *Pv*DBPII variants, longitudinal studies should be performed in conjunction with identification of both linear and conformational B-cell epitopes of *Pv*DBPII.

ACKNOWLEDGEMENTS

The authors thank staff of the Clinical Malaria Research Unit, Faculty of Tropical Medicine, Mahidol University for provision of *P. vivax*

plasma samples. The research was supported by the Faculty of Tropical Medicine, Mahidol University and Mahidol University through the National Research Universities Initiative.

CONFLICTS OF INTEREST DISCLOSURE

The authors declare no conflicts of interest.

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