รายงานวิจัยฉบับสมบูรณ์

การศึกษาเชิงลึกภูมิคุ้มกันต่อต้าน *Plasmodium vivax* Duffy binding protein II เพื่อการผลิตวัคซีนป้องกันโรคมาลาเรียชนิด *Plasmodium vivax* ในประเทศไทย

โดย

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สัญญาเลขที่: MRG5580091

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

Abstract

Project Code: MRG5580091

Project Title: Understanding protective immunity against *Plasmodium vivax* Duffy binding protein II (PvDBPII) for discovery vivax vaccine in Thailand

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Project Period: 2 July 2012-2 July 2014

Plasmodium vivax Duffy Binding Protein II (DBPII) plays an important role in reticulocyte invasion and is a potential vaccine candidate against vivax malaria. However, polymorphisms in DBPII are a challenge for the successful design of a broadly protective vaccine. In this study, the genetic diversity of DBPII among Thai isolates was analyzed from *P. vivax*-infected blood samples and polymorphism characters were defined with the MEGA4 program. Sequence analysis identified 12 variant residues that are common among Thai DBPII haplotypes with variant residues L333F, L424I, W437R and I503K having the highest frequency. Variant residue D384K occurs in combination with either E385K or K386N/Q. Additionally, variant residue L424I occurs in conjunction with W437R in most Thai DBPII alleles and these variants frequently occur in combination with the I503K variant. The polymorphic patterns of Thai isolates were defined into 9 haplotypes (Thai DBL-1, -2, -3, etc...). Thai DBL-2, -5, -6 haplotypes are the most common DBPII variants in Thai residents. To study the association of these Thai DBPII polymorphisms with antigenic character, the functional inhibition of anti-DBPII monoclonal antibodies against a panel of Thai DBL variants was characterized by an *in vitro* erythrocyte binding inhibition assay. The functional inhibition of anti-DBPII monoclonal antibodies 3C9, 2D10 and 2C6 against Thai variants was significantly different, suggesting that polymorphisms of Thai DBPII variants alter the antigenic character of the target epitopes. In contrast, anti-DBPII monoclonal antibody 2H2 inhibited all Thai DBPII variants equally well. Our results suggest that the immune efficacy of a DBPII vaccine will depend on the specificity of the anti-DBPII antibodies induced and that it is preferable to optimize responses to conserved epitopes for broadly neutralizing protection against *P. vivax*.

Keywords: Duffy Binding Protein II, Polymorphism, Thailand.

บทคัดย่อ

รหัสโครงการ: MRG5580091

ชื่อโครงการ: การศึกษาเชิงลึกภูมิคุ้มกันต่อต้าน *Plasmodium vivax* Duffy binding protein II (PvDBPII) เพื่อการผลิตวัคซีนป้องกันโรคมาลาเรียชนิด *Plasmodium vivax* ในประเทศไทย

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Plasmodium vivax Duffy Binding Protein II (PVDBPII) มีบทบาทในการจับกับโมเลกุลตัวรับเพื่อการบุกรุกเข้าสู่ Reticulocyte ดังนั้นจึงทำให้ PvDBPII เป็นแอนติเจนที่มีความสำคัญในการผลิตวัคซีนต่อต้านโรคมาลาเรียชนิด *Plasmodium vivax* โดยการกระตุ้นให้ระบบภูมิคุ้มกันให้สร้างแอนติบอดียั้บยั้งการจับกันระหว่าง PvDBPII และโมเลกุลตัวรับบน Reticulocyte แต่อย่างไรก็ตามการกลายพันธุ์และการเกิดความหลากหลายของสายพันธุ์ PVDBPII เป็นอุปสรรคที่สำคัญในการผลิตวัคซีน ในการศึกษาครั้งนี้ผู้วิจัยจึงได้ทำการวิเคราะห์ความหลากหลายของการกลายพันธุ์ของโมเลกุล PVDBPII ในกลุ่มเชื้อ *Plasmodium vivax* ที่ระบาดในกลุ่มคนไข้ไทยโดยใช้โปรแกรม MEGA4 ผลการทดลองสามารถวิเคราะห์ตำแหน่งของกรดอะมิโนที่เกิดการกลายพันธุ์ได้จำนวน 12 ตำแหน่งโดยพบว่าตำแหน่งที่ L333F L424I W437R และ I503K พบความถี่สูงสุดในการเกิดการการพันธุ์ ตำแหน่งการกลายพันธุ์ที่ D384K พบร่วมกับตำแหน่ง E385K และ K386N/Q นอกจากนี้พบว่าตำแหน่งการกลายพันธุ์ที่ L424I จะเกิดร่วมกับตำแหน่งการกลายพันธุ์ W437R ในทุกสายพันธุ์ Thai PvDBPII และตำแหน่งการกลายพันธุ์ที่ L424I และ W437R จะพบร่วมกับตำแหน่งการกลายพันธุ์ I503K รูปแบบการกลายพันธุ์ในกลุ่ม Thai PvDBPII สามารถจำกัดความหลากหลายของการการพันธุ์ออกเป็น 9 สายพันธุ์ คือ PvDBPII สายพันธุ์ Thai DBL-1, -2, -3, -4, -5, -6, -7, -8 และ -9 สายพันธุ์ที่พบความถี่สูงสุดในกลุ่มคนไข้ไทยคือสายพันธุ์ Thai DBL-2, -5 และ -6 การศึกษาในครั้งนี้ยังได้ทำการศึกษาความเชื่อมโยงระหว่างการการพันธุ์ของ Thai PvDBPII และคุณลักษณะความเป็นแอนติเจน หน้าที่การยับยั้งของโมโนโคลนัลแอนติบอดีจำเพาะต่อ Thai PvDBPII โดยใช้การทดลอง In vitro erythrocyte binding inhibition assay ผลการทดลองพบว่าโมโนโคลนัลแอนติบอดี 3C9 2D10 และ 2C6 มีประสิทธิภาพในการยับยั้งการจับกันระหว่าง Thai PvDBPII แต่ละสายพันธุ์และเม็ดเลือดแดงแตกต่างกันอย่างมีนัยสำคัญทางสถิติบ่งชี้ให้เห็นถึงการเปลี่ยนแปลงคุณลักษณะความเป็นแอนติเจนของ Epitopes ที่เป็นเป้าหมายของแอนติบอดีหลังจากที่มีการกลายพันธุ์ของ Thai PvDBPII ในขณะที่โมโนโคลนัลแอนติบอดี 2H2 มีความสามารถในการยับยั้งการจับกันของทุกสายพันธุ์ Thai PvDBPII และเม็ดเลือดแดง การศึกษาในครั้งนี้สามารถสรุปได้ว่าการผลิตวัคซีน PvDBPII ควรจะกระตุ้นระบบภูมิคุ้มกันให้สร้าง Anti-PvDBPII antibody ที่มีความจำเพาะต่อ conserved epitopes เพื่อการกระตุ้น broadly neutralizing antibody ในการป้องกันการติดเชื้อ Thai PvDBPII ทุกสายพันธุ์

คำหลัก: พลาสโมเดียมไวแวกซ์ ความหลากหลายของการกลายพันธุ์ ประเทศไทย

Introduction

Plasmodium vivax is the major cause of malaria in most regions where this disease is endemic outside Africa, and it causes substantial morbidity worldwide, including Thailand (Sattabongkot J et al 2004). *Plasmodium* microneme proteins, such as Duffy binding protein (DBP), have important roles in the merozoite invasion of reticulocytes during asexual blood-stage infection (Batchelor JD et al 2011). DBP is a member of the Duffy binding-like erythrocyte binding protein (DBL-EBP) family expressed in the micronemes and on the surface of *P. vivax* merozoites and is associated with the decisive junction formation step during the invasion process (5). It is this critical interaction of DBP with its cognate receptor that makes DBP an important anti-malaria vaccine candidate. The critical receptor binding motif of DBP is in a 330-amino-acid cysteine rich domain referred to as DBP region II (DBPII) or the DBL domain, which is the minimal domain responsible for binding to Duffy-positive human reticulocytes (Adams JH, et al 1990). The central portion of the DBP domain is hypervariable compared to other DBP regions, and polymorphisms occur frequently at certain residues in a pattern consistent with selection pressure on DBP, suggesting that allelic variation functions as a mechanism for immune evasion (Tolia NH et al, 2005, Singh SK et al, 2006). Naturally acquired antibodies to DBP are prevalent in residents of areas where malaria is highly endemic, but individuals show significant quantitative and qualitative differences in their anti-DBP serological responses (Tsuboi T, et al 1994, Cole-Tobian J et al 2013). Generally, serological responses to DBP and the inhibition of DBP-erythrocyte binding activity increase with a person's age, suggesting that there is a boosting effect due to repeated exposure through recurrent infection (King CL et al, 2008). The initial antibody response to a single *P. vivax* infection is a response to conformational epitopes and is not broadly protective, while an immunity that transcends strain specificity develops only after repeated exposure (Michon PA et al, 1998). There is a consensus that long-lasting, broadly inhibitory immunity correlate with development of antibodies to more conserved epitopes in the critical erythrocyte binding region of DBP.

Consequently, several evidences support that DBPII is an important anti-*P.vivax* vaccine candidate since antibodies against this molecule: (i) inhibit *in vitro* their binding to Duffy antigen/receptor for Chemokines (DARC); (ii) reduce merozoite invasion of human erythrocytes; and (iii) confer protection against blood-stage infection. However, the genetic variability of DBP from field parasites that result in antigenic differences among DBPII haplotype is a challenge for finding an effective vaccine design. In Thailand, one study has demonstrated a high rate of nonsynonymous polymorphism of 30 Thai vivax isolates. The highest frequency of polymorphism was found in five variant amino acids, D384G, R390H, L424I, W437R and I503K. Phylogenetic analysis of PVDBPII Thai isolates to others found six district allele groups, alleles group 4 and 6 were unique in Thailand (Gosi P, et al 2008). However, no assessment of immune response was reported in the study.

Our preliminary data showed anti-DBPII was significantly higher in vivax Thai patients (n=54) than immune villager and naive controls, indicating the present of protective role of anti-DBPII during vivax infection. The anti-DBPII levels showed difference in individual patient and could be assigned into 3 groups, high responders (HR), low responder (LR) and non responder (NR). The inhibitory function of anti-DBPII against DBPII binding to human erythrocyte was tested by *in vitro* COS7 assay that measure DBP erythrocyte binding function. Only 5 samples, 3 samples from HR and 2 samples from LR showed completely or almost completely inhibition against DBPII binding to Duffy positive erythrocyte. The mapping target epitopes of inhibitory antibody showed H1: ENNLRSIFGTDEKAQQRRKQ, M1: TNLVNNTDTNFHRDITFRKL and M2: EGDLLLKLNNYRYNKDWWN are the highest potential B-cell epitopes associated with the inhibitory activity of neutralizing antibody. However, our preliminary study has investigated naturally antibody using a Central American DBP strain, Sal1, which is not representative DBP haplotype in Thai residents.

Therefore in this study, we seek to understand the specificity of natural anti-DBP immunity that develops during *P. vivax* infection using Thai variant alleles. (1) Identify dominant dbpII alleles in Thai vivax malaria patients and assess the inhibition of neutralizing antibody against these variant alleles. (2) Determine the immunogenicity of Thai haplotype and identify which Thai DBPII determine antigenicity different DBPII haplotype.

วัตถุประสงค์ของโครงการ (Specific aims)

1. To defined *Plasmodium vivax* Duffy binding protein II (dbpII) alleles in Thai isolates
2. To evaluate the efficiency of neutralizing antibody from Thai vivax patients in protection against PvDBPII-Thai strain binding to human erythrocyte
3. To define which polymorphism determine antigenically different DBPII haplotypes in Thai endemic areas

การดำเนินงานวิจัยตามวัตถุประสงค์ (Experiments)

1. *P. vivax* Sample collection

The study was pursued in the malaria endemic areas of Southern Thailand where both major species of malaria, *P. vivax* and *P. falciparum*, are common. Blood samples (n=44) will be collected from acute *P. vivax* infected volunteers at malaria clinics, Chumphon province, which is in the Southern part of Thailand. The areas are malaria endemic having high prevalence of *P. vivax* infection. Acutely *P. vivax*-infected volunteers who register at Malarial Clinics were basked for informed consent under the protocol approve by the Ethic Committee on Human Rights Related to Human Experimentation, Mahidol University (MUIRB2012/162.0510). Selecting criteria of the projects are as followings: (1) systolic blood pressure is not less than 90 mm, (2) body temperature was not higher than 40°C, (3) Hematocrit was not less than 25% and (4) all patients have to be the age of 18 or above. Those who were not fitting the criteria and pregnant patients will be excluded. Fifty samples of heparinized blood was used for preparation of parasite isolates which are collected in

the spot filter for genotyping of dbpII alleles, 10 clones for one parasite isolate, and will be used for preparation of human plasma. The human plasma was used for determination of antibody levels and inhibition efficiency against DBPII. Giemsa-stained thick blood smears was used to examine for parasites and PCR assays for malaria diagnosis was conducted later in laboratory.

2. Genotyping of dbpII allele in Thai isolates

The parasite isolates (n=44), 10 clones for each isolate, from acute patients were collected from *P. vivax* patients. The DBP genes were PCR amplified and the products were purified and sequenced. The alignment of complete sequences of PVDBPII genes from 44 isolates were analyzed by CLUSTAL and the percent similarity was assessed using BioEdit software. A phylogenetic analysis was used to investigate the association of PvDBPII gene with sequence elucidated from different geographic regions. The gene tree was constructed using regions common to all available PvDBPII sequences. The *P. vivax* laboratory reference clone Sal I sequence was used as a reference and compared with sequences available in GenBank (i.e., Thai isolates were compared to sequences from other endemic countries of Asia (Vietnam, Indonesia, India, Bangladesh, South Korea and Papua New Guinea), other area (Brazil, Colombia).

3. The pEGFP-DBPII constructs and site-directed mutagenesis

The pEGFP-DBPII construct contains the Sal I DBPII allele cloned into the pEGFP-N1 plasmid with flanking signal sequences from the herpes simplex virus glycoprotein D1 allowing expression of a GFP fusion protein on the surface of transiently transfected COS7 cells (Michon, 2000, Michon, 2001). Site-directed mutagenesis was used to create a panel of pEGFP-DBPII constructs with identical polymorphisms to PvDBPII alleles from Thai field isolates using the pEGFP-DBPII-Sal I construct as the parent template (See Table 1). Mutagenesis was performed using the Stratagene Quickchange mutagenesis kit (Stratagene, La Jolla, CA) as previously described [VanBuskirk, 2004a; VanBuskirk, 2004b]. Primers used for mutagenesis can be seen in Table 2. Recombinant plasmid DNA was purified using an endotoxin-free plasmid DNA purification system (Qiagen, Valencia, CA).

4. Evaluate the efficiency of anti-DBPII neutralizing antibody in blocking against Thai PvDBPII haplotype

To evaluate the inhibitory activity of neutralizing antibodies against Thai DBPII haplotypes and DBPII Sal I binding to erythrocyte, the experiment made the plasmid, pEGFP-DBPII constructs and site-directed mutagenesis. Salvador I (Sal I) DBPII was cloned into the pEGFP-N1 plasmid with flanking signal sequences from the herpes simplex virus glycoprotein D1 allowing expression of a GFP fusion protein on the surface of transiently transfected COS7 cells (American Type Culture Collection, Manassas, VA). Mutagenesis to create a panel of Thai DBPII variants was performed using the Stratagene Quickchange mutagenesis kit (Stratagene, La Jolla, CA). The pEGFP-DBPII plasmid containing DBPII cloned from the Sal I strain of *P. Vivax* was used as the parent template.

Recombinant plasmid DNA was purified using an endotoxin-free plasmid DNA purification system (Qiagen, Valencia, CA)

COS-7 cell erythrocyte assay was carried out to quantify the inhibition of DBPII-erythrocyte binding. The monoclonal antibodies (Mabs 3C9, 2D10, 2C6 and 2H2) against recombinant DBPII 7.18 haplotype was provides by Prof. John H Adams, University of South Florida, USA were tested against Thai DBPII and Sal I haplotypes. Expression construct that express Thai DBPII and Sal I on the surface of transiently transfected COS have been used extensively as described (Michon 2010). This targeted expression efficiently presents the parasite ligand on the surface of the COS cells and expression was confirmed by detection of C-terminal GFP tag using epifluorescence. The inhibition assay was performed 42 hr. after initial transfection. Diluted Mabs and transfected COS7 cells were incubated 1 hr. Human erythrocytes subsequently added to each well in a 10% suspension and incubated at room temperature for 2 hr. The binding was quantified by counting rosettes. Percent inhibition was calculated for each serum sample relative to binding.

5. Evaluate the naturally acquired anti-DBPII antibodies in Thai *P. vivax* patients

Anti-DBP response from acutely infected *P. vivax* (n=33) were quantified by ELISA. The recombinant DBP regions II (rDBPII) was produced as described previously [Fraser et al]. Briefly, rDBPII was expressed as a GST fusion protein in *E. coli*, affinity purified on glutathione and cleaved from GST with thrombin using standard methods (Fraser et al, 1994). Recombinant 2 mg/mL of purified rDBPII-IV was added to 96-well plates, respectively, incubated for 30 min at room temperature, and washed three times with wash buffer (0.2%Tween-20 in PBS). Wells were incubated with 200 mL block buffer (2% skim milk in PBS) for 30 min, washed three times with was buffer, allowed to dry and stored overnight at 4 $^{\circ}$C. Serum diluted 1:400 in block buffer was added to pre-wetted wells and incubated for 90 min at 37 $^{\circ}$C. Plates were rinsed 3x in wash buffer, incubated with 1:1000 goat anti-human IgG-alkaline phosphatase, rinsed 3x, and substrate added. Absorbance was recorded at 405 nm at 45 min after addition of developer reagent. A baseline was established using control sera from non-exposed Thai residents and this control value was subtracted from the test OD values. ELISA data was classified into three groups: High responders (OD=0.71–0.51); Low responders (OD=0.50–0.32); and Non responders (OD<0.23). The samples were considered positive when OD value is ≥mean +SD of naive controls

6. Evaluate the efficiency of naturally acquired anti-DBPII antibody in blocking against Thai PvDBPII haplotype

To evaluate the inhibitory activity of naturally acquired anti-DBPII antibodies against Thai DBPII haplotypes and DBPII Sal I binding to erythrocyte, COS-7 cell erythrocyte assay was carried out to quantify the inhibition of DBPII-erythrocyte binding. The Thai vivax serum from high responders were tested against Thai DBPII and Sal I haplotypes. Expression construct that express Thai DBPII and Sal I on the surface of transiently transfected COS have been used extensively as described (Michon

et al 2010). This targeted expression efficiently presents the parasite ligand on the surface of the COS cells and expression was confirmed by detection of C-terminal GFP tag using epifluorescence. The inhibition assay was performed 42 hr. after initial transfection. Diluted Mabs and transfected COS7 cells were incubated 1 hr. Human erythrocytes subsequently added to each well in a 10% suspension and incubated at room temperature for 2 hr. The binding was quantified by counting rosettes. Percent inhibition was calculated for each serum sample relative to binding.

ผลงานวิจัยที่ได้รับ (Results)

1. Genetic polymorphism and amino acid changes of DBPII in *P.vivax* Thai isolates

Nucleotide sequence analysis of PVDBPII among 44 *P. vivax* Thai isolates showed 12 mutation residues compared to the reference sequence of Sal I. The sequence analysis of deduced amino acid sequence classified them into 9 haplotypes, DBL-TH1,-TH2,-TH3,-TH4,-TH5,-TH6,-TH7,-TH8 and -TH9. The haplotypes, DBL-TH2,-TH5, -TH6 are the most common DBPII variants in Thai residents.

The variant L333F, D384G, L424I, H437 and I503K having the highest frequency among Thai isolates. The variant D384K occurs in combination with either E385K or K386N/Q. The variant L424I occurs in conjunction with W437R in most Thai DBPII alleles and these variants frequently occur in combination with variant I503K.

(A)

Reference Name	Amino acid position →	308	333	371	384	385	386	390	417	424	437	475	503
Sal I	Sal I	R	L	K	D	E	K	R	N	L	W	P	I
DBL-7.18	AAL79051.1	S			G		Q		K	I	R		K
DBL-TH1	Thailand Haplotype 1	R	F							I	R	A	K
DBL-TH2	Thailand Haplotype2	R	F	E	G	K	Q		K	I	R		
DBL-TH3	Thailand Haplotype3	R	F		G			H					K
DBL-TH4	Thailand Haplotype4	R	F		G	K	Q	H	K	I	R		K
DBL-TH5	Thailand Haplotype5	R		E	G		N		K	I	R		K
DBL-TH6	Thailand Haplotype6	R			G		H						
DBL-TH7	Thailand Haplotype7	R			G								
DBL-TH8	Thailand Haplotype8	R	F										
DBL-TH9	Thailand Haplotype9	R	F							I	R		K

(B)

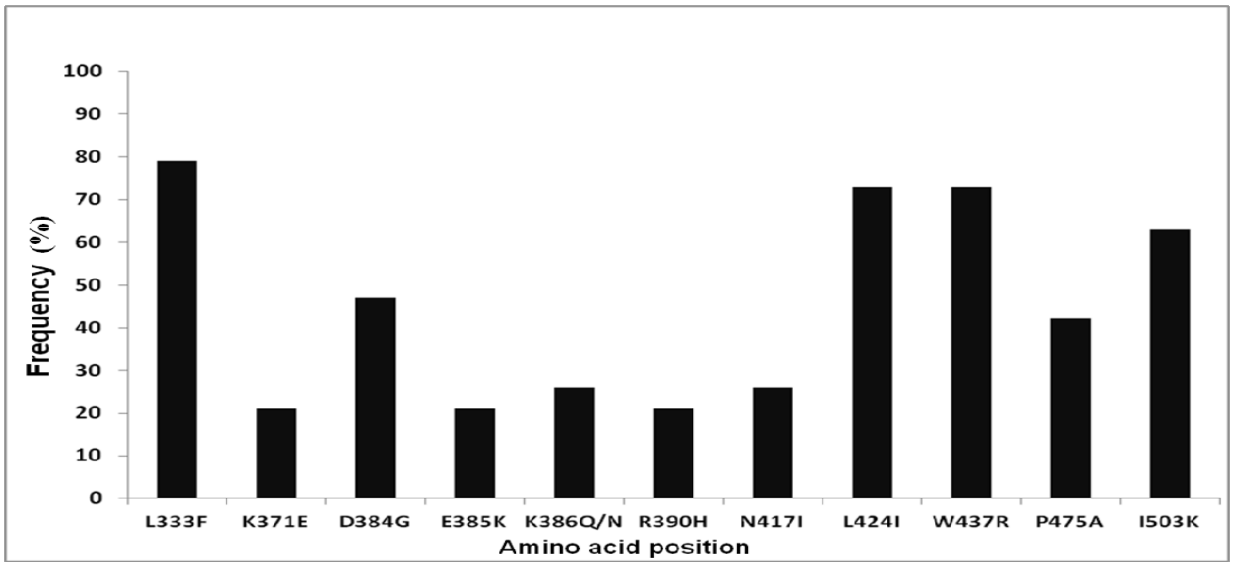


Figure 1: The genetic polymorphism of PvDBPII among Thai isolates. (A) Polymorphic amino acid changes are listed for each Thai DBL haplotype compare to reference sequencing Sal I (DQ156512). (B) Frequency of amino acid change at variant residues found in Thai DBL haplotypes.

2. Phylogenetic analysis of Thai DBPII haplotypes

The phylogenetic tree for the 9 haplotypes of PVDBPII was constructed with a neighbor-joining method using MEGA4 program. Number on the branches indicates bootstrap proportions. The result shows the diversity of Thai DBPII haplotypes.

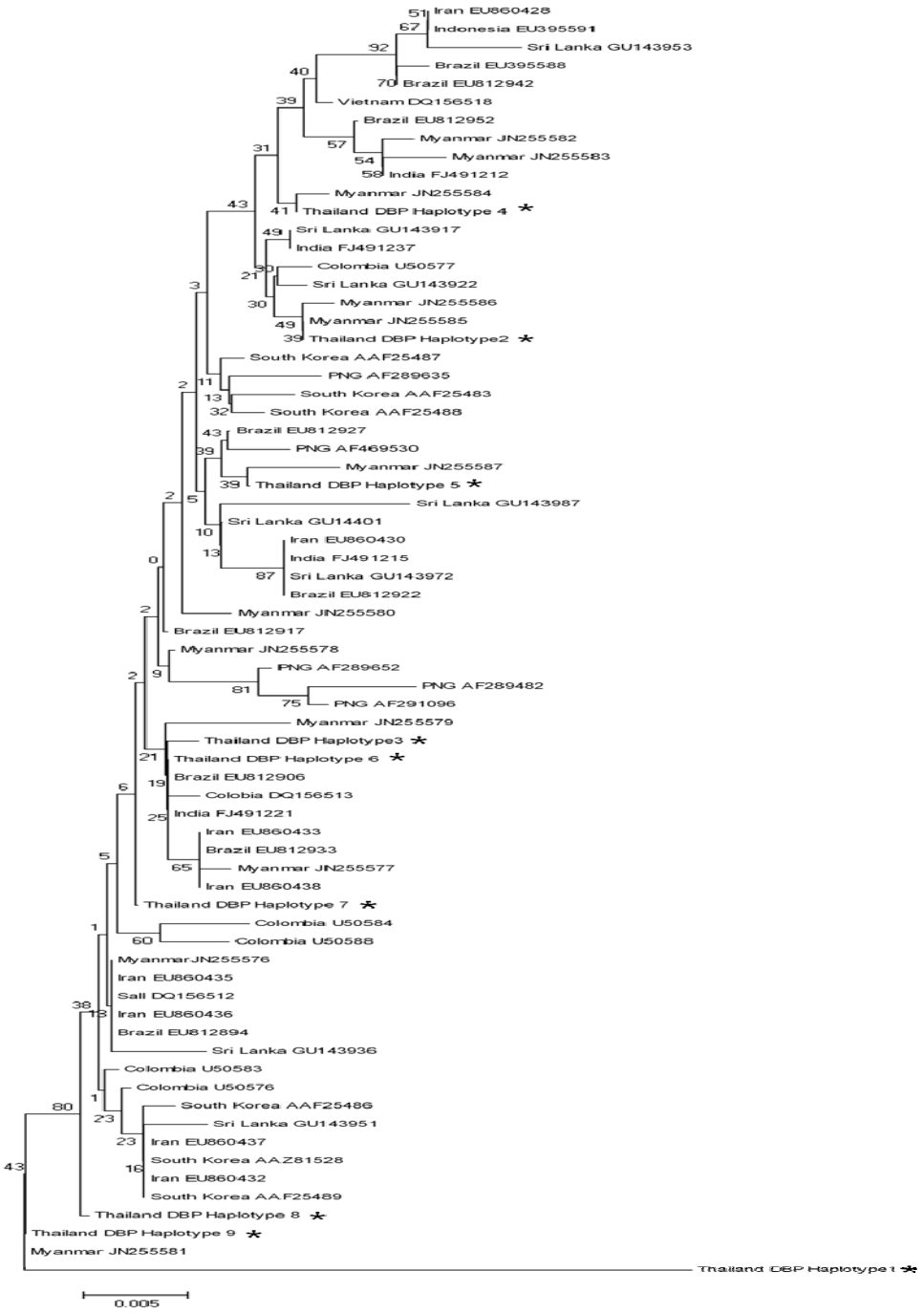


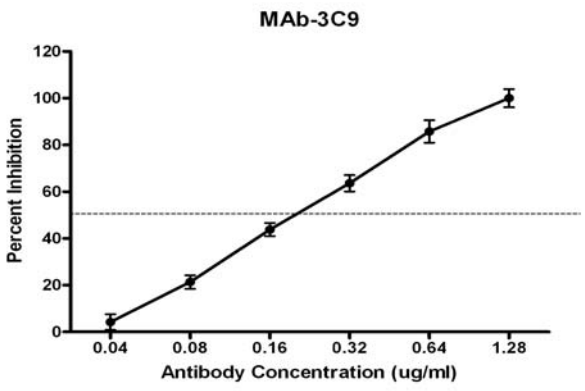
Figure 2: Phylogenetic analysis. The phylogenetic tree of 9 haplotypes of Thai DBPII was constructed with a neighbor-joining method using MEGA4 program. Numbers of the braches indicated bootstrap proportion (1000 replicates). The new Thailand DBPII haplotypes are marked with asterisks.

3. The inhibition of Thai-DBPII neutralizing antibody-human erythrocyte binding

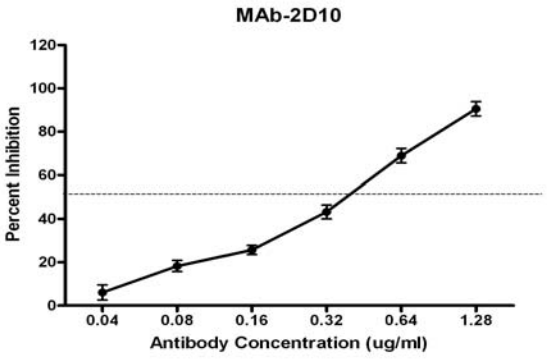
Functional activity of naturally acquired antibodies and vaccine elicited anti-DBPII antibodies that block *P. vivax* merozoite invasion correlates with levels of inhibition in the vitro COS7 assay of DBPII erythrocyte binding. Therefore, the COS7 DBPII-erythrocyte binding assay serves as a useful surrogate to evaluate potential invasion inhibitory effect of anti0DBPII antibodies. Limiting dilution of

anti-DBP monoclonal antibodies, 3C9, 2D10, 2C6 and 2H2 were tested in a range of concentration to give 100% to 0% DBPII erythrocyte binding inhibition against DBPII7.18. DBPII binding to erythrocytes was inhibited in a dose dependent manner and a 50% inhibitory concentration (IC50) of each antibody was determined. The 50% inhibitory concentrations (IC50) of 3C9, 2D10, 2C6 and 2H2 monoclonal antibodies were 0.21, 0.38, 2.61 and 0.64 ug/ml, respectively (Fig 3A-3D). We used the IC50 of each monoclonal antibody to evaluate the functional activity of anti-DBPII monoclonal antibody against the panel of Thai DBPII variants and homologous DBL7.18 expressed in the COS7 cell assay. The results revealed little variation in the inhibitory activity of monoclonal antibody 2H2 (Fig 3D), suggesting that the epitope target of this anti-DBPII neutralizing antibody was conserved among DBL-TH variants. In contrast, there was a significant difference of inhibitory activity of monoclonal antibodies 3C9, 2D10 and 2C6 against the panel of Thai DBL variants ($P<0.005$) (Fig 3A-3C). The results indicate that variant epitopes among Thai isolates were the target of most anti-DBPII neutralizing antibodies.

A



B



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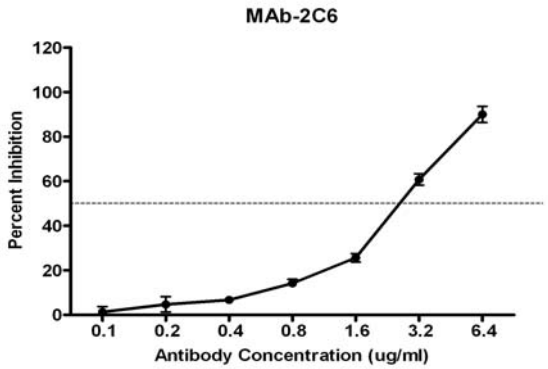

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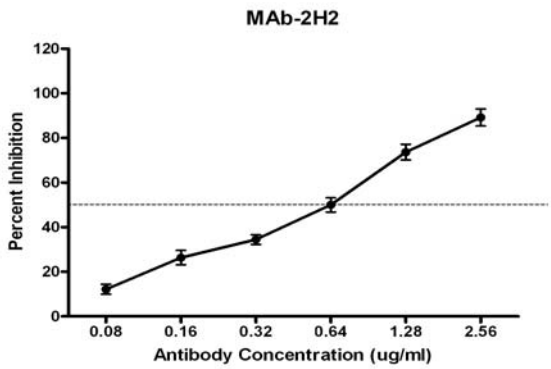
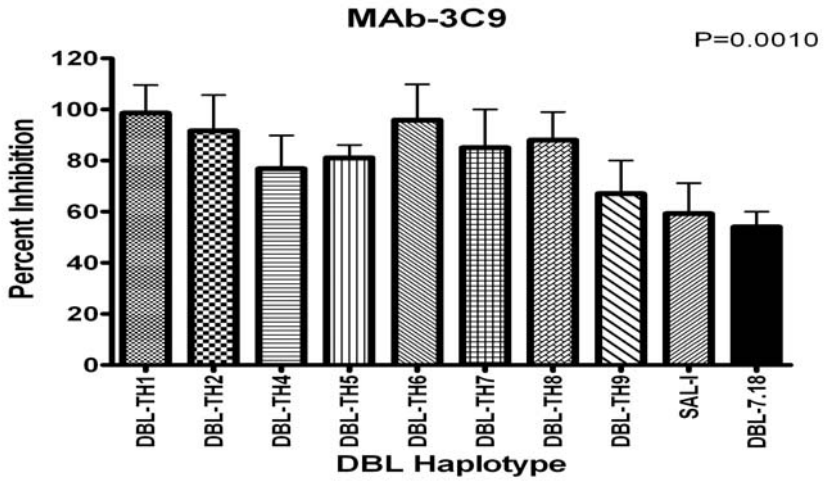


Figure 3: Determination of 50% inhibitory concentration (IC50) of anti-DBPII monoclonal antibodies. The monoclonal antibodies 3C9, 2C6, 2D10 and 2H2 were tested for their inhibitory function against PVDBPII 7.18 haplotype. The transfected COS7 cells expressing homologous DBPII 7.18 was incubated with monoclonal antibodies at difference concentrations and added human erythrocytes. The number of rosette was compared between wells of transfected cell incubated with monoclonal antibodies relative to well without antibodies. Curves represent mean of percent inhibition of two experiments tested in duplicate. Dotted line shows point on curve where IC50 of each antibody is achieved.

Each Mabs (3C9, 2D10, 2C6 and 2H2) was tested at a concentration equal to the IC50 of homologous antigen for inhibition of DBPII- erythrocyte binding to the panel of homologous and heterologous Thai DBPII alleles. The result showed that functional inhibition of Mabs 2H2 against Thai DBL varied little, indicating the conserved B-cell epitopes among Thai DBL variants. However significant functional differences in inhibitory efficiency were shown for Mabs 3C9, 2D10, and 2C6 against the different Thai DBL alleles. The result indicated that variant epitopes of Thai DBL haplotypes were the target of anti-DBPII inhibitory antibodies.

A



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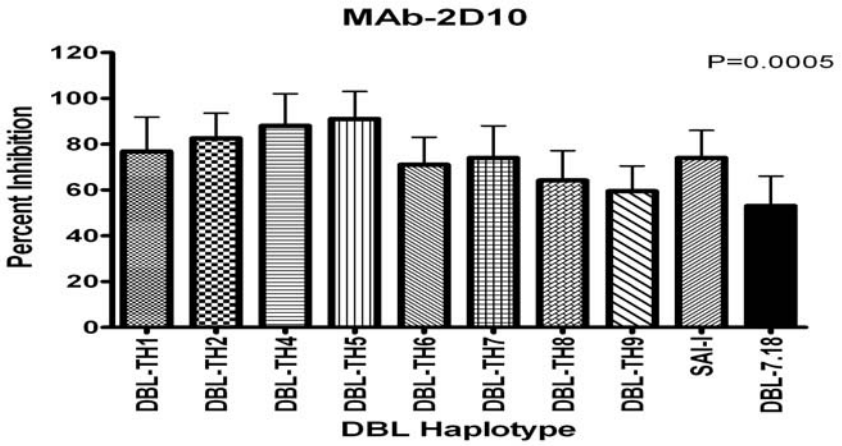
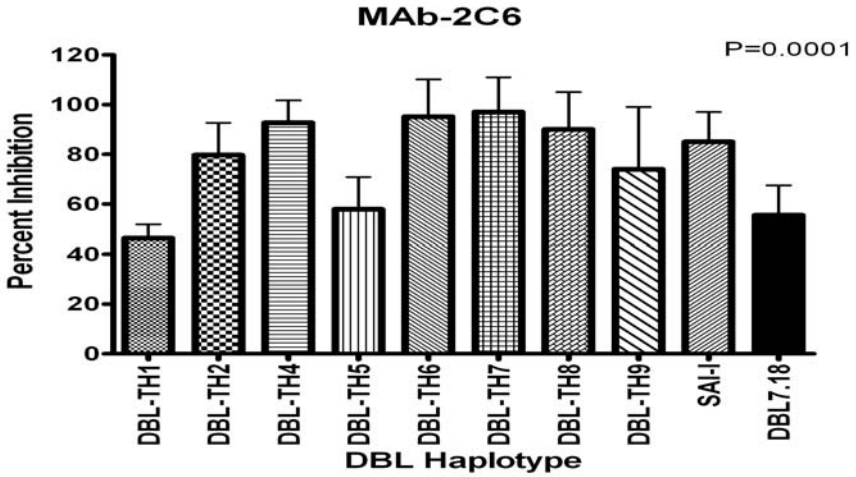

MAb-2D10
P=0.0005
Percent Inhibition
120
100
80
60
40
20
0
DBL-TH1
DBL-TH2
DBL-TH4
DBL-TH5
DBL-TH6
DBL-TH7
DBL-TH8
DBL-TH9
SAI-I
DBL-7.18
DBL Haplotype

C


MAb-2C6
P=0.0001
Percent Inhibition
120
100
80
60
40
20
0
DBL-TH1
DBL-TH2
DBL-TH4
DBL-TH5
DBL-TH6
DBL-TH7
DBL-TH8
DBL-TH9
SAI-I
DBL7.18
DBL Haplotype

D

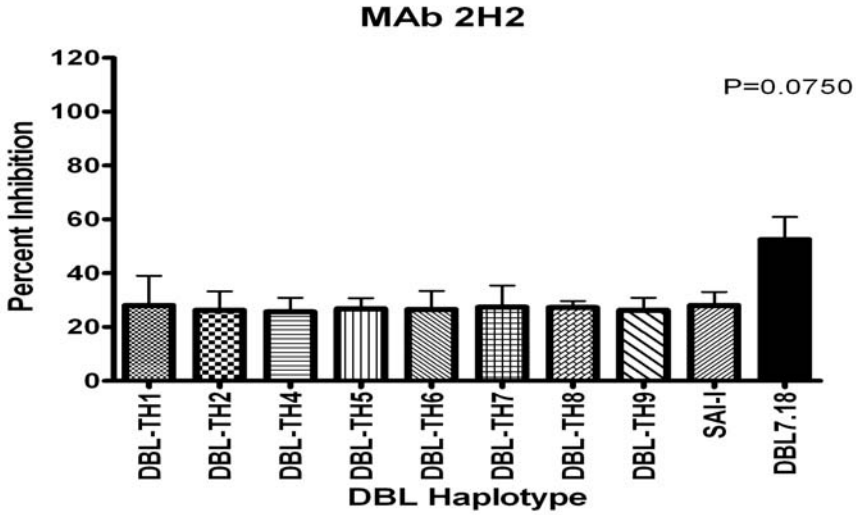

MAb 2H2
P=0.0750
Percent Inhibition
120
100
80
60
40
20
0
DBL-TH1
DBL-TH2
DBL-TH4
DBL-TH5
DBL-TH6
DBL-TH7
DBL-TH8
DBL-TH9
SAI-I
DBL7.18
DBL Haplotype

Figure 4: The functional inhibition of anti-DBPII monoclonal antibodies against the panel of DBL-TH variants. The transfected COS7 cell expressing DBPII reference Sal I or 7.18 or DBL-TH variants were pre-incubated with at concentration to the IC 50 of the homologous 7.18 antigen for inhibiton DBPII-erythrocyte binding a panel of homologous and heterologous DBL-TH alleles. The mean inhibition ratio of each monoclonal antibody against all DBPII alleles is represented by a horizontal. The various symbols above and below the mean is represented the variability of the inhibitory response against the different DBPII alleles.

5. The naturally acquired anti-DBPII antibody in Thai patients

To assess the immunological responses during *P. vivax* infection, the reactivity of naturally acquired antibodies were tested against the recombinant DBPII. The antibody titer specific to anti-DBPII responses in individual patient's plasma samples were significantly elevated during P. vivax infections (average OD=0.32, figure 5) when compared with that of healthy controls (average OD= 0.13, figure 5). There were 7 high responders among Thai residents. The wide range of antibody responses to the recombinant DBPII antigen suggested a potential protective role of higher titer anti-DBP antibodies during *P. vivax* infection.

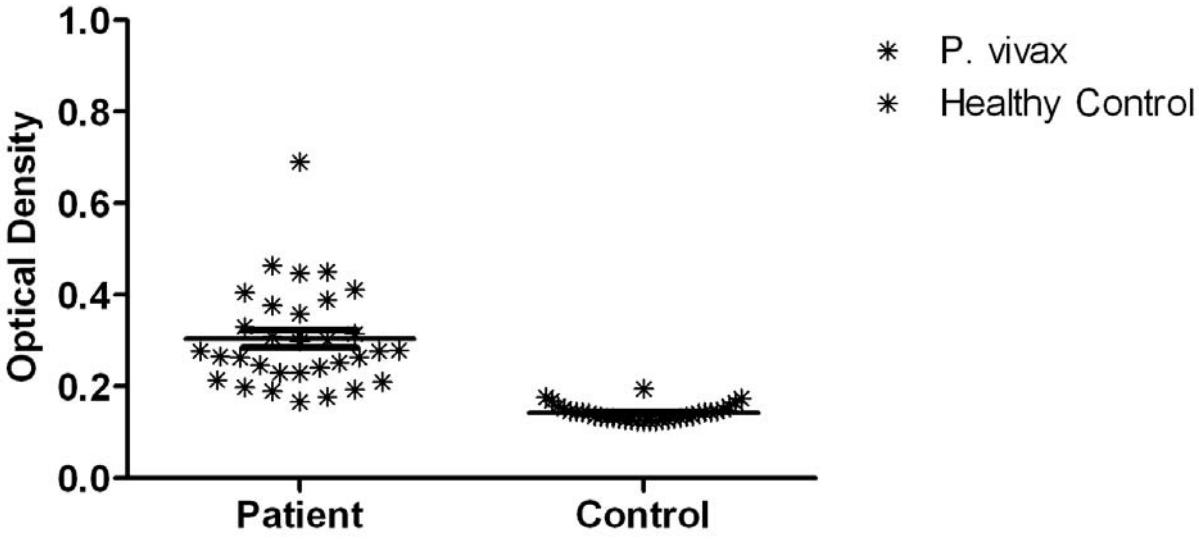


Figure 5: The antibody levels specific to recombinant PvDBPII antigen. Graphical display of antibody levels anti-DBPII in Thai *P. vivax* patients (n=33) and healthy controls. Dots represent the mean optical density value in triplicate wells for each sample. Bars represent mean value.

6. The inhibitory efficiency of naturally acquired anti-DBPII antibody against Thai DBL variants

Because PvDBPII is highly polymorphic, it is important to determine whether anti-PvDBPII neutralizing antibody recognizes PvDBPII variants and whether inhibition of receptor-binding is strain specific. To examine this possibility, plasma with in high responders (HR) were tested for inhibition of binding of 8 different Thai PvDBPII variants, including reference Sal I. There are 42.86%, 57.14%, 28.57%, 57.14%, 42.86% and 85.71% of HR had high inhibitory function against PvDBPII Thai variants DBL-TH1, -TH4, -TH5, -TH7, -TH8 and DBL-TH9, respectively. Interestingly, all HR samples strongly

inhibited Thai variants DBL-TH2 and DBL-TH6 haplotype. Only one sample inhibited PvDBPII reference Sal I.

Using HR4 and HR5 samples to compare the inhibition activity against a panel Thai PvDBPII variants, the result showed significantly difference of blocking function ($P<0.05$, Fig 6A -6B). These data suggested that immune response against PvDBPII-erythrocyte binding is strain specific inhibitory activity. Contrastly, HR3 and HR6 sample inhibited binding of all Thai PvDBPII variants by >80%, indicating the anti-PvDBPII neutralizing antibody may recognize conserved epitopes in Thai PvDBPII variants ($P>0.05$, Figure 6C-6D).

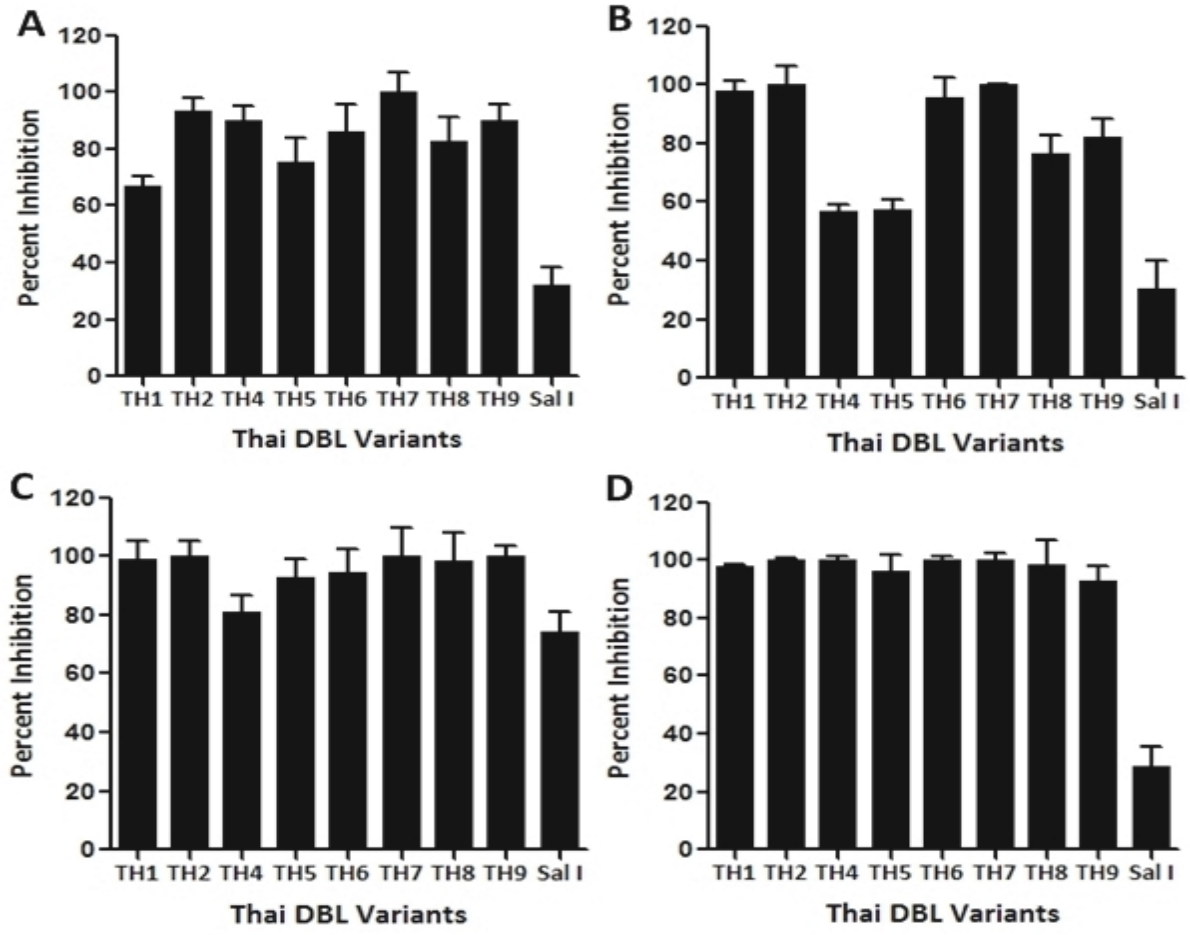


Figure 6: Functional inhibition of anti-DBPII antibodies in high responder sample against the panel of DBL-TH variants. The transfected COS7 cell expressing DBPII reference Sal I or DBL-TH variants were pre-incubated with plasma at dilution 1:100 for inhibition of DBPII-erythrocyte binding. The charts show the mean inhibition of each DBL-TH variant. The variation in means across DBL-TH variants shows significant differences in the inhibitory responses of HR4 **(A)** and HR5 sample **(B)** whereas A little variation of inhibitory function against the panel of DBL-TH variants of HR3 **(C)** and HR6 sample **(D)**. COS7 experiments for each plasma sample were done in duplicate wells of each DBL variant and were repeated two times. Statistical significance was determined using one-way analysis of variance (ANOVA).

Conclusion and Discussion

Malaria is a major public health problem in Thailand. The surveillance data show that malaria transmission is found in nearly all areas of the country but is most prevalent along the border regions, especially the Thai-Myanmar border. Current public health surveys indicate vivax malaria prevalence has been on the rise in Thailand (Sattabongkot J et al, 2004). A significant portion of malaria cases in Thailand occur among temporary migrant workers from bordering countries presenting a major challenge to prevention and control of malaria in Thailand. The genetic polymorphism in the Reticulocyte Binding Protein (RBP), Merozoite Surface Protein 5 (MSP-5) and Merozoite Surface Protein 3 α/β of *P. vivax* Thai isolates were analysed in previous studies and showed high levels of genetic polymorphism (Rungsihirunrat K et al, 2011). However, the information on the nature and extent of population diversity as well as the antigenic character of malaria parasites in Thailand is still limited. In this study, genetic polymorphism and its potential relationship to antigenicity of Thai PvDBPII haplotypes were analyzed in order to support development of a strategy for *P. vivax* vaccine design in Thailand.

The 44 PvDBPII Thai isolates in this study were classified into 9 different haplotypes. Haplotype DBL-TH1 was identical to the PvDBPII sequence in a Myanmar isolate whereas the other 8 haplotypes were novel. A total of 12 polymorphic residues were identified which result in significant amino acid changes in PvDBPII Thai isolates. All variant residues of Thai PvDBPII have been previously reported in global vivax isolates (Ju HL et al, 2012, Hwang SY et al, 2009). The most prevalent polymorphic variants in Thai PvDBPII variants were L333F (78.9%), L424I (73.7%), W437R (73.4%) and I503K (63.2%) variants. Analysis of the L424I, W437R and I503K variants showed that the L424I-W437R, L424I-I503K and L424I-W437R-I503K combinations occurred with high frequencies of 73.7%, 57.9% and 57.2%, respectively. A previous study demonstrated that the W437R variant combined with the N417K or I503K variants can alter DBP antigenic character and can affect the efficiency of inhibitory antibodies against DBPII-erythrocyte binding (VanBuskirk KM et al, 2004). Therefore, the combination of the L424I, W437R and I503K variants may also alter antigenicity and thus, the anti-DBPII response. Additional studies of naturally-acquired immunity against Thai DBPII protein antigens will be important for effective vaccine design.

Phylogenetic analysis revealed that Thai DBPII isolates had broad genetic diversity. A total of 9 Thai PvDBPII haplotypes were distributed among other geographic regions: Brazil, Papua New Guinea, Myanmar, Vietnam, Indonesia, Iran, Colombia, Sri Lanka, South Korea and India. The PvDBPII phylogeny showed that Thai PvDBPII isolates shared a common ancestor close to Myanmar isolates. The highest prevalence L333F, L424I, W437R and I503K variants were common in Thai and Myanmar isolates (Ju HL et al, 2012). However, there are 11 polymorphisms (I310L, F344S, R391H, K455I, K473R, C477G, R490K, D528G, V533M, K541T and A545V) found only in Myanmar isolates and not present in Thai isolates (Ju HL et al, 2012). Taken together, these results indicate that immigration and/or travel are likely to influence the frequency of PvDBPII haplotypes between Thailand and Myanmar.

A significant challenge for DBPII vaccine development is its high level of polymorphism and the resulting strain-specific immunity. Previous studies have shown that individuals exposed to *P. vivax* parasites develop naturally acquired immunity (Fraser T et al, 1997, Chootong P et al 2010). However, most individuals develop strain specific immunity, with only a few individuals capable of producing

broadly neutralizing anti-DBPII antibodies (Cole-Tobian JL et al, 2009). Vaccine design needs to identify neutralizing antibodies against conserved epitopes while avoiding presentation of immunogenic variant epitopes in a potential DBPII vaccine. Our objective was to evaluate the activity of broadly inhibitory anti-DBPII monoclonal antibodies against the diverse Thai DBPII variants identified in this study.

We analyzed the ability of anti-PvDBPII antibodies to inhibit Thai PvDBPII variant- erythrocyte binding by using an *in vitro* COS-7 erythrocyte-binding-inhibition assay. The concentration of monoclonal antibodies to achieve 50% inhibition against the homologous strain (IC50) was used to compare the inhibition efficiency against Thai PvDBPII variants. Monoclonal antibody 3C9 (IC50=0.21) had the strongest inhibition activity against homologous DBP7.18, followed by 2D10 (IC50=0.38), 2H2 (IC50=0.64) and 2C6 (IC50=2.61), respectively. A significant difference in inhibitory function against heterologous Thai variants was found in the 3C9, 2D10 and 2C6 monoclonal antibodies (Fig 4A-4C, $P<0.005$), indicating that the these monoclonal antibodies may bind to different epitopes of Thai DBPII variants. Immune activity of 3C9, 2D10 and 2C6 monoclonal antibodies showed a trend toward variant-specific antibody with the individual Thai DBPII alleles. These results strongly support that the genetic polymorphisms of PvDBPII can alter antigenic character and thus may confer significant changes in their sensitivity to inhibitory function of anti-DBPII neutralizing antibodies as previously reported (VanBuskirk KM et al 2004, Ntumngia FB et al 2012).

Immunization with PvDBP7.18 antigen showed a higher inhibitory response against Thai DBPII variants than against the homologous 7.18 haplotype. This data is supported by our observation that Mab 2C6 showed stronger inhibitory activity against DBPII-Sal I than against homologous DBPII7.18 (2C6; IC50=1.01 ug/ml in DBPII Sal I, IC50=2.61 ug/ml in DBPII7.18, data not shown). Our data is also in line with a previous study which showed that the alteration of variant residues on DBPII enhanced sensitivity to heterologous anti-DBPII antibody while other amino acid changes in the same residues increased refractoriness to antibody inhibition (VanBuskirk KM et al, 2004).

Interestingly, a number of DBPII neutralizing epitopes are shared among Thai DBPII variants. We have shown that a conserved epitope shared among Thai PvDBPII variants appears to be the target of the 2H2 anti-DBPII neutralizing monoclonal antibody (Fig 4D). This data strongly support that DBPII immunity has the potential to generate neutralizing antibodies against a range of variant haplotypes for broader strain recognition. The development of a broadly protective anti-DBPII vaccine to the dominant DBPII variants in field parasites will require induction of antibodies targeting epitopes that are conserved among variant DBPII alleles, such as that recognized by the 2H2 monoclonal antibody indicated in this study.

ภาคผนวก

Manuscript

Elsevier Editorial System(tm) for Parasitology International
Manuscript Draft

Manuscript Number: PARINT-D-14-00031R2

Title: The Association of Duffy Binding Protein II Polymorphisms and its Antigenicity in Plasmodium vivax Isolates from Thailand

Article Type: Research Paper

Keywords: Duffy Binding Protein II, Polymorphism, Thailand

Corresponding Author: Dr. patchanee chootong, ph.D

Corresponding Author's Institution: Mahidol University

First Author: patchanee chootong, ph.D

Order of Authors: patchanee chootong, ph.D

Abstract: Plasmodium vivax Duffy Binding Protein II (DBPII) plays an important role in reticulocyte invasion and is a potential vaccine candidate against vivax malaria. However, polymorphisms in DBPII are a challenge for the successful design of a broadly protective vaccine. In this study, the genetic diversity of DBPII among Thai isolates was analyzed from P. vivax-infected blood samples and polymorphism characters were defined with the MEGA4 program. Sequence analysis identified 12 variant residues that are common among Thai DBPII haplotypes with variant residues L333F, L424I, W437R and I503K having the highest frequency. Variant residue D384K occurs in combination with either E385K or K386N/Q. Additionally, variant residue L424I occurs in conjunction with W437R in most Thai DBPII alleles and these variants frequently occur in combination with the I503K variant. The polymorphic patterns of Thai isolates were defined into 9 haplotypes (Thai DBL-1, -2, -3, etc…). Thai DBL-2, -5, -6 haplotypes are the most common DBPII variants in Thai residents. To study the association of these Thai DBPII polymorphisms with antigenic character, the functional inhibition of anti-DBPII monoclonal antibodies against a panel of Thai DBL variants was characterized by an in vitro erythrocyte binding inhibition assay. The functional inhibition of anti-DBPII monoclonal antibodies 3C9, 2D10 and 2C6 against Thai variants was significantly different, suggesting that polymorphisms of Thai DBPII variants alter the antigenic character of the target epitopes. In contrast, anti-DBPII monoclonal antibody 2H2 inhibited all Thai DBPII variants equally well. Our results suggest that the immune efficacy of a DBPII vaccine will depend on the specificity of the anti-DBPII antibodies induced and that it is preferable to optimize responses to conserved epitopes for broadly neutralizing protection against P. vivax.

4th February 2014

T. Shuboi, Ph.D.
Editor
Parasitology International

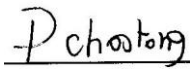
Dear Editor,

We are submitting a manuscript entitled "The Association of Duffy Binding Protein II Polymorphisms and its Antigenicity in *Plasmodium vivax* Isolates from Thailand" to you for consideration to publish in Parasitology International. This manuscript has been seen and approved by all authors and we have agreement to submit our paper. The manuscript is an original research study intended to gain a better understanding the basis of protective immunity to vivax malaria targeting the blood-stage vaccine candidate Duffy binding protein region II (PvDBPII). Strain-specific immunity has severely limited efficacy of other blood-stage malaria vaccine candidates, which has often occurred due the extensive geographic and allergic diversity of the targets. In this study we are first describing the association of Thai DBPII polymorphisms in the expression of antigenic character and the functional inhibition of anti-DBPII neutralizing antibodies against a panel of Thai DBL variants. Sequence analysis identified 12 variant residues that are common among Thai DBPII haplotypes. The polymorphic patterns of Thai isolates were defined into 9 haplotypes (Thai DBL-1, -2, -3, etc…). The inhibitory function of anti-DBPII neutralizing antibodies recognized variant residues among Thai DBPII alleles, indicating the immunodominant of variant Thai DBPII epitope induce strain specificity immunity. However, the anti-DBPII neutralizing antibody is capable of broadly inhibiting diverse Thai vivax strain. Certainly, the focusing of immunodominant Thai DBPII alleles in multi-alleles vaccine or the optimization of conserved epitopes sharing among Thai DBPII alleles may serve as important data for effective *P. vivax* DBPII vaccine design in Thailand and other endemic areas of SE Asia.

I would like to recommend the reviewers for kindly providing me the good comments and evaluation:
Prof. Liwang Cui, who has experience in *P. vivax* epidemiology in Thailand;
Prof. Peter Preiser, who has extensive experience studying ligands of malaria parasites; and Prof. Rachanee Udomsangpetch who has experience in malaria immunity.

I would like to thank you in advance for kindly spending your time to read and evaluate our manuscript. If any problem, please don't hesitate to contact me.

Best regards,



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Reviewers' comments:

Reviewer 1*: The Authors addressed all the comments appropriately. However, there are two more points need to be clarified by the Authors.*

1) Highlights "The anti-DBPII monoclonal antibody 2H2 inhibited strongly all Thai DBLII variants." The vertical axis in both graphical abstract and Figure 4 indicated that 100% Percent Inhibition (no erythrocyte binding) means strongest mAb efficacy. If my understanding is correct, Mab 2H2 is the "weakest" mAb than the others. So, please carefully revise both related text and Figures.

The highlights were revised as suggested.

Text was revised to address the reviewer's comment. Inhibition assays were performed at the IC50 concentration (point at which 50% inhibition occurs) determined in Figure 3 for each monoclonal antibody. The 50% inhibitory concentrations (IC50) of 3C9, 2D10, 2C6 and 2H2 monoclonal antibodies were 0.21, 0.38, 2.61 and 0.64 ug/ml, respectively (Fig 3A-3D). (See section 3.3). Monoclonal antibody 3C9 (IC50=0.21) had the strongest inhibition activity against homologous DBP7.18, followed by 2D10 (IC50=0.38), 2H2 (IC50=0.64) and 2C6 (IC50=2.61), respectively. (See discussion.)

2) Style of the reference is not fit for that of Parasitology International. For example, please remove "PubMed PMID: 22403626. Pubmed Central PMCID: 3293883" etc.

The reference format was revised

Reviewer2*: The revised manuscript "The Association of Duffy Binding ProteinII Polymorphisms and its Antigenicity in Plasmodium vivax Isolates from Thailand" by Chootong et al is a much improved version of the original manuscript that addresses the strain specificity of DBPII monoclonal antibodies raises against a Papua New Guinean haplotype with respect to a panel of Thai isolates. The manuscript is now much clearer and easier to read. Minor points*

1) The"highlights" section needs to be re-written for clarity and correct usage of English.

The highlights were revised.

2) The results section (inhibition assay)

i) The authors explain that, as there is little variation in the Thai isolates' binding in the presence of monoclonal H2, that this must target a conserved domain. Actually, looking at the data, there is little inhibition (~20%) with this monoclonal. I think an appropriate control here would be the addition of monoclonal antibodies raised against something else, as this could be a very non-specific inhibition. The fact that the antibody inhibits the homologous strain to a much higher degree than any of the heterologous strains suggests that this monoclonal targets a specific epitope unique to the 7.18 haplotype, doesn't it? Is it possible that it targets the area including amino acid position 308, which is the only amino acid that is different in 7.18, but

conserved completely in the Thai isolates? That would explain why inhibition was less in the Thai isolates, but that there was no difference between them. In which case calling this monoclonal target "conserved" is true only with respect to those particular Thai isolates considered here.

Before we evaluated the inhibition activity of monoclonal antibodies 3C9, 2C6, 2D10 and 2H2 against Thai DBP variant, the specificity of monoclonal antibodies to recombinant DBPII was characterized by ELISA assay using a *P. falciparum* apical membrane antigen 1 (PfAMA1)-specific monoclonal antibody as a negative control (Reference 29). Monoclonal antibody 2H2 specifically reacted to recombinant DBPII but no reactivity of (PfAMA1)-specific monoclonal antibody was detected in ELISA assay. This data suggests that Mab 2H2 specifically recognizes DBPII and that its inhibition activity is not non-specific.

In our inhibition assays, we included controls for positive inhibition and negative or “no inhibition” for each COS7 experiment. The positive inhibition control was the maximum concentration of each mAb tested to determine the IC50 (50% inhibition level) at which almost 100% inhibition was seen (2.56 ug/mL for Mab 2H2, shown in Figure 3). “No inhibition” control wells contained medium alone. The inhibition activity of Mab 2H2 against homologous DBL7.18 and Thai variant haplotypes were tested in each experiment. The inhibition activity of Mab 2H2 at the IC50 concentration (0.64 ug/ml) was tested against the panel of Thai DBL-TH variants and DBL.7.18 in triplicate wells in two experiments.

For the COS7 results that show greater inhibition activity of anti-DBP Mab 2H2 against homologous DBP7.18 than heterologous Thai DBP variants, our previous study (Reference 19) mapped the target epitopes of anti-DBP inhibitory antibody from Papua New Guinea plasma. We showed that epitope H1: FH**R**DITFRKLYLKRKL, H2: EGDLLLKLNNYRYN and H3: DEKAQQRRKQWWNESK were the highest potential target epitopes of naturally acquired inhibitory antibody. These epitopes were localized in the dimer interface for DBP-DARC binding. In that study, the variant amino acid R308S had high levels of genetic polymorphism whereas it is conserved among Thai isolates in this study. The single amino acid change at R308S increased refractoriness to antibody inhibition (Chootong et al, PLoS ONE, 2012). Here, we aim to identify conserved epitope among Thai DBP variants and in this regard our result revealed little variation of inhibitory activity of Mab 2H2 against the panel of Thai DBP variants. Therefore, these data suggest that epitope recognized by Mab 2H2 is conserved in variant DBPII alleles of the Thai isolates. However, our data could not conclude that amino acid position R308S, which is the only amino acid conserved completely in the Thai isolates, is the target of anti-DBPII monoclonal antibody 2H2. The identification of the specific target of Mab 2H2 that inhibited all Thai DBPII variants needs to be determined in a future study.

ii) The fact that the Thai haplotypes are inhibited to a greater degree than the homologous haplotype with the other three monoclonal is very interesting, and a bit strange. The authors do, to be fair, allude to this in their discussion, but the explanation is a bit unclear. Could they, perhaps, offer a stronger explanation for this phenomenon? How consistent are these results between experiments? In figure 4, are the error bars standard errors of the means for the three wells in only one experiment? Can the authors perhaps provide the data from the second experiment as supplementary material?

The inhibition assays were performed in triplicate wells in two experiments. The average and standard error of inhibition activity of two experiments is shown in Figure 4A-4D. The discussion was revised.

From the inhibition assay, monoclonal antibody 3C9 (IC50=0.21) had the strongest inhibition activity against DBP7.18, followed by 2D10 (IC50=0.38), 2H2 (IC50=0.64) and 2C6 (IC50=2.61), respectively. Our results showed a significant difference in inhibitory response to different alleles of COS7-expressed DBPII. It suggests that these monoclonal antibodies likely bind to different epitopes of Thai DBPII variants. Anti-DBP7.18 monoclonal antibody strongly inhibited Thai DBPII variants more than the homologous DBP7.18 haplotype. This data is supported by our observation that Mab 2C6 showed stronger inhibitory activity against DBPII-Sal I than against homologous DBPII7.18 (2C6; IC50=1.01 ug/ml in DBPII Sal I, IC50=2.61 ug/ml in DBPII7.18, data not shown in manuscript) indicating that the change in amino acid of DBPII enhanced sensitivity to heterologous DBPII Sal I. This is also in line with a previous study that showed that anti-DBPII 7.18 antibody from single allele DBP7.18 immunization had greater inhibitory responses against DBPII SaI and DBPII variants AH (AAY34130.1) than against homologous DBPII 7.18. Therefore, our data suggests that the presence of immunogenic variant epitopes in DBPII presenting in *P. vivax* endemic area can produce strain-specific anti-DBPII neutralizing antibody that would limit effectiveness against different DBPII alleles. We believe that development of a broadly protective anti-DBPII vaccine to the dominant DBPII variants in field parasites will require induction of antibodies targeting epitopes that are conserved among variant DBPII alleles, such as that recognized by the 2H2 monoclonal antibody indicated in this study.

***Suggested Reviewers**

The suggested reviewer

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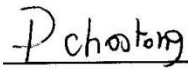
Best regards,



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Highlights

- Twelve polymorphic residues were identified in Thai PvDBPII compared to reference strain Sal I.
- Sequence analysis indicates 9 different haplotypes (DBL-TH1, -TH2, -TH3, -TH4, -TH5, -TH6, -TH7, -TH8 and TH9).
- The low level of variation in the inhibitory activity of anti-DBPII monoclonal antibody 2H2 against Thai DBPII variants suggests that the epitope target of this antibody is conserved among Thai DBPII variants.
- Targeting of conserved epitopes will increase the ability of a DBPII vaccine to induce broadly neutralizing protection against *P. vivax.*

RUNNING TITLE: Duffy Binding Protein II Polymorphism in Thailand

Title: The Association of Duffy Binding Protein II Polymorphisms and its Antigenicity in *Plasmodium vivax* Isolates from Thailand

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ABBREVIATIONS:

DBPII, Duffy binding protein region II; DBL, Duffy binding ligand domain; DBL-TH, Duffy binding ligand Thai haplotypes

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Abstract

Plasmodium vivax Duffy Binding Protein II (DBPII) plays an important role in reticulocyte invasion and is a potential vaccine candidate against vivax malaria. However, polymorphisms in DBPII are a challenge for the successful design of a broadly protective vaccine. In this study, the genetic diversity of DBPII among Thai isolates was analyzed from *P. vivax*-infected blood samples and polymorphism characters were defined with the MEGA4 program. Sequence analysis identified 12 variant residues that are common among Thai DBPII haplotypes with variant residues L333F, L424I, W437R and I503K having the highest frequency. Variant residue D384K occurs in combination with either E385K or K386N/Q. Additionally, variant residue L424I occurs in conjunction with W437R in most Thai DBPII alleles and these variants frequently occur in combination with the I503K variant. The polymorphic patterns of Thai isolates were defined into 9 haplotypes (Thai DBL-1, -2, -3, etc…). Thai DBL-2, -5, -6 haplotypes are the most common DBPII variants in Thai residents. To study the association of these Thai DBPII polymorphisms with antigenic character, the functional inhibition of anti-DBPII monoclonal antibodies against a panel of Thai DBL variants was characterized by an *in vitro* erythrocyte binding inhibition assay. The functional inhibition of anti-DBPII monoclonal antibodies 3C9, 2D10 and 2C6 against Thai variants was significantly different, suggesting that polymorphisms of Thai DBPII variants alter the antigenic character of the target epitopes. In contrast, anti-DBPII monoclonal antibody 2H2 inhibited all Thai DBPII variants equally well. Our results suggest that the immune efficacy of a DBPII vaccine will depend on the specificity of the anti-DBPII antibodies induced and that it is preferable to optimize responses to conserved epitopes for broadly neutralizing protection against *P. vivax.*

Introduction

Plasmodium vivax is a major cause of malaria worldwide leading to >50% of the disease is outside Africa, mainly afflicting Asia and the Americas with approximately 2.5 billion people at risk from vivax malaria (1). The re-emergence of *P. vivax* in areas where it was considered eradicated, the emergence of drug resistance, and cases of severe and fatal vivax malaria are evidence that it persists as a significant public health problem. Therefore, an important part of control strategy will be an implementation of a vaccine capable of inducing protective immunity against *P. vivax*.

P. vivax Duffy binding protein (PvDBP) is a 140-kDa type 1 integral membrane protein which belongs to a family of homologous Duffy binding-like erythrocyte binding proteins (DBL-EBP) located within the micronemes of *Plasmodium* merozoites (2, 3). The critical erythrocyte binding motif of DBP is in a 330-amino-acid cysteine rich domain referred to as DBP region II (DBPII) or the DBL domain. DBPII binds Duffy antigen/receptor for chemokines (DARC) on red blood cells. The DBP invasion ligand is considered a strong potential vaccine candidate against *P. vivax* infection in part because anti-DBP antibodies inhibit *in vitro* DBP-erythrocyte binding, reduce merozoite invasion of human erythrocytes and confer protection against blood stage infection (4-8). Serological responses to DBP and the inhibitory effect of anti-DBP antibodies against DBP-erythrocyte binding increase with a person's age, suggesting that there is a boosting effect due to repeated exposure through recurrent infection (4, 19, 7). These data strongly support that DBP can induce a protective immune response during *P. vivax* infection.

However, PvDBPII is highly polymorphic and *dbpII* alleles have a very high ratio of nonsynonymous to synonymous mutation, suggesting a mechanism consistent with high selection pressure driving DBP allelic diversity as a means for immune evasion (9-11). Analysis of genetic diversity of *dbpII* alleles among *P. vivax* isolates from different geographical regions, including Brazil, Colombia, South Korea and Papua New Guinea, shows that polymorphic residues are mostly concentrated in the ligand domain and vary by geographic region (12-14). A study of *dbp* alleles in Papua New Guinea (PNG) found that the substitution rate within region II was 10 times greater than that found within the *dbp* gene overall (9) and that 93% of DBP polymorphisms were within the central segment of DBPII between cysteines 4 and 7 (9). Polymorphic residues at position 417, 437 and 503 either singly or in combination changed DBP antigenic character, which significantly changed sensitivity to inhibitory antibodies directed against DBPII (15).

Analysis of field parasites shows that some polymorphic residues in DBPII are unique to one population or geographic region, while some variant amino acids, K371E, D384G, E385K, K386N, N417K, L424I, W347R and I503K are common among global vivax isolates (12, 13, 16, 17). However, only a few individuals produce anti-DBP responses that broadly inhibit against multiple allelic variants (18, 19). Consequently, the polymorphic nature of PvDBPII represents a major impediment to the successful design of a DBPII protective vaccine against diverse *P. vivax* haplotypes. Better understanding the nature of genetic polymorphisms in DBPII of *P. vivax* isolates from distinct geographic areas, particularly where a large proportion of *P. vivax* infections occur, as well as determining the correlation between DBPII polymorphisms and antigenic character are important for the rational design of a broadly protective vaccine against

vivax malaria. In this study we analyzed the genetic polymorphisms of Thai DBPII variants and their effects on antigenic character by using a set of murine monoclonal antibodies.

2. Materials and Methods

2.1. Blood Samples and DNA preparation

The study was pursued in the malaria endemic areas of Southern Thailand where both major species of malaria, *P. vivax* and *P. falciparum*, are common. The 44 blood samples used in this study were collected from acute *P. vivax*-infected volunteers at malaria clinics, Chumphon province, which is in the southern peninsular part of Thailand. The areas are malaria endemic with high prevalence of *P. vivax* infection. The confirmation of *P. vivax* infection was performed by microscopic examination of thin and thick Giemsa-stained blood smears. Acute *P. vivax*-infected volunteers who registered at Malarial Clinics were asked for informed consent under the protocol approved by the Ethic Committee on Human Rights Related to Human Experimentation, Mahidol University (MUIRB2012/162.0510). Ten 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).

2.2. Gene amplification and sequencing of PvDBPII

DBPII genes were PCR amplified by the polymerase chain reaction (PCR) using specific primers, PvDBPII F: 5'-TTTGGATCCACGATCTCTAGTGCTATT-3' and PvDBPII R: 5'-AAACTCGAGTGTCACAACTTCCTGAG 3'. The amplification reaction was performed using the following thermal cycling condition: 94ºC for 90 sec, 30 cycles at 94ºC for 15 sec, 60ºC for 35 sec and 68ºC for 60 sec, followed by a 68ºC extension for 2 min. HiFi platinum Taq DNA polymerase was used in all PCR reactions. PCR products were analysed on 1.2% agarose gel. PCR products were purified and ligated into the T&A cloning vector (Invitrogen, USA). Each ligation mixture was transformed into *Escherichia coli* DH5α competent cells and positive clones were screened. Sequence analysis was performed on two clones for each parasite isolate. Nucleotide sequences were obtained using the dideoxynucleotide chain termination method (Applied Biosytems, Foster City, CA).

2.3 Analysis of PvDBPII gene sequences

The alignment of complete sequences of PVDBPII genes from 44 isolates were analyzed by CLUSTAL and percent similarity was assessed using BioEdit software. Phylogenetic analysis was used to investigate the association of PvDBPII gene with sequences from different geographic regions. The phylogenetic gene tree was constructed using regions common to all available PvDBPII sequences. Sixty PvDBPII gene sequences found in GenBank were compared to Thai PvDBPII isolates including the sequence from reference strain Sal I (DQ156512), the sequences from Vietnam (DQ156518), Indonesia (EU395591), India (FJ491215, FJ491221 and FJ491237), Iran (EU860428 and EU860432-438), South Korea (AAF25483, AAF25486-489 and AAZ81528), Myanmar (JN255576-587), Sri Lanka (GU143917, GU143922, GU143936, GU1439951, Gu143953, GU143972, GU143987 and GU144012), Papua New Guinea

(AF289482, AF289635, AF289652, AF291096 and AF469530), Brazil (EU812894, EU812906, EU812917, EU812922, EU812927, EU812933, EU812952, EU812933, EU812942 and EU395588) and Colombia (DQ156513, U50576, U50577, U50583, U50584 and U50588).

2.4 pEGFP-DBPII constructs and site-directed mutagenesis

The pEGFP-DBPII construct contains the Sal I DBPII allele cloned into the pEGFP-N1 plasmid with flanking signal sequences from the herpes simplex virus glycoprotein D1 allowing expression of a GFP fusion protein on the surface of transiently transfected COS7 cells (7). Site-directed mutagenesis was used to create a panel of pEGFP-DBPII constructs with identical polymorphisms to PvDBPII alleles from Thai field isolates using the pEGFP-DBPII-Sal I construct as the parent template. Mutagenesis was performed using the Stratagene Quickchange mutagenesis kit (Stratagene, La Jolla, CA) as previously described (7, 15, 20). The recombinant plasmid DNA was purified using an endotoxin-free plasmid DNA purification system (Qiagen, Valencia, CA).

2.5. Evaluating the inhibitory efficiency of anti-DBPII neutralizing antibodies against Thai PvDBPII haplotypes

We used the COS-7 cell erythrocyte assay to evaluate the ability of previously described monoclonal antibodies to inhibit binding of Thai DBPII and reference Sal I haplotypes human erythrocytes. Our previous study describes the production and inhibitory efficiency of monoclonal antibodies 3C9, 2C6, 2D10 and 2H2 raised against the DBL7.18 haplotype (DBL variant allele; accession number AAL79051.1). These antibodies strongly inhibit binding of DBL7.18 to erythrocytes (29). In this study, monoclonal antibodies 3C9, 2C6, 2D10 and 2H2 were tested against Thai DBPII and reference Sal I haplotypes. The construct used to express Thai DBPII or reference Sal I haplotype on the surface of transiently transfected COS has been used extensively as described (7). This targeted expression efficiently presents the parasite ligand on the surface of the COS cells and expression was confirmed by detection of the C-terminal GFP tag using epifluorescence.

To evaluate the ability of monoclonal antibodies to inhibit binding of erythrocytes to DBP expressed on the surface of transfected COS7 cells, various concentrations of monoclonal antibodies were preincubated with the transfected cells for 1 hr at 37ºC before addition of a 10% suspension of human erythrocytes in each well and then incubated at room temperature for 2 hr. Percent binding-inhibition of each monoclonal antibody was determined by assessing the number of rosettes in wells of transfected COS7 cells in the presence of monoclonal antibodies relative to rosettes in wells of transfected cells in presence of medium control. The differences in inhibitory efficiency of each monoclonal antibody to the different Thai DBPII alleles were compared by one-way analysis of variance (ANOVA) using SPSS software. Experiments for each monoclonal antibody were done in triplicate wells and were repeated two times.

% inhibition = [1-(number of rosettes in the presence of monoclonal antibodies/ number of rosettes in the presence of medium control)] x 100.

3. Results

3.1. Genetic polymorphism of PvDBPII among Thai isolates

The analysis and comparison of amino acid sequences of Thai PvDBPII against the reference Sal I strain (P22290.2) showed 12 polymorphic residues in Thai vivax isolates (Fig 1A). The sequence analysis classified the mutation pattern into 9 different haplotypes (DBL-TH1, -TH2, -TH3, -TH4, -TH5, -TH6, -TH7, -TH8 and TH9). Most amino acid polymorphisms were dimorphic. Only one position K386Q/N showed trimorphic polymorphism. All variant residues described in this study have been reported in other *P. vivax* areas but the pattern of polymorphism was novel. Haplotype DBL-TH1 was the predominant haplotype among Thai vivax isolates (26.3%). The highest frequencies (>50%) of variant DBL-TH amino acids as compared to the reference Sal I sequence were found at positions L333F (78.9%), L424I (73.7%) W437R (73.4%) and I503K (63.2%) (Fig1B). Variant L424I occurred in conjunction with W437R in most Thai DBPII alleles and these variants frequently occurred in combination with variant I503K. The comparison of DBL-TH haplotypes with the previously identified PvDBPII sequences revealed that haplotypes DBL-TH2-TH3,-TH4,-TH5,-TH6,-TH7 and haplotype DBL-TH9 were novel in Thai isolates. Only the DBL-TH1 haplotype was common between Thai and Myanmar isolates.

3.2. Phylogenetic analysis of PvDBPII

Phylogenetic trees were constructed from aligned nucleotide sequences based on the Neighbour-Joining method using Tamura's three-parameter distance (Fig. 2). The Sal I haplotype served as the reference for each alignment and for constructing phylogenetic trees. The trees showed that the 9 DBL-TH haplotypes were widely distributed among different *P. vivax* isolates from distinct geographic regions (Fig. 2). PvDBPII DNA sequences of Thai isolates were related to isolates from Myanmar whereas they differed from Vietnam, Indonesia, India, South Korea, Colombia, Iran, Sir Lanka, Brazil and PNG isolates. The Sal I reference strain was closely related with Mynmar, Iran, Brazil and Sri Lanka isolates. However, the bootstrap analysis showed that Thai isolates were distinct from the Sal I reference strain. These results indicated that there were common DBPII haplotypes circulating in *P. vivax* endemic areas of Thailand and Myanmar.

3.3. Inhibitory function of anti-DBPII neutralizing antibodies against Thai DBPII variants

To determine if there are common epitopes associated with inhibition among Thai DBPII variants, the inhibitory function of anti-DBPII neutralizing antibodies against Thai DBPII haplotypes was assessed by *in vitro* COS7 assay of Thai DBPII-erythrocyte binding. The inhibitory efficiency of monoclonal antibodies 3C9, 2D10, 2C6 and 2H2 was measured against DBPII-DBL7.18-erythrocyte binding. First, the limiting dilution of each monoclonal antibody in the range of concentration to get 100% to 0% inhibition activity against PvDBPII7.18-erythrocyte binding was determined. The results showed inhibitory activity against DBPII-DBL7.18-erythrocyte binding in a dose dependent manner (Fig 3). The 50% inhibitory concentrations (IC50) of 3C9, 2D10, 2C6 and 2H2 monoclonal antibodies were 0.21, 0.38, 2.61 and 0.64 ug/ml, respectively (Fig 3A-3D). We used the IC50 of each monoclonal antibody to evaluate the functional activity of anti-DBPII monoclonal antibody against the panel of Thai DBPII variants and homologous DBL7.18

expressed in the COS7 cell assay. The results revealed little variation in the inhibitory activity of monoclonal antibody 2H2 (Fig 4D), suggesting that the epitope target of this anti-DBPII neutralizing antibody was conserved among DBL-TH variants. In contrast, there was a significant difference of inhibitory activity of monoclonal antibodies 3C9, 2D10 and 2C6 against the panel of Thai DBL variants ($P<0.005$) (Fig 4A-4C). The results indicate that variant epitopes among Thai isolates were the target of most anti-DBPII neutralizing antibodies.

4. Discussion

Malaria is a major public health problem in Thailand. The surveillance data show that malaria transmission is found in nearly all areas of the country but is most prevalent along the border regions, especially the Thai-Myanmar border. Current public health surveys indicate vivax malaria prevalence has been on the rise in Thailand (21, 22). A significant portion of malaria cases in Thailand occur among temporary migrant workers from bordering countries presenting a major challenge to prevention and control of malaria in Thailand. The genetic polymorphism in the Reticulocyte Binding Protein (RBP), Merozoite Surface Protein 5 (MSP-5) and Merozoite Surface Protein 3 α/β of *P. vivax* Thai isolates were analyzed in previous studies and showed high levels of genetic polymorphism (23-26). However, the information on the nature and extent of population diversity as well as the antigenic character of malaria parasites in Thailand is still limited. In this study, genetic polymorphism and its potential relationship to antigenicity of Thai PvDBPII haplotypes were analyzed in order to support development of a strategy for *P. vivax* vaccine design in Thailand.

The 44 PvDBPII Thai isolates in this study were classified into 9 different haplotypes. Haplotype DBL-TH1 was identical to the PvDBPII sequence in a Myanmar isolate whereas the other 8 haplotypes were novel. A total of 12 polymorphic residues were identified which result in significant amino acid changes in PvDBPII Thai isolates. All variant residues of Thai PvDBPII have been previously reported in global vivax isolates (12, 13, 17, 27). The most prevalent polymorphic variants in Thai PvDBPII variants were L333F (78.9%), L424I (73.7%), W437R (73.4%) and I503K (63.2%) variants. Analysis of the L424I, W437R and I503K variants showed that the L424I-W437R, L424I-I503K and L424I-W437R-I503K combinations occurred with high frequencies of 73.7%, 57.9% and 57.2%, respectively. A previous study demonstrated that the W437R variant combined with the N417K or I503K variants can alter DBP antigenic character and can affect the efficiency of inhibitory antibodies against DBPII-erythrocyte binding (15). Therefore, the combination of the L424I, W437R and I503K variants may also alter antigenicity and thus, the anti-DBPII response. Additional studies of naturally-acquired immunity against Thai DBPII protein antigens will be important for effective vaccine design.

Phylogenetic analysis revealed that Thai DBPII isolates had broad genetic diversity. A total of 9 Thai PvDBPII haplotypes were distributed among other geographic regions: Brazil, Papua New Guinea, Myanmar, Vietnam, Indonesia, Iran, Colombia, Sri Lanka, South Korea and India. The PvDBPII phylogeny showed that Thai PvDBPII isolates shared a common ancestor close to Myanmar isolates. The highest prevalence L333F, L424I, W437R and I503K variants were common in Thai and Myanmar isolates (12). However, there are 11 polymorphisms (I310L, F344S, R391H, K455I, K473R, C477G, R490K, D528G, V533M, K541T and A545V) found only in Myanmar isolates and not

present in Thai isolates (12). Taken together, these results indicate that immigration and/or travel are likely to influence the frequency of PvDBPII haplotypes between Thailand and Myanmar.

A significant challenge for DBPII vaccine development is its high level of polymorphism and the resulting strain-specific immunity. Previous studies have shown that individuals exposed to *P. vivax* parasites develop naturally acquired immunity (5, 18, 19). However, most individuals develop strain specific immunity, with only a few individuals capable of producing broadly neutralizing anti-DBPII antibodies (28). Vaccine design needs to identify neutralizing antibodies against conserved epitopes while avoiding presentation of immunogenic variant epitopes in a potential DBPII vaccine. Our objective was to evaluate the activity of broadly inhibitory anti-DBPII monoclonal antibodies against the diverse Thai DBPII variants identified in this study.

We analyzed the ability of anti-PvDBPII antibodies to inhibit Thai PvDBPII variant-erythrocyte binding by using an *in vitro* COS-7 erythrocyte-binding-inhibition assay. The concentration of monoclonal antibodies to achieve 50% inhibition against the homologous strain (IC50) was used to compare the inhibition efficiency against Thai PvDBPII variants. Monoclonal antibody 3C9 (IC50=0.21) had the strongest inhibition activity against homologous DBP7.18, followed by 2D10 (IC50=0.38), 2H2 (IC50=0.64) and 2C6 (IC50=2.61), respectively. A significant difference in inhibitory function against heterologous Thai variants was found in the 3C9, 2D10 and 2C6 monoclonal antibodies (Fig 4A-4C, $P<0.005$), indicating that the these monoclonal antibodies may bind to different epitopes of Thai DBPII variants. Immune activity of 3C9, 2D10 and 2C6 monoclonal antibodies showed a trend toward variant-specific antibody with the individual Thai DBPII alleles. These results strongly support that the genetic polymorphisms of PvDBPII can alter antigenic character and thus may confer significant changes in their sensitivity to inhibitory function of anti-DBPII neutralizing antibodies as previously reported (8, 15, 19, 28).

Immunization with PvDBP7.18 antigen showed a higher inhibitory response against Thai DBPII variants than against the homologous 7.18 haplotype. This data is supported by our observation that Mab 2C6 showed stronger inhibitory activity against DBPII-Sal I than against homologous DBPII7.18 (2C6; IC50=1.01 ug/ml in DBPII Sal I, IC50=2.61 ug/ml in DBPII7.18, data not shown). Our data is also in line with a previous study which showed that the alteration of variant residues on DBPII enhanced sensitivity to heterologous anti-DBPII antibody while other amino acid changes in the same residues increased refractoriness to antibody inhibition (15).

Interestingly, a number of DBPII neutralizing epitopes are shared among Thai DBPII variants. We have shown that a conserved epitope shared among Thai PvDBPII variants appears to be the target of the 2H2 anti-DBPII neutralizing monoclonal antibody (Fig 4D). This data strongly support that DBPII immunity has the potential to generate neutralizing antibodies against a range of variant haplotypes for broader strain recognition. The development of a broadly protective anti-DBPII vaccine to the dominant DBPII variants in field parasites will require induction of antibodies targeting epitopes that are conserved among variant DBPII alleles, such as that recognized by the 2H2 monoclonal antibody indicated in this study.

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Figure Legend

Fig 1. The genetic polymorphism of PvDBPII among Thai isolates. (A) Polymorphic amino acid changes are listed for each Thai DBL haplotype relative to reference sequence Sal I (P22290.2). (B) Frequency of amino acid change at variant residues found in Thai DBL haplotypes.

A

Reference Name	Amino acid position →	308	333	371	384	385	386	390	417	424	437	475	503
Sal I	Sal I	R	L	K	D	E	K	R	N	L	W	P	I
DBL-7.18	AAL79051.1	S			G		Q		K	I	R		K
DBL-TH1	Thailand Haplotype 1	R	F							I	R	A	K
DBL-TH2	Thailand Haplotype2	R	F	E	G	K	Q		K	I	R		
DBL-TH3	Thailand Haplotype3	R	F		G			H					K
DBL-TH4	Thailand Haplotype4	R	F		G	K	Q	H	K	I	R		K
DBL-TH5	Thailand Haplotype5	R		E	G		N		K	I	R		K
DBL-TH6	Thailand Haplotype6	R			G		H						
DBL-TH7	Thailand Haplotype7	R			G								
DBL-TH8	Thailand Haplotype8	R	F										
DBL-TH9	Thailand Haplotype9	R	F							I	R		K

B

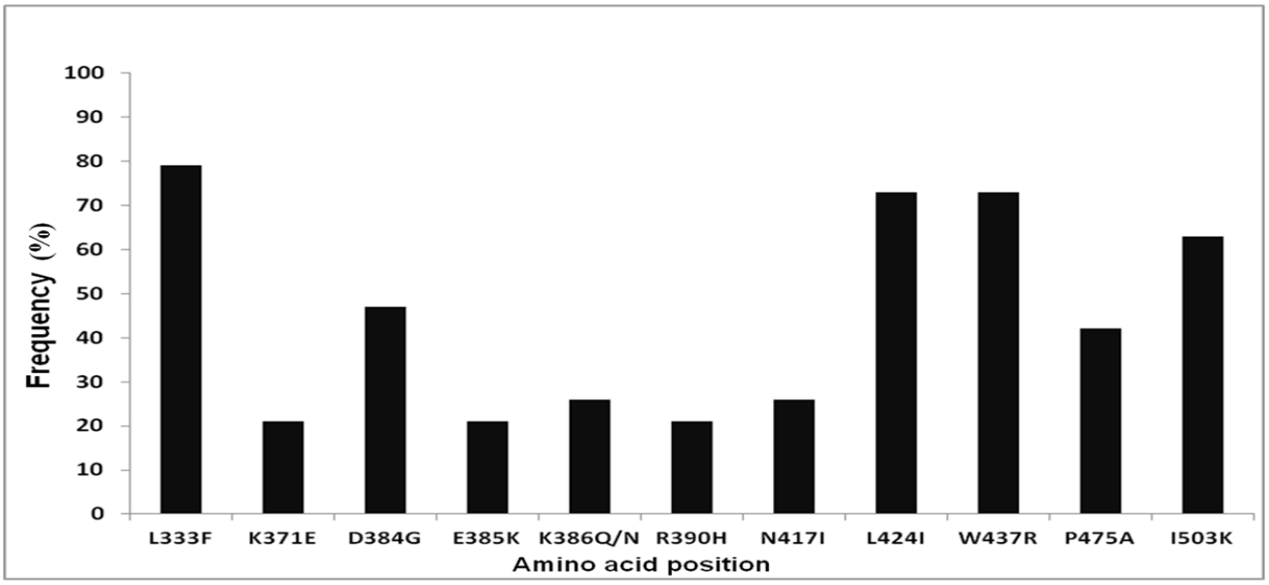


Fig 2. Phylogenetic analysis. The phylogenetic tree of the 9 Thai DBPII haplotypes was constructed with a neighbor-joining method using MEGA4 program. Numbers of the branches indicate bootstrap proportion (1000 replicates). The novel Thailand DBPII haplotypes are marked with asterisks.

Fig 3. Determination of 50% inhibitory concentration (IC50) of anti-DBPII monoclonal antibodies. Monoclonal antibodies 3C9, 2D10, 2C6 and 2H2 specific to PvDBPII 7.18 were tested for their inhibitory function against the homologous PvDBPII 7.18 haplotype. The transfected COS7 cells expressing PvDBPII 7.18 allele were incubated with monoclonal antibodies at different concentrations and with human erythrocytes. The number of rosettes was compared between wells of transfected cells incubated with monoclonal antibodies relative to wells without antibodies. Curves represent mean percent inhibition of two experiments tested in triplicate. Dotted line shows point on curve where IC50 of each antibody is achieved.

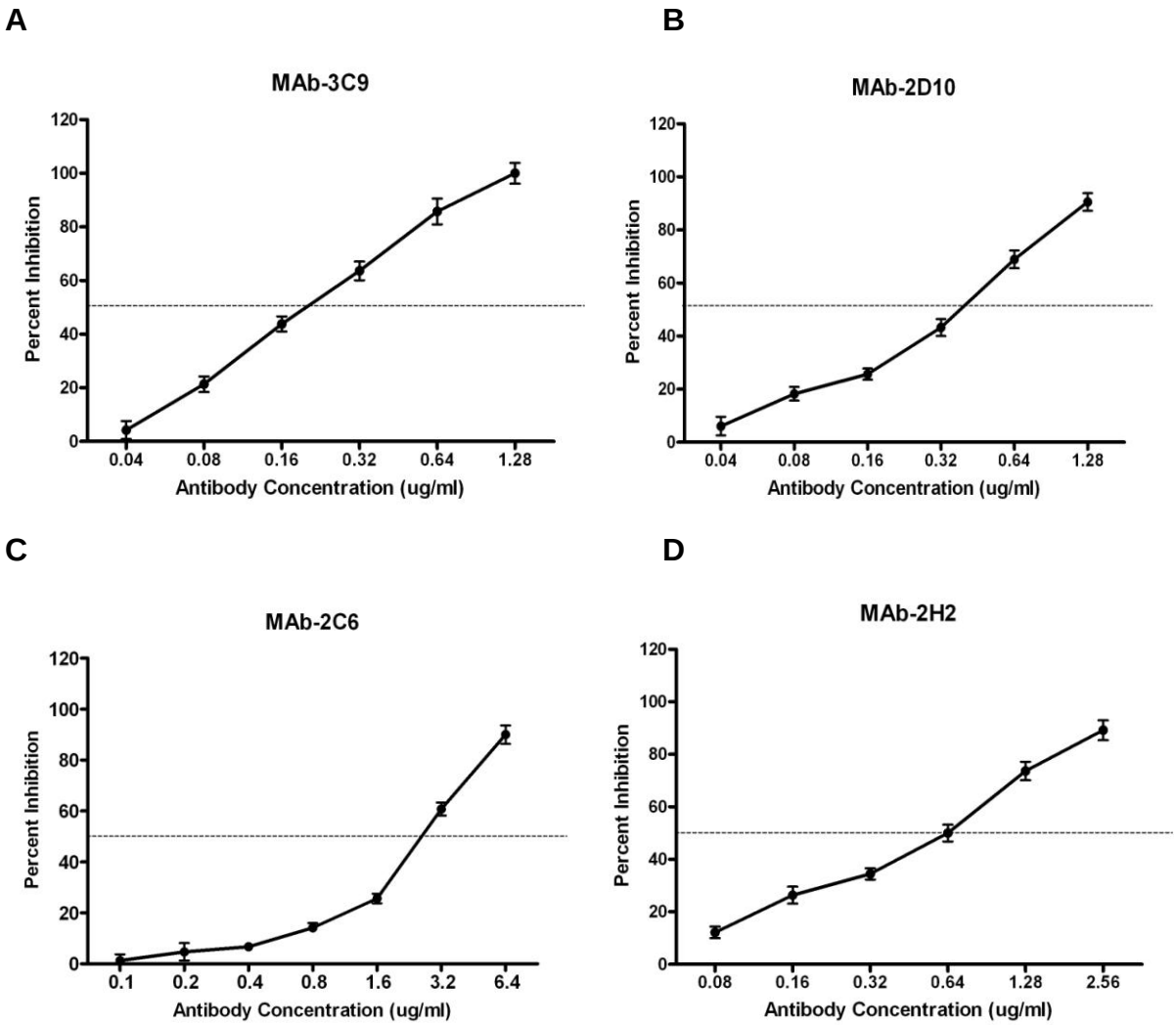
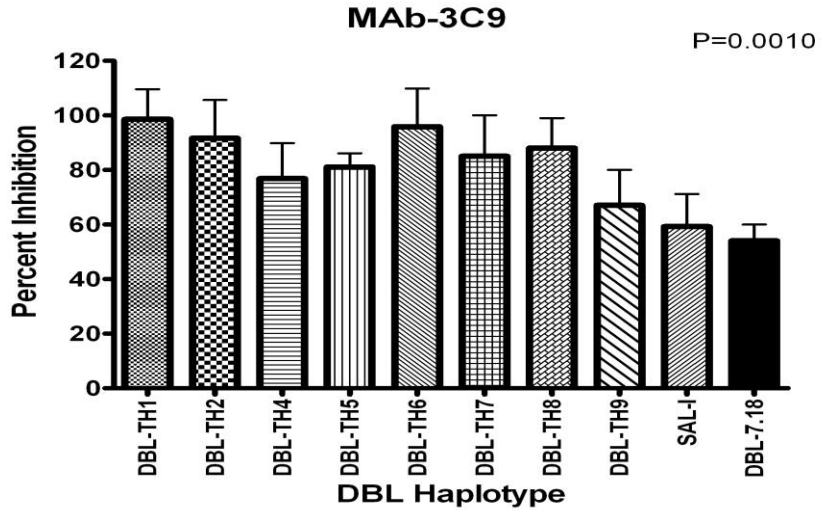


Fig 4. Functional inhibition of anti-DBPII monoclonal antibodies against the panel of DBL-TH variants. The transfected COS7 cell expressing DBPII reference Sal I or 7.18 or DBL-TH variants were pre-incubated with monoclonal antibodies at concentrations set to the IC50 of the DBP7.18 haplotype antigen for inhibition of DBPII-erythrocyte binding. The charts show the mean inhibition of each DBL-TH variant. The variation in means across DBL-TH variants shows significant differences in the inhibitory responses of 3C9, 2D10 and 2C6 monoclonal antibodies. 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).

A



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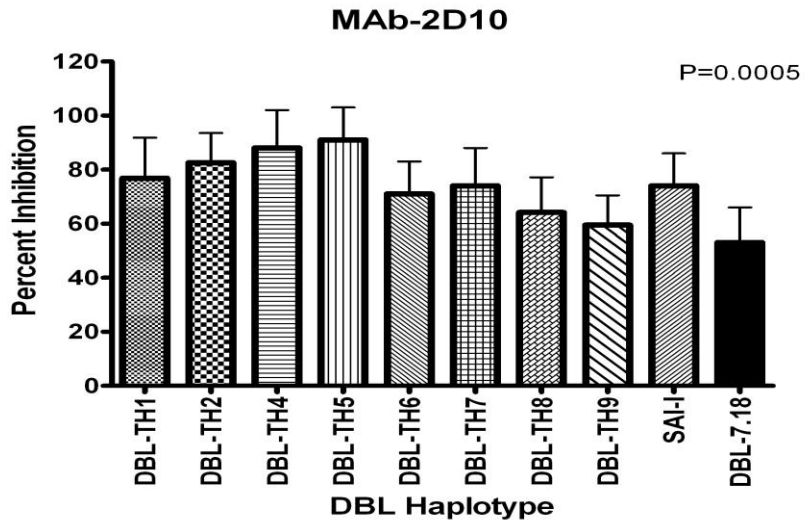

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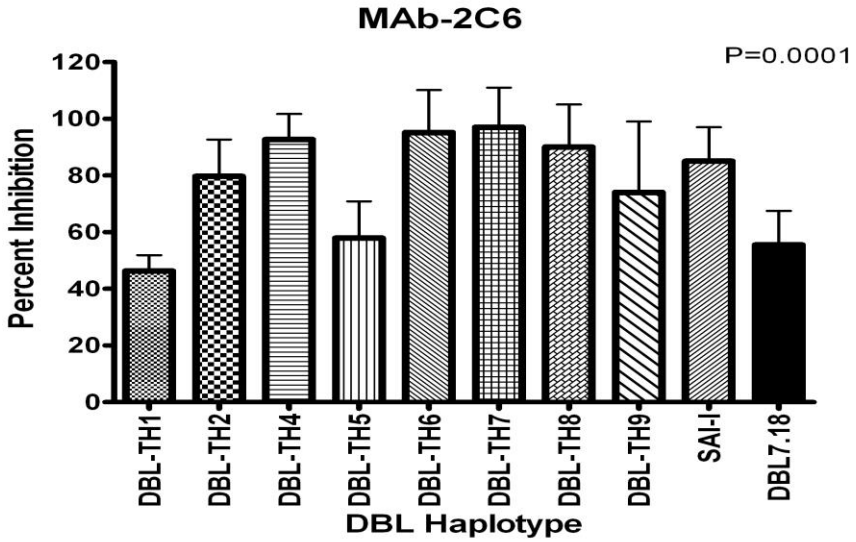
MAb-2C6
P=0.0001
Percent Inhibition
120
100
80
60
40
20
0
DBL-TH1
DBL-TH2
DBL-TH4
DBL-TH5
DBL-TH6
DBL-TH7
DBL-TH8
DBL-TH9
SAI-I
DBL7.18
DBL Haplotype

D

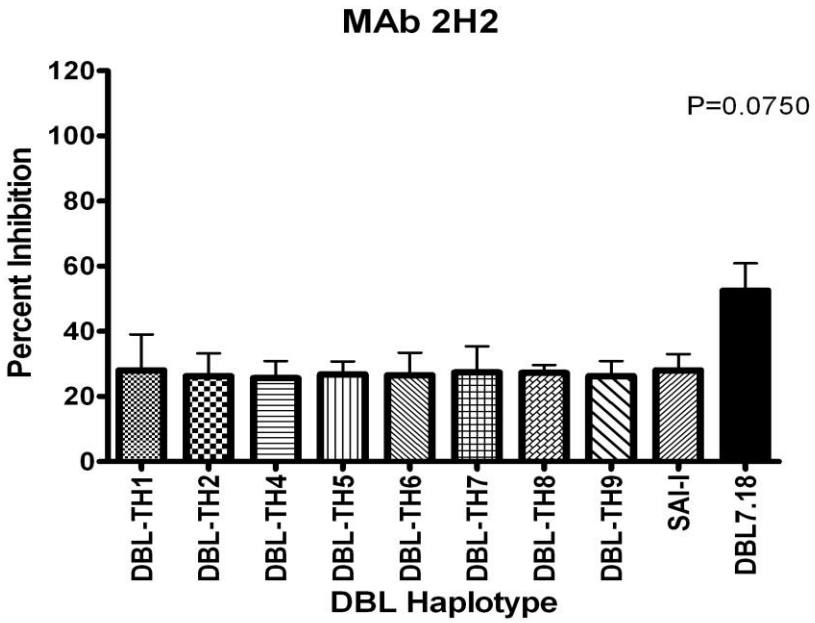
MAb 2H2
P=0.0750
Percent Inhibition
120
100
80
60
40
20
0
DBL-TH1
DBL-TH2
DBL-TH4
DBL-TH5
DBL-TH6
DBL-TH7
DBL-TH8
DBL-TH9
SAI-I
DBL7.18
DBL Haplotype

Figure1A

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Reference Name	Amino acid position →	308	333	371	384	385	386	390	417	424	437	475	503
Sal I	Sal I	R	L	K	D	E	K	R	N	L	W	P	I
DBL-7.18	AAL79051.1	S			G		Q		K	I	R		K
DBL-TH1	Thailand Haplotype 1	R	F							I	R	A	K
DBL-TH2	Thailand Haplotype2	R	F	E	G	K	Q		K	I	R		
DBL-TH3	Thailand Haplotype3	R	F		G			H					K
DBL-TH4	Thailand Haplotype4	R	F		G	K	Q	H	K	I	R		K
DBL-TH5	Thailand Haplotype5	R		E	G		N		K	I	R		K
DBL-TH6	Thailand Haplotype6	R			G		H						
DBL-TH7	Thailand Haplotype7	R			G								
DBL-TH8	Thailand Haplotype8	R	F										
DBL-TH9	Thailand Haplotype9	R	F							I	R		K

Figure1B

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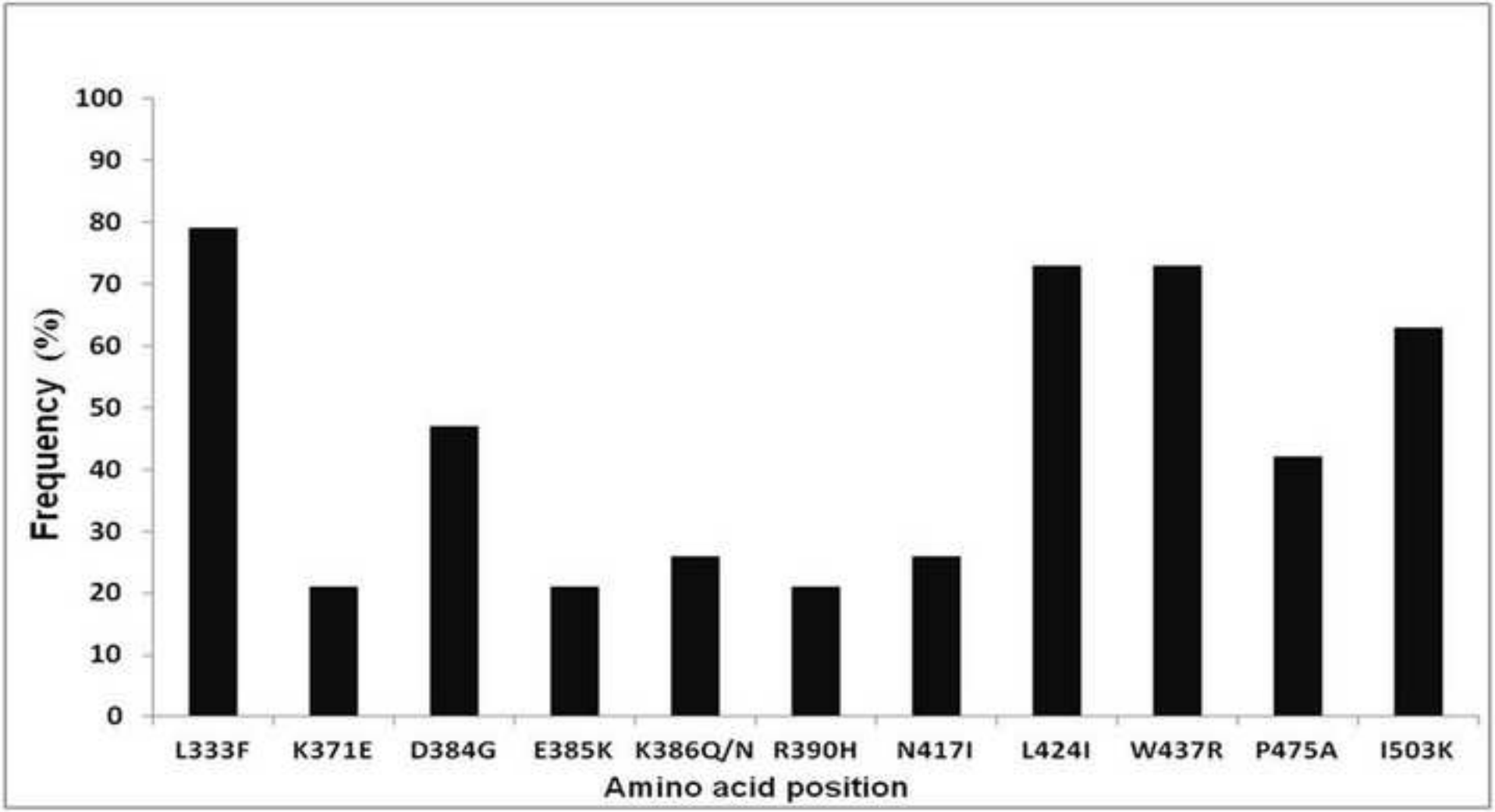

Figure2

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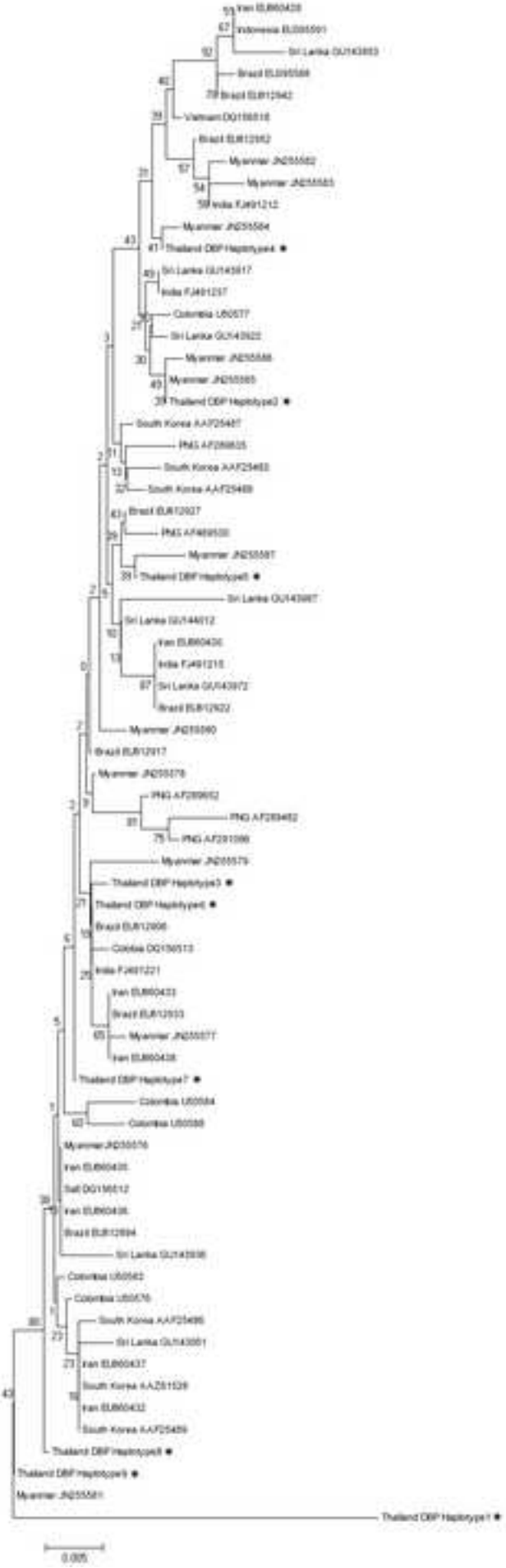

Figure3A

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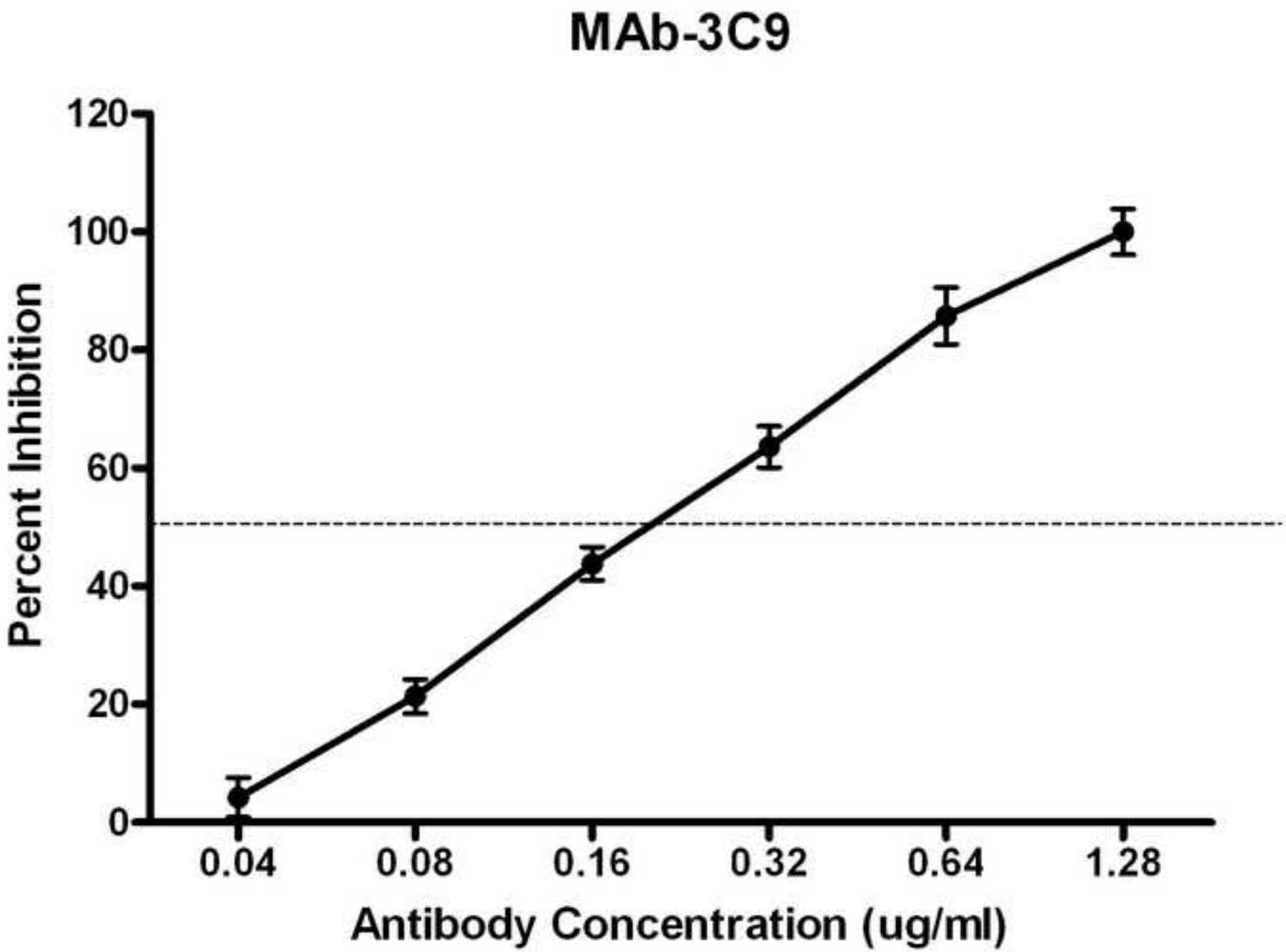

Figure3B

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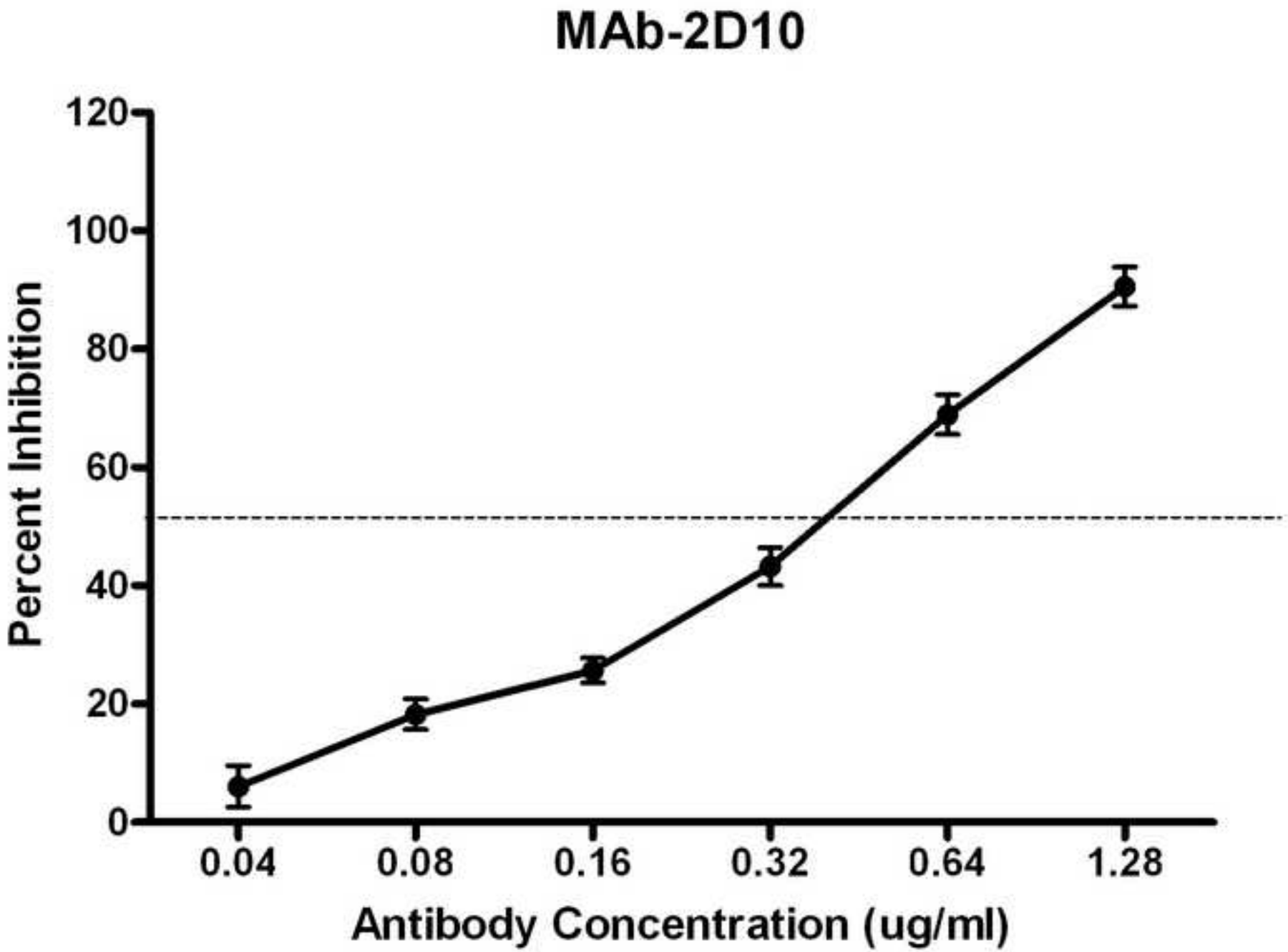

Figure3C

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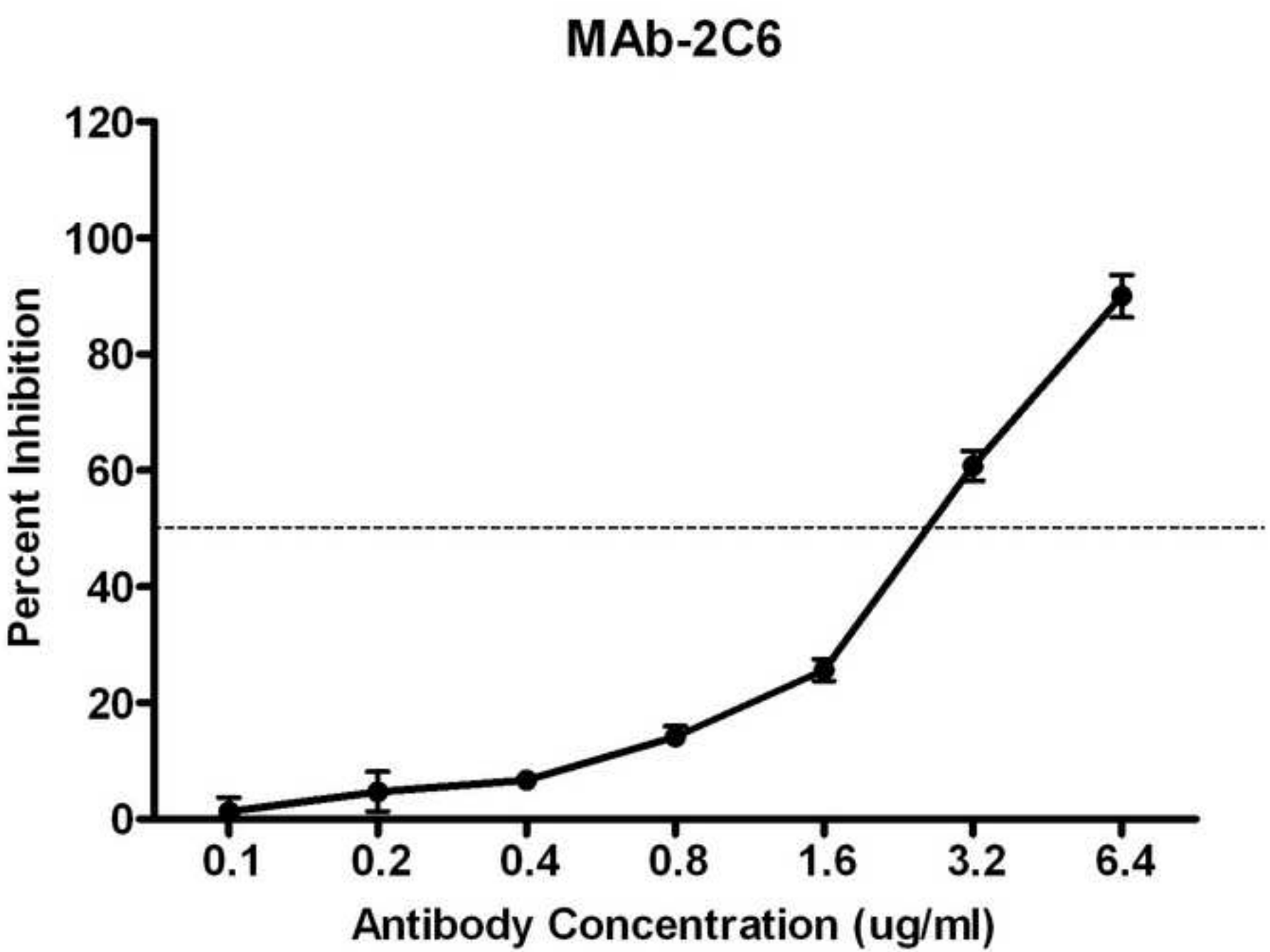

Figure3D

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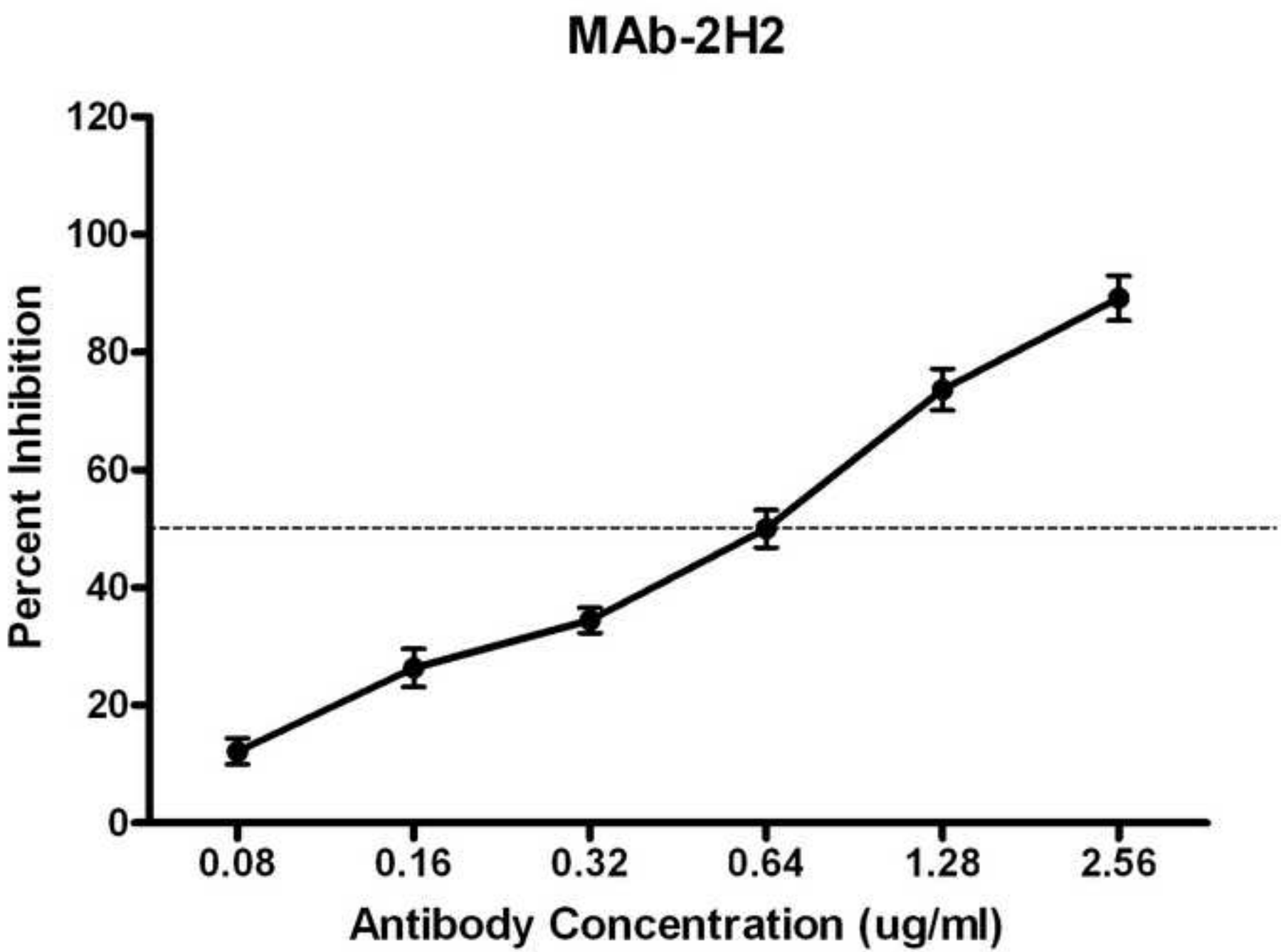

Figure4A

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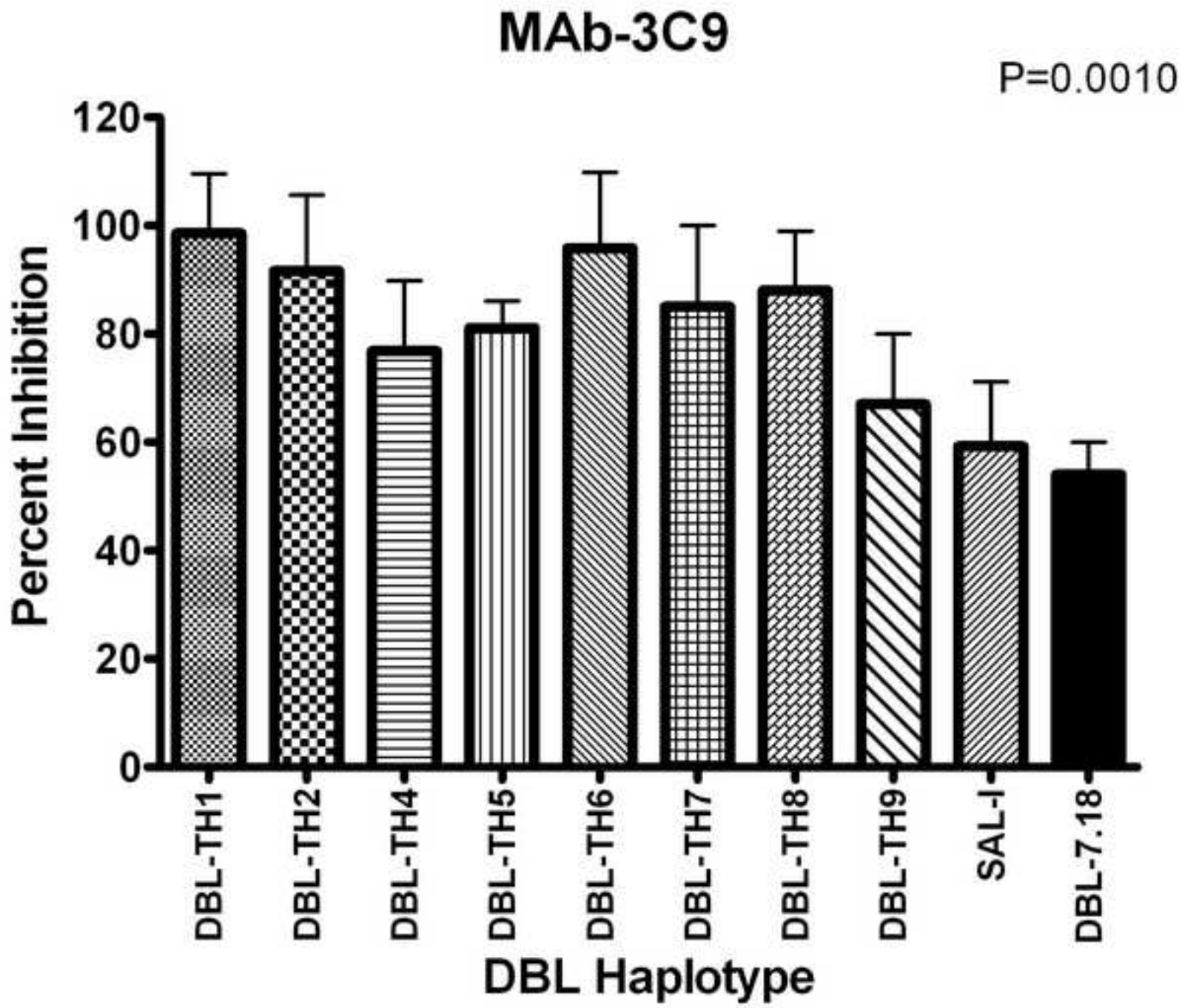

Figure4B

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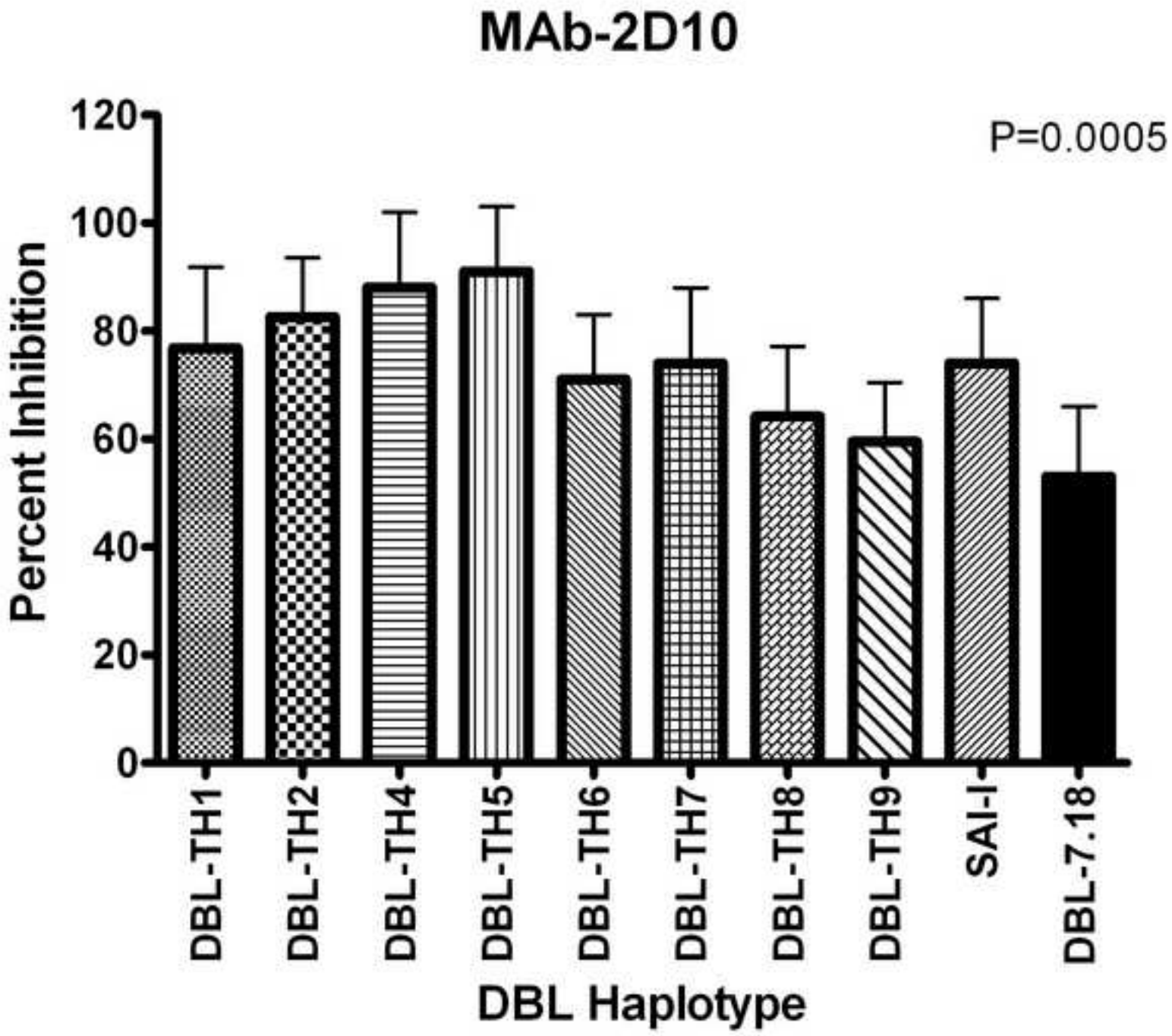

Figure4C

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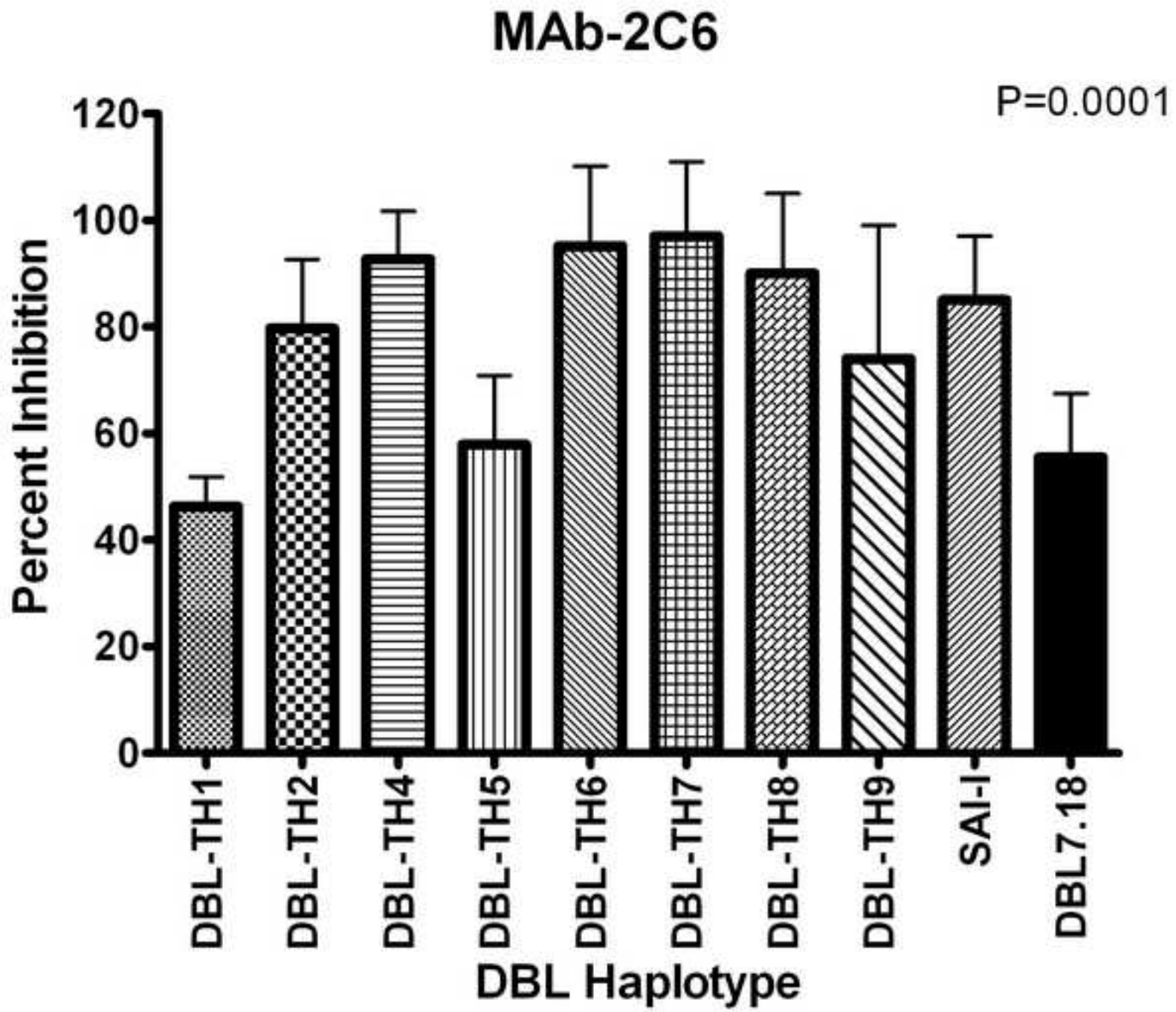

Figure4D

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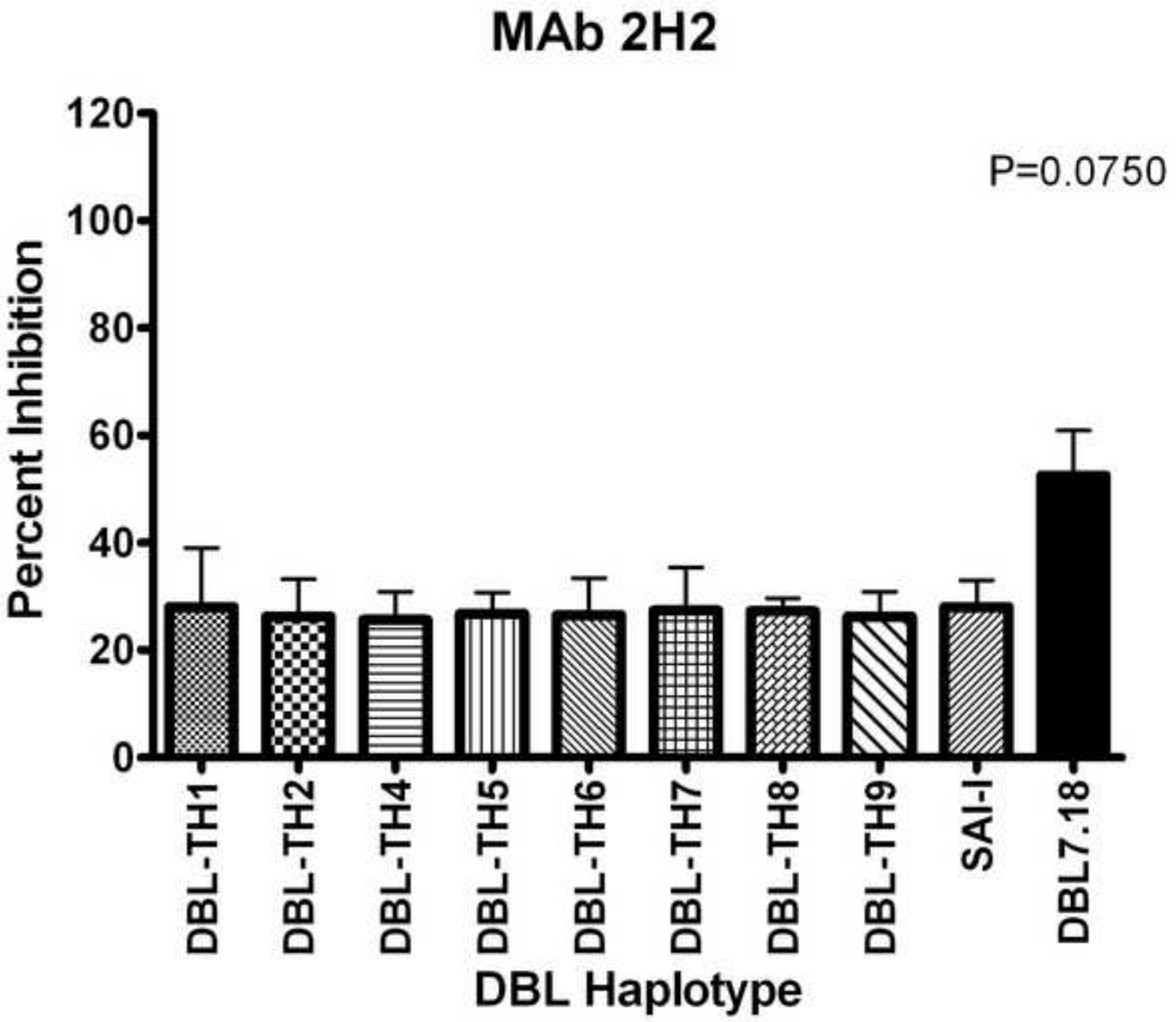

Graphical abstract (for review)

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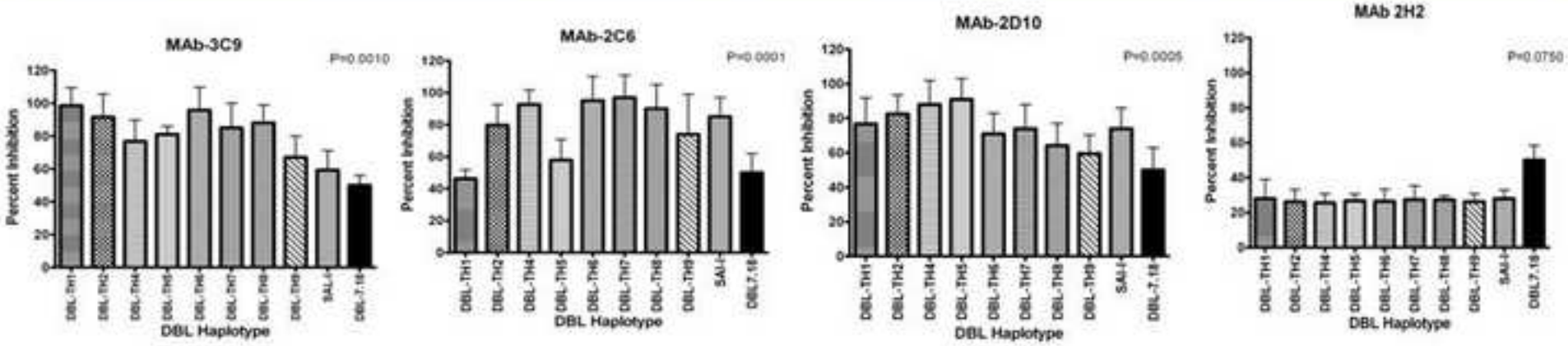
Inhibitory Function of Anti-DBPII Neutralizing Antibody Against Thai DBPII Variants



Genetic Polymorphism of *Plasmodium vivax* Duffy Binding Protein II Among Thai Isolates

Amino Acid Position / Reference Name	308	333	371	384	385	386	390	417	424	437	475	503
Sal I	R	L	K	D	E	K	R	N	L	W	P	I
DBL7.18	S			G		Q		K	I	R		K
DBL-TH1	R	F							I	R	A	K
DBL-TH2	R	F	E	G	K	Q		K	I	R		
DBL-TH3	R	F		G			H					K
DBL-TH4	R	F		G	K	Q	H	K		R		K
DBL-TH5	R		E	G		N		K	I	R		K
DBL-TH6	R			G		H			I			
DBL-TH7	R			G								
DBL-TH8	R	F										
DBL-TH9	R	F							I	R		K

The Association of Duffy Binding Protein II Polymorphisms in *Plasmodium vivax* Isolates from Thailand

Patchanee Chootong[1], Amy M. McHenry[2], Francis B Ntumngia[3], Jetsumon Sattabongkot[4], and John H Adams[3]

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Department of Biology, Southwestern Adventist University, Texas, USA

Global Health Infectious Disease Research Program, University of South Florida, Tampa, Florida, USA

Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

Plasmodium vivax Duffy Binding Protein II (DBPII) plays an important role in reticulocyte invasion and is a potential vaccine candidate against vivax malaria. However, polymorphism of DBPII is a potential challenge for the successful design of broadly protective vaccine. In this study, the genetic diversity of DBPII among Thai isolates were analysed from Thai *P. vivax* infected blood samples and polymorphism characters were defined by MEGA4 program. Sequence analysis identified 12 variant residues that are common among Thai DBPII haplotypes with the variant D384G, L424I, H437 and I503K having the highest frequency. The variant D384K occurs in combination with either E385K or K386N/Q. Moreover, the variant L424I occurs in conjunction with W437R in most Thai DBPII alleles and these variants frequently occur in combination with variant I503K. The polymorphic patterns of Thai vivax isolates are defined into 9 haplotypes, Thai DBL-1, -2, -3, -4, -5, -6, -7, -8 and -9. Thai DBL-2,-5, -6 are the most common DBPII variants. To study the association of Thai DBPII polymorphisms in the expression of antigenic character, the functional inhibition of anti-DBPII monoclonal antibodies against a panel of Thai DBL-2,-5,-6 variant was characterized by *in vitro* erythrocyte binding inhibition assay. The functional inhibition of anti-DBPII against variant Thai DBL 2,-5,-6 was significantly different. Our results indicate that amino acid mutation in variant Thai DBP-2, -5 and -6 change antigenic characters suggesting that the variant epitopes act as a target of anti-DBPII inhibitory antibodies. Therefore, to design protective vaccine in Thai residents should contain the variant B-cell epitope represent in Thai DBPII haplotypes.

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08036 Barcelona (Spain)
Tel.: 0034 93 227 5400 (ext. 42.01)
Email: anne-sophie.gresle@cresib.cat

Barcelona, 7 May 2013

REF.: Dr. Patchanee Chootong Visa Request

To whom it may concern,

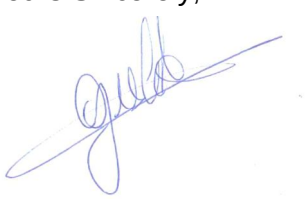
I am writing to you on behalf of Dr. Ivo Mueller, chair of the scientific organizing committee, regarding the attendance of Dr. Patchanee Chootong to **"the Advances in Plasmodium vivax Malaria Research"**, an international conference that will be organized on 28-29 May, and directly followed by **"Assessing the Plasmodium vivax Research Agenda: Six Interdisciplinary Workshops"**, on 30 May. Both events will take place in Barcelona, at the Cosmocaixa facilities.

Plasmodium Vivax (P.vivax) Malaria accounts for approximately 250 million clinical cases and nearly 1 million deaths annually, mostly in children, and in 109 countries. There has been growing evidence that *P. vivax* is the most widely distributed malaria parasite, responsible for a significant burden of global disease and accounting for the majority of malaria cases in Latin America and Asia. However, despite the increasing awareness, the overall burden, economic impact, and severity of *P. vivax* disease is still underestimated.

This 2-day conference plus 6 Workshops will convene a global community of researchers to discuss *P. vivax* molecular biology and genomics, host-parasite interactions, novel research techniques (e.g., 'omics) to overcome barriers to in vitro, in vivo, and clinical study of *P. vivax*; drug resistance and drug discovery; and recent clinical trial and in-field efforts in *P. vivax* prevention, treatment, control, and elimination.

Given that Dr. Patchanee Chootong will be presenting a poster during the conference and taking into consideration the need of a wide geographical representation, we consider his participation in this meeting as critical.

Yours Sincerely,



Anne-Sophie Gresle
Conference Coordinator