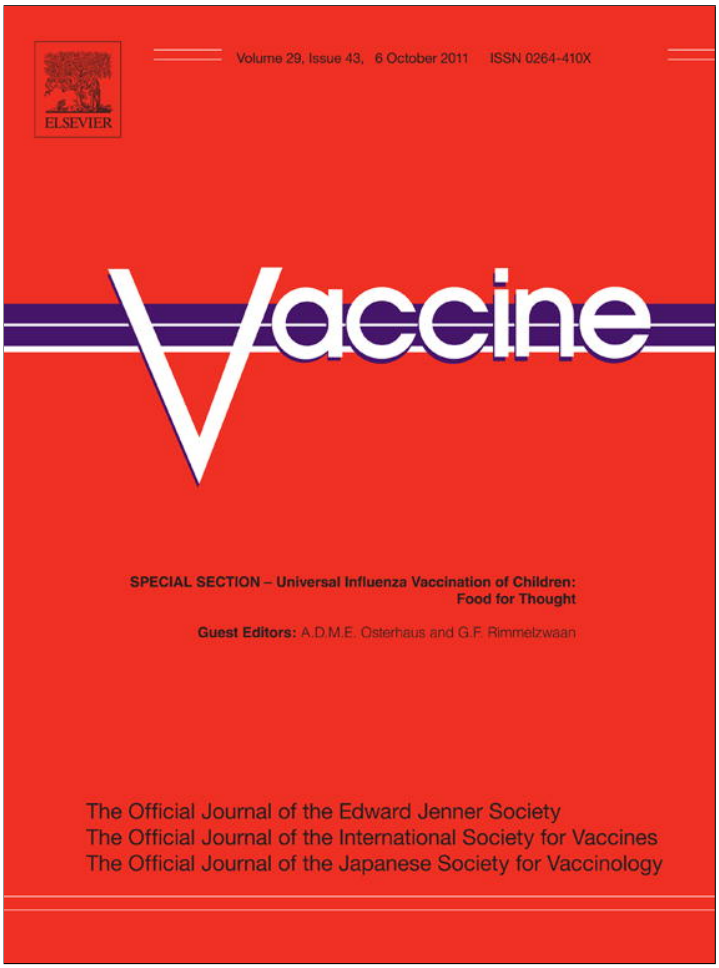

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Evaluation of the genetic diversity of domain II of *Plasmodium vivax* Apical Membrane Antigen 1 (PvAMA-1) and the ensuing strain-specific immune responses in patients from Sri Lanka

Sajani Dias[a], Maheshika Somarathna[b], Aresha Manamperi[c], Ananias A. Escalante[d], Anusha M. Gunasekera[e], Preethi V. Udagama[a,*]

[a] *Department of Zoology, Faculty of Science, University of Colombo, No 94, Cumaratunga Munidasa Mawatha, Colombo 03, Sri Lanka*
[b] *Institute of Biochemistry Molecular Biology and Biotechnology, University of Colombo, Colombo 03, Sri Lanka*
[c] *Molecular Medicine Unit, Faculty of Medicine, University of Kelaniya, Ragama, Sri Lanka*
[d] *Center for Evolutionary Medicine & Informatics, The Biodesing Institute, Arizona State University, Tempe, AZ, USA*
[e] *Sanaria Inc., 9800 Medical Center Drive, Suite A209, Rockville, MD 20850, USA*

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ABSTRACT

Antigenic polymorphism displayed by malaria parasites is a skewed schema to escape the host immune system. The prevailing genetic diversity at domain II of the *Plasmodium vivax* Apical Membrane Antigen-1 (*Pvama-1DII*) was characterized in 64 single clone *P. vivax* isolates from Sri Lanka, where unstable malaria prevails with low intensity.

In Sri Lanka, the *Pvama-1DII* gene showed meager meiotic recombination with the enclosure of single nucleotide polymorphisms (SNPs). Eleven amino acid (a.a.) variant positions defined 21 a.a. haplotypes with 9 unique to the island, where the predominant haplotype, H1, was identical to the reference Salvador I strain. A further 376 globally dispersed isolates defined 38 a.a. haplotypes (H22–H59), with 4 and 26 haplotypes exclusive to India and Thailand, respectively. The phylogenetic tree revealed no clustering, where most isolates had a very recent common origin.

The polymorphism detected in PvAMA-1DII B and T cell epitopes evidenced an immune evasion mechanism exploited by the parasite. Majority of Sri Lankan patients developed antibody responses to both conformational and linear B cell epitopes.

The ensuing strain-specific immunity due to extensive antigenic polymorphism was evaluated by aligning a.a. sequences of PvAMA-1DII with the homologous total (IgM + IgG) antibody responses assayed by in-house established indirect ELISAs against 7 PvAMA-1DII overlapping synthetic peptides, P01–P07. While the antibody responses to P01–P03, P06, P07 harbouring *P. vivax* clinical isolates with polymorphic a.a. haplotype to Sal I was clearly strain-transcending (cross-reactive), individuals with isolates identical to the Sal I strain observed varying antibody prevalence against the seven PvAMA-1DII Sal-I synthetic peptides, with the highest prevalence detected against P04.

Synthetic peptide P04, spanning a.a. positions 302–324 of the PvAMA-1DII of the Sal I strain that included the epitope recognized by the invasion inhibitory 4G2 monoclonal antibody of PfAMA-1, was highly conserved in all 440 local and global *P. vivax* isolates examined. A functional role for this region is reinforced by the highly immunogenic nature of P04, and could point towards a presumably "protective" anti-P04 antibody response that elicited an isotype switch from IgM to IgG, with increasing exposure to malaria exclusively in endemic residents. Thus the conserved and seemingly "protective" nature of the domain II loop of PvAMA-1 makes it a putative contender to be included in a cocktail vaccine against *P. vivax* asexual erythrocytic stages in Sri Lanka.

1. Introduction

Malaria caused by *Plasmodium vivax* accounts for 25–40% of the 515 million annual cases of malaria worldwide [1] and nearly 2.5 billion people, mostly outside Africa, are at risk from *P. vivax* infection [2]. In Sri Lanka, the majority of the reported malaria cases (72–96%) is due to *P. vivax* [3]. According to the World Health

* Corresponding author. Tel.: +94 11 2503399; fax: +94 11 2503148.
E-mail address: dappvr@yahoo.com (P.V. Udagama).

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Organization, Sri Lanka entered the malaria pre-elimination stage in 2008, where from 2000 until 2007, a dramatic decrease in *P. vivax* cases was reported [4].

Studies on population diversity of malaria parasites have practical significance on the development for strategies of disease control, including vaccine development [5]. The primary factors affecting genetic diversity at such loci are natural selection [6], and genetic drift [7]. The knowledge on the latter together with genetic diversity which reflects on the population history is important to fully understand the epidemiology of potential malaria vaccine candidates in their diverse natural populations.

An effective malaria vaccine should display protective immune responses that either destroy the parasite or its infected host cell, or inhibit a function that is crucial to its survival [8]. *Plasmodium* merozoites are a major target of blood-stage malaria vaccine development as they are uniquely exposed to the host immune system. Inhibition of erythrocyte invasion by merozoites would in principle block the erythrocytic stage of the parasite life cycle and prevent clinical manifestations [9]. One such antigen that shows promise as a vaccine candidate is the Apical Membrane Antigen-1 (AMA-1), a protein secreted from micronemes and associated with the parasite surface during invasion, involving in the tight interaction between the host cell and the parasite surface, called the "moving junction" [10]. As antigenic diversity is one of the main mechanisms used by malaria parasites to evade the host immune system, detailed genotyping to elucidate the alleles found in the population and to determine which of the polymorphisms allow evasion of vaccine induced protective immune responses, may be useful for the development of a future AMA-1 based vaccine.

In Sri Lanka, under low transmission and unstable malaria conditions, the signature of diversifying selection was seen most strongly in domain II of *Pvama-1* [11]. Therefore, using comparative sequence analysis, the level of genetic diversity and potential natural selection at domain II of *Pvama-1* in field isolates from Sri Lanka was analyzed. These were further compared with sequences of global isolates and evolutionary relationships traced. Moreover, in order to detect population sub division at this locus, the differences in allele frequencies among diverse geographic regions world wide were analyzed. Further, as point mutations due to genetic diversity can alter the sequences coding for B or T-cell epitopes of malarial antigens [12], the resultant polymorphisms acting on the predicted B and T cell epitopes were recognized with altering binding scores at this locus in the Sri Lankan test population.

An immuno-epidemiological study in Sri Lanka [13] revealed 45% non-responsiveness to recombinant PV66/AMA-1, representing the native PvAMA-1 ectodomain, in residents from areas endemic to malaria. A plausible explanation may be that, due to the high polymorphism in merozoite antigens, antibodies induced against one particular allelic form may not be effective in controlling growth of parasites expressing alternative forms [14]. Therefore, an immunological assay was carried out to analyze the most immunogenic region within the PvAMA-1 domain II and to analyze the ensuing strain-specific immune responses generated due to different alleles among PvAMA-1 domain II in *P. vivax* infected Sri Lankan individuals.

2. Materials and methods

2.1. Study population

This study was approved by the Ethics Review Committee of the University of Colombo, Sri Lanka, (EC/04/103). Following informed consent, 5 ml of blood from age and gender matched patients with microscopically confirmed *P. vivax* infections were collected. Patients were recruited for the study from two malaria endemic areas, the General Hospital, Anuradhapura (8°22′N, 80°20′E, $N = 52$) and the Malaria Research station, Kataragama (6°25′N, 81°20′E, $N = 91$) and from a non-endemic area, the National Hospital Sri Lanka in Colombo (7°55′N, 79°50′E, $N = 58$), presented during December 1998–March 2000 [15]. Those patients from Colombo contracted the disease during occupational visits to *P. vivax* endemic areas of the country [16–18]. Parasitemia ranged from 0.0001 to 0.025%.

2.2. Amplification and sequencing of Pvama-1 domain II

Parasite DNA was extracted from 5 ml of venous blood as previously described [11]. Each of the DNA samples were genotyped at the polymorphic *Pvmsp-3α* locus using a combined nested polymerase chain reaction (PCR) and restriction fragment length polymorphism to select out single clone infections [15].

The selected single clone isolates were amplified at the domain II locus of *Pvama-1* (792 bp) as described previously [11]. The amplified products resolved on 1% agarose gels were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and subjected to sequencing at Macrogen, Korea. Some isolates were subjected to 4-fold sequence coverage to confirm the polymorphisms observed.

2.3. Data analysis

The consensus sequences were generated and aligned using the Seqman II (DNAStar, Madison, WI) and ClustalW of MEGA 4.1 [19] software, respectively.

Three measures of genetic polymorphism were used at *Pvama-1DII* in the Sri Lankan local population; polymorphic sites (S), nucleotide diversity (π) and haplotype diversity (h) [DnaSP 4.50; 20]. Natural selection was determined by the difference between the non-synonymous and synonymous substitutions ($Dn - Ds$) estimated by Nei and Gojobori's method (MEGA 4.1 [19]) and the McDonald Kreitman test using a single *P. cynomolgi* sequence as the out group [20]. Statistical analyses of recombination and linkage disequilibrium (LD) were tested by DnaSP 4.50 [20].

A phylogenetic tree constructed by MEGA 4.1 software was used to investigate the relatedness of *ama-1* alleles in different parts of the world, while Fixation indices (F_{ST}), which is the measure of the proportion of overall allele diversity attributable to differences in populations, were calculated using DnaSP 4.50 software [19,20].

The differential binding of linear B and T cell epitopes spanning domain II of PvAMA-1, related to a.a. polymorphism of this domain, were predicted by Bcpred Server 1.0 [21] and ProPred MHC class II binding peptide server [22], respectively, as described previously [18].

2.4. Serum samples

One hundred and sixty-two serum samples positive for total (IgM + IgG) anti-AMA-1 antibodies assayed against the recombinant PvAMA-1 protein (PV66/AMA-1) representing the Salvador I strain from the three study areas, Colombo ($N = 56$), Anuradhapura ($N = 39$) and Kataragama ($N = 67$) [13,23], were used to assay the total (IgM + IgG) and isotype specific antibody responses against peptides representing domain II of PvAMA-1. Residents in Colombo ($N = 30$) who were previously not exposed to malaria and were not experiencing any illness at the time, were used as healthy normal controls. Malaria episodes are routinely confirmed by thick or thin blood smear microscopy prior to treatment, the self-reported number of previous malaria infections (including *P. vivax* and *P. falciparum*) was used to obtain the previous cumulative exposure to malaria of each patient [24].

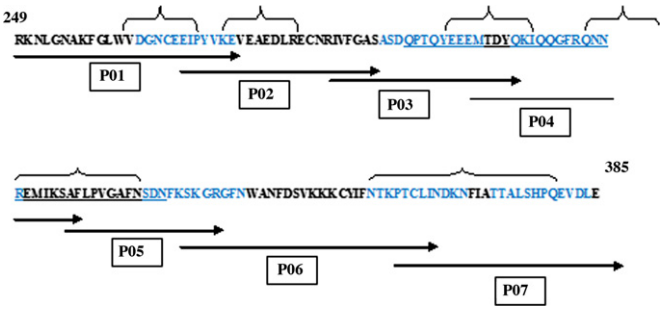

Fig. 1. Schematic representation of the seven overlapping synthetic peptides of PvAMA-1 domain II. Amino acid sequences highlighted in blue and in brackets correspond to predicted linear B and T cell epitopes, respectively. Underlined sequence represents the domain II loop [29].

2.5. Synthetic peptides

Seven overlapping synthetic peptides (P01–P07), were designed to represent domain II of PvAMA-1 Salvador I strain (Fig. 1) with predicted linear B and T cell epitopes [21,22]. These were synthesized by Mimotopes Private Limited, Australia. Lyophilized peptides were dissolved in nuclease-free water, dilute acetic acid or dilute aqueous ammonia according to their solubility.

2.6. Analysis of the antibody responses to synthetic peptides

2.6.1. IgM + IgG indirect microplate ELISA

The total (IgM + IgG) antibody responses against the seven synthetic peptides were assayed by indirect microplate enzyme linked immunosorbant assay (ELISA) as previously described [13] with modifications. Flat-bottom Immulon-2HB ELISA plates (Dynatech Laboratories, USA) were coated at 4 °C overnight with 100 μl of capture antigen (P01: 4.5 μg/ml, P02: 3.5 μg/ml, P03: 8 μg/ml, P04: 10 μg/ml and P05: 3 μg/ml, P06: 4 μg/ml and P07: 6 μg/ml) in 1X phosphate buffer (PB, pH 7.4) and then washed with 1X PBS (1 M NaCl) and 0.2% Tween-20 (PBS-T). The uncovered reactive sites in the wells were blocked by incubation for 2 h at room temperature (RT) with a 5% solution of non-fat dried milk powder in PBS. All sera to be tested were diluted 1/50 (P01, P02, P03, P04 and P06) and 1/25 (P05 and P07) in PBS-T containing 5% nonfat dried milk powder and incubated in the capture antigen-coated wells for 2 h at 37 °C in a humid chamber. The unbound antibodies were washed off with PBS-T. Horseradish peroxidase (HRP) linked rabbit anti-human IgM and IgG antibody (DAKO Immunoglobulins, Denmark) conjugate was used (P01-1:1250, P02-1:1500, P03-1:2250, P04-1:2500, P05-1:2500, P06-1:2000 and P07-1:750) as the second antibody. The plates were incubated 1 h at 37 °C and then washed with PBS-T. The enzyme reaction was developed with 1 mg/ml O-phenylenediamine dihydrochloride (Sigma Chemicals, USA) as the chromogen in substrate buffer with 0.03% hydrogen peroxide. After 15 min at RT the reaction was stopped with 50 μl of 2 N sulfuric acid, the reaction product in the wells was recorded by using a Microplate reader at OD_{490} (Model 680, BioRad International, USA.).

2.6.2. IgM and IgG isotype specific antibody sandwich ELISA

Serum of responders for total anti-P04 and anti-P05 antibodies from each test area was used to assay for IgM and IgG isotypes [13]. Microtiter plates were coated with 50 μl antigen (P04, 14 μg/ml for IgM and IgG; P05, 15 μg/ml for IgM and 11 μg/ml for IgG) in 1X PB and blocked by incubation 2 h at RT in a humid chamber. Serum samples to be tested were diluted 1/15 in PBS-T (1 M NaCl, 1% Tween 20) containing 5% non-fat milk powder and incubated for 2 h at 37 °C. Isotype-specific mouse anti-human monoclonal antibodies (Sigma Chemicals, USA) were used at dilutions of 1:1500 and 1:3500 for IgM P04 and P05, respectively, and 1:750 for both IgG P04 and P05, and incubated overnight at RT. Affinity purified HRP linked goat anti-mouse IgG (Sigma Chemicals, USA) (IgM-P04 1:1500, P05 1:3000; IgG-P04 1:600 and P05 1:750) was added to each well and incubated at 37 °C for 1 h. The enzyme reaction was developed and measured as described above, where the colour reaction was allowed to proceed for 30 min at RT.

The cutoff value for a positive response was defined as an OD greater than the mean plus 2 SD of sera from 30 human donors who had never been exposed to malaria.

2.7. Statistical analysis

Statistical analysis of data was performed using SPSS evaluation version 15 for windows (SPSS Inc., USA) and Epi Info 6 (version 6.04b to c upgrade; CDC, USA and WHO, Switzerland). Proportions of responders (both total and isotype specific) for independent samples were tested using the Chi squared test. Antibody magnitudes of independent variables were compared using the *T*-test, ANOVA, and the Wilcoxon Signed Rank test as appropriate. Dependable variables were tested using the paired *T*-test and Kruskal–Wallis test. Associations between antibody responses and host factors were tested by using bivariate correlations (Spearman and Pearson correlation coefficients), and Chi square for linear trends as appropriate. The significant level was set at $P < 0.05$.

3. Results

Sixty-four *Pvama-1* domain II DNA sequences were examined in this study, of which 23 were previously reported [11], while the rest of the 41 sequences obtained from single clone isolates resulted from this study (submitted to Gene Bank database under the accession numbers GU433899–GU433939).

3.1. Polymorphism, evidence of selection, recombination and genetic differentiation at Pvama-1 domain II in Sri Lanka

The 64 *Pvama-1* sequences (corresponding to 619–1029 bp of the *P. vivax* AMA-1 domain II Salvador I strain [23]) of Sri Lankan isolates revealed 16 polymorphic positions with a single singleton site and 15 parsimony informative sites giving rise to 17 point mutations. The overall nucleotide diversity ($\pi = 0.0095 \pm 0.0005$ S.D.) and the haplotype diversity ($Hd = 0.928 \pm 0.015$ S.D.) in Sri Lanka, was greater than those previously published [25–27]. Analysis with the sliding window option revealed a nucleotide diversity ranging from 0.007 to 0.026, with the maximum diversity observed between nucleotide positions 869 and 1029 representing the latter part of domain II.

The difference between non-synonymous ($Dn = 0.0111 \pm 0.0035$ S.D.) and synonymous ($Ds = 0.0036 \pm 0.0022$ S.D.) mutations in the entire Sri Lankan population demonstrated a significant balancing selection acting on this domain ($Dn - Ds = 0.0075 \pm 0.0043$ S.D.; *Z* test $P < 0.05$) confirming previous observations [11,26]. However, the McDonald–Kreitman test using a single *P. cynomolgi* [Accession no. X86099; 27] sequence as the out group, provided evidence for departure from neutrality where the ratio of non-synonymous to synonymous mutations within *P. vivax* (14/3) was significantly greater than between species (13/31; $P < 0.05$).

The minimum number of recombination events between adjacent polymorphic sites (Rm), the recombination parameter (*C*) between adjacent sites and per gene in the local Sri Lankan population was 4, 0.1483 and 60.8, respectively. Low recombination parameter value may suggest a meager meiotic recombination occurring in this gene in the local population. Analysis of linkage disequilibrium (LD) index (R^2) for the entire local population

Table 1
Amino acid polymorphisms in the *P. vivax* AMA-1 domain II among geographically diverse malaria endemic areas.

Amino acid haplotype	Sri Lanka (*N* = 64)	Philippine	Brisbane	India (*N* = 72)	Venezuela (*N* = 73)	Thailand (*N* = 231)	252	277	288	313	352	368	370	371	380	382	383	384	385
P. vivax AMA-1 Salvador I							G	E	G	R	K	K	F	I	Q	V	D	L	E
H1	11			9	–	8	.	.	.		.	.	.	.	.	.	.	.	.
H2[a]	2			–	–	–	.	.	.		.	I	.	.	.	.	.	.	.
H3	5			2	–	5	.	K	E		.	.	.	.	.	.	.	.	.
H4	5			2	–	–	.	K	E		E	.	.	.	.	.	.	.	.
H5[a]	6			–	–	–	.	.	.		.	.	.	.	K	.	.	P	.
H6	3			1	–	13	.	K	E		N	.	.	.	R	.	.	.	.
H7	1			–	16	–	.	K	.		.	I	.	.	.	.	V	P	.
H8	1			–	–	5	.	K	E		.	.	.	.	.	.	.	P	.
H9	9			1	–	–	E	.	.		.	I	.	.	.	.	.	R	D
H10[a]	1			–	–	–	.	K	.		.	I	.	.	.	.	V	R	D
H11	1			1	–	5	.	.	.		.	.	.	.	.	.	.	P	.
H12[a]	2			–	–	–	.	K	E		E	.	L	.	.	.	.	.	.
H13[a]	6			–	–	–	.	K	.		.	I	.	.	.	.	.	R	D
H14	2			1	6	–	.	K	.		N	I	.	.	.	.	.	P	.
H15[a]	1			–	–	–	E	.	.		.	I	.	.	.	.	V	R	D
H16	3			10	6	–	.	K	.		.	I	.	.	.	.	.	P	.
H17[a]	1			–	–	–	.	.	.		.	.	.	F	.	.	V	.	.
H18[a]	1			–	–	–	.	.	.		.	.	.	.	.	.	V	.	.
H19	1			14	–	6	.	K	.		.	.	.	.	.	.	.	P	.
H20	1			–	–	–	.	K	.		N	.	.	.	R	.	.	.	.
H21	1			–	9	–	.	K	.		.	.	.	.	K	.	.	P	.
H22	–	1		1	–	–	.	K	.		N	.	.	.	.	.	.	P	.
H23	–		1	8	–	31	.	K	.		.	.	.	.	.	.	.	R	D
H24	–			8	–	1	.	K	.		.	.	.	.	.	E	.	P	.
H25	–			4	–	60	.	K	E		.	.	.	.	.	.	.	R	.
H26	–			3	24	2	.	K	.		.	.	.	.	.	.	.	.	.
H27	–			1	1	–	.	K	.		.	I	.	.	.	.	.	.	Q
H28	–			2	–	15	.	K	.		.	.	.	.	.	.	.	R	.
H29[b]	–			1	–	–	.	K	.		E	.	.	.	.	.	.	R	.
H30[b]	–			1	–	–	.	K	E		E	.	.	.	.	.	.	P	D
H31[b]	–			1	–	–	.	K	.		E	.	.	.	K	.	.	P	.
H32[b]	–			1	–	–	.	K	.		.	.	.	.	K	.	.	P	.
H33	–			–	6	1	.	K	.		N	.	.	.	.	.	.	.	.
H34[c]	–			–	–	1	.	K	.	.	.	I			.	.		R	.
H35[c]	–			–	–	1	.	K	E	.	.	.			.	E		R	.
H36[c]	–			–	–	5	.	K	E	.	.	.			R	.		.	.
H37[c]	–			–	–	4	K	.	.	.	.	R			.	.		.	
H38[c]	–			–	–	2	.	K	E	.	N	.			.	.		R	D
H39[c]	–			–	–	2	.	K	E	.	.	.			R	.		P	.
H40[c]	–			–	–	1	.	K	.	.	.	.			.	.		P	D
H41[c]	–			–	–	1	.	K	E	.	N	.			.	.		R	.
H42[c]	–			–	–	1	.	.	.	.	N	.			.	.		P	K
H43[c]	–			–	–	2	.	.	.	.	.	.			.	.		R	.
H44[c]	–			–	–	1	.	.	E	.	.	.			.	.		R	.
H45[c]	–			–	–	1	.	K	E	I	.	.			.	.		P	K
H46[c]	–			–	–	1	E	K	.	.	.	.			R	.		P	Q

H47[c]	–	–	–	2	.	.	.	.	.	.	.	.	R	D
H48[c]	–	–	–	1	.	K	E	.	.	.	.	.	R	D
H49[c]	–	–	–	21	.	K	E	.	.	.	.	E	P	.
H50[c]	–	–	–	2	.	K	E	.	N	.	.	.	P	K
H51[c]	–	–	–	1	.	K	E	.	N	.	.	.	P	.
H52[c]	–	–	–	1	.	K	.	.	.	.	R	.	P	Q
H53[c]	–	–	–	1	.	K	E	.	E	.	.	.	P	.
H54[c]	–	–	–	2	.	.	.	.	.	.	R	.	.	.
H55[c]	–	–	–	1	.	K	E	.	.	I	.	.	P	.
H56[c]	–	–	–	2	.	K	.	.	E	.	.	.	R	D
H57[c]	–	–	–	1	.	K	.	.	N	.	.	.	P	K
H58[c]	–	–	–	16	E	K	E	.	.	.	.	.	P	.
H59[c]	–	–	–	5	.	K	E	.	E	.	.	.	R	.

Amino acid haplotypes H1–H21 are based on the 64 Sri Lankan *P. vivax* clinical isolates sequenced in the current study.

[a] Haplotypes exclusive to Sri Lanka.

[b] Haplotypes exclusive to India [26].

[c] Haplotypes exclusive to Thailand [27].

[*] Amino acid positions of SNPs with respect to domain II of *Pvama-1* Salvador I sequence (accession no Y16950).

indicated equilibrium inter-site linkage with increasing nucleotide distance within the *Pvama-1* sequences.

F_{ST} values, which calculates the genetic differentiation between two areas evidenced a moderate (F_{ST} = 0.1–0.15) genetic differentiation between Sri Lanka/Venezuela (F_{ST} = 0.1472), Sri Lanka/Thailand (F_{ST} = 0.153), India/Thailand (F_{ST} = 0.115) and Venezuela/Thailand (F_{ST} = 0.15), whereas, F_{ST} values between Sri Lanka/India (F_{ST} = 0.0692) and India/Venezuela (F_{ST} = 0.0641) suggested meager genetic differentiation between these geographically dispersed areas.

3.2. *Classification of amino acid haplotypes and phylogenetic analysis of Pvama-1 domain II in Sri Lanka and geographically different countries*

Eleven amino acid (a.a.) variant positions at PvAMA-1 domain II defined 21 a.a. haplotypes from the Sri Lankan cohort, where a.a. haplotype H1, 100% identical to the Salvador I strain, was found to be predominant (17.2%, *N* = 11) in all three test areas (Tables 1 and 2). Haplotypes H1, H3 and H5 were common to all three test areas, while H4 was exclusive to the two endemic areas. Area restricted haplotypes were also detected; Anuradhapura (H2 and H7), Kataragama (H8, H10, H11 and H15) and Colombo (H17–H21) (Table 2). Haplotype H6 was distinctive to isolates from Anuradhapura and Colombo, while H9, H12, H13, H14 and H16 were restricted to Kataragama and Colombo.

Alignment of all available *Pvama-1* sequences (*N* = 376) from Sri Lanka, India, Venezuela and Thailand resulted in 59 amino acid haplotypes (Table 1) and except for a single substitution at a.a. position 313 from R/I in a single isolate from Thailand, all local and global isolates were identical between amino acid positions 295–334 corresponding to the domain II loop of PvAMA-1.

Of the 21 amino acid haplotypes observed from the 64 Sri Lankan clinical isolates, 9 were unique to the island (Table 1), of which 4 (H5, H12, H13 and H20) were previously published [11], while 5 new haplotypes (H2, H10, H15, H17 and H18) are reported for the first time. These 9 unique Sri Lankan haplotypes were encountered from 11 endemic and 10 non-endemic isolates (Table 2). Globally, India (*N* = 4) and Thailand (*N* = 26) produced exclusive haplotypes, while none were unique to Venezuela.

Haplotypes common to different geographical regions worldwide were observed; H1, H3, H6, H11 and H19 among Sri Lanka, India and Thailand; H14 and H16 among Sri Lanka, India and Venezuela; H4 and H9 common to Sri Lankan and India; H7 and H21 to Sri Lanka and Venezuela; H8 restricted to Sri Lanka and Thailand; PH84 from the Philippines was similar to an Indian isolate, and PVQ from Brisbane was similar to 8 and 31 previously published Indian and Thai isolates, respectively. A majority (15%) of the isolates represented H25 that included 3 Indian and 60 Thai isolates (Table 1).

Of the 13 polymorphic amino acid positions identified in this and previous studies [24–26], 4 were trimorphic (K352E/N, Q380R/K, L384P/R and E385Q/D), while the rest were dimorphic (Table 1). A single amino acid change at position 382 found in PV4 and PV12 Indian isolates, from Valine (V) to Glutamate (E) was not recorded in the Sri Lankan and Venezuelan isolates. Moreover, position 382 was the only amino acid position where all the Sri Lankan isolates were similar to isolates from the Philippines (PH84), Brisbane (PVQ) and Sal-I. At positions 370 and 371, where non-synonymous changes occurred from Phenylalanine (F) to Leucine (L) and Isoleucine (I) to Phenylalanine (F) in 02 and 01 Sri Lankan isolates, respectively, the rest of the Sri Lankan, Indian, Venezuelan and Thai isolates were similar to PH84, PVQ and Sal-I. Thus, amino acid positions 370 and 371 were exclusively distinct to Sri Lanka while position 382 was unique to India (Table 1). Worldwide distribution of isolates in the phylogenetic tree revealed no clustering of isolates, where all

Table 2
Distribution of the PvAMA-1 domain II amino acid haplotypes among the three study areas of Sri Lanka.

Haplotype	Study area		
	Anuradhapura (*N* = number of isolates)	Kataragama (*N* = number of isolates)	Colombo (*N* = number of isolates)
H1	√(01)	√(06)	√(04)
H2	√(02)	–	–
H3	√(02)	√(02)	√(01)
H4	√(02)	√(03)	–
H5	√(01)	√(01)	√(04)
H6	√(01)	–	√(02)
H7	√(01)	–	–
H8	–	√(01)	–
H9	–	√(04)	√(05)
H10	–	√(01)	–
H11	–	√(01)	–
H12	–	√(01)	√(01)
H13	–	√(04)	√(02)
H14	–	√(01)	√(01)
H15	–	√(01)	–
H16	–	√(02)	√(01)
H17	–	–	√(01)
H18	–	–	√(01)
H19	–	–	√(01)
H20	–	–	√(01)
H21	–	–	√(01)

isolates had a very recent common origin and most of the changes were singletons (Supplymentary data).

3.3. Polymorphism at predicted linear B and T cell epitopes at PvAMA-1 domain II in Sri Lanka

Six linear B cell epitopes spanning 12 a.a. in length were predicted at the PvAMA-1 domain II of the Salvador I strain (Fig. 1), of which, two (**NTKPTCLINDKN** and **TTALSHPQEVDL**) recorded polymorphic substitutions at a.a. positions 368, Lysine/Isoleucine (K/I); 380, Glutamine/Lysine/Arginine (Q/K/R); 383, Aspartic acid/Valine (D/V); and 384, Leucine/Proline/Arginine (L/P/R). A single polymorphism occurring from K to I at a.a. position 368 increased the binding score of epitope **NTKPTCLINDKN** from 0.6 to 0.65. Substitutions occurring from Q/K and L/P at a.a. positions 380 and 384, respectively, resulted in the loss of the predicted B cell epitope **TTALSHPQEVDL** at a.a. position 373. Polymorphic substitution from L/R and L/P at position 384 decreased the score to 0.598 and 0.646, respectively, while polymorphism at 383 from D/V increased the score to 1. The binding score of each epitope represents the prediction of the mean score of several combined propensity scales (e.g. flexibility or hydrophilicity). Though the polymorphic substitution from Q/K at 380 lacked the predicted B cell epitope **TTALSHPQEVDL**, the substitution from Q/R decreased the score of the B cell epitope from 0.678 to 0.423. Nevertheless, the B cell epitope **TTALSHPQEVDL** was lost due to the polymorphism occurring at positions 380 and 383 from Q/K and D/V, respectively, the score of the predicted B cell epitope increased to 0.979 due to the polymorphism of Q/R and D/V at positions 380 and 383 and shifted the epitope to amino acid position 374. The binding score of the predicted B cell epitope **TTALSHPQEVDL** increased due to the subsequent polymorphisms of D/V, L/P and D/V, L/R at positions 383 and 384.

The ProPred MHC class II binding peptide prediction server predicted 4 and 3 T cell epitopes to HLA alleles DRB1*0101, 0701 and 0401, 1101, respectively (Fig. 2). Except for one linear B cell epitope (**SDNFKSKGRGFN**), all other predicted B cell epitopes overlapped either partially or entirely with the predicted T cell epitopes (Fig. 1). Fig. 2A illustrates the T cell epitopes predicted for domain II of PvAMA-1 Sal I strain for the four HLA alleles. Of the three polymorphic a.a. substitutions, one at position 288 with a substitution from Glutamic acid (E)/Glycine (G) was only observed in the epitope predicted to HLA-DRB1*0401, while the remaining two substitutions at positions 370 from Leucine (L)/Phenylalanine (F) and 371 from Phenylalanine (F)/Isoleucine (I) were observed in only three HLA alleles (Fig. 2A). However, due to the E/G substitution, the previously predicted T cell epitope **IVFEASASD** to HLA-DRB*0401 was lost (Fig. 2B). The substitution from L/F at a.a. position 370 lost the T cell epitope predicted to HLA-DRB*0101, transferred the position of the obligatory P1 anchor residue in HLA alleles DRB*0401 and 0701, which assumed to be obligatory for high affinity binding to these HLA alleles, and predicted a new epitope to DRB*1101 (Fig. 2B).

3.4. Antibody responses against PvAMA-1 domain II synthetic peptides P01–P07

3.4.1. Prevalence and magnitudes of anti-PvAMA-1 domain II total (IgM + IgG) antibodies against seven PvAMA-1 peptides among the three study areas

Peptide P04 recorded the highest antibody prevalence for all three test areas followed by P05 (Table 3). It was evident that groups previously exposed (PE) and previously not exposed (PNE) to malaria from Colombo responded mainly against peptide P04 with a prevalence of 61% and 73%, respectively. A significantly high antibody prevalence was recorded against peptides P03 ($\chi^2 = 8.34$), P05 ($\chi^2 = 31.48$) and P07 ($\chi^2 = 7.47$) in individuals from Anuradhapura and against P04 ($\chi^2 = 35.89$) in individuals from Kataragama compared with their counterparts from the other two test areas (Table 3, Chi square test, $P < 0.05$). No significant difference in antibody prevalence to each of these seven peptides was evident between the acute PNE and PE patients from Colombo (Table 3).

Among the 162 serum samples tested, only a single sample from Kataragama responded to all seven peptides, while, 9 from Colombo and one each from Anuradhapura and Kataragama lacked anti-PvAMA-1 antibodies to all seven peptides. Peptide P04 was preferentially recognized by individuals in all three test areas (25% Colombo, 3% Anuradhapura and 12% Kataragama) including the two exposure groups of Colombo (27% PNE and 28% PE). Total antibody responses in the three study areas against the seven peptides of PvAMA-1 domain II demonstrated that individuals tend to preferentially respond more to peptides P04 and P05 with a significantly high responding prevalence recording exclusively from Kataragama (68%) compared to Colombo (24%) and Anuradhapura (31%) (Chi square test, $P < 0.05$).

Fig. 2. Amino acid sequence of domain II of PvAMA-1 with the predicted T cell epitopes obtained for the HLA-DRB1*0101, HLA-DRB1*0401, HLA-DRB1*0701 and HLA-DRB*1101 molecules. T cell epitopes are highlighted in blue. Obligatory P1 anchor residues (high affinity binding residues) are highlighted in red. Polymorphic sites on epitopes are highlighted in green: (A) represents the epitopes on the PvAMA-1 Salvador I strain and (B) represents the epitopes originated within the polymorphic sites in domain II of PvAMA-1.

Table 3
Prevalence of responders (IgM + IgG) and magnitudes with acute *P. vivax* infections against PvAMA-1 domain II peptides (P01–P07) among the three study areas.

Area	Peptide P01			Peptide P02			Peptide P03			Peptide P04		
	Antibody			Antibody			Antibody			Antibody		
	Prevalence (%)	Magnitude		Prevalence (%)	Magnitude		Prevalence (%)	Magnitude		Prevalence (%)	Magnitude	
		Mean[a]	SEM[b]		Mean[a]	SEM[b]		Mean[a]	SEM[b]		Mean[a]	SEM[b]
Non-endemic												
Colombo (Total) *N* = 56	29	0.189	0.008	21	0.289	0.013	13	0.222	0.039	64	0.154	0.014
PE[c] *N* = 36	19	0.19	0.011	14	0.292	0.018	6	0.328	0.124	61	0.14	0.02
PNE[d] *N* = 15	40	0.179	0.014	33	0.294	0.025	13	0.177	0.014	73	0.154	0.018
Endemic												
Anuradhapura *N* = 39	46	0.232	0.017	13	0.279	0.026	33	0.2	0.007	42	0.18	0.052
Kataragama *N* = 67	40	0.239	0.015	31	0.299	0.016	13	0.209	0.009	95	0.22	0.021

	Peptide P05			Peptide P06			Peptide P07		
	Antibody			Antibody			Antibody		
	Prevalence (%) Mean[a]	Magnitude SEM[b]		Prevalence (%)	Magnitude Mean[a]	SEM[b]	Prevalence (%)	Magnitude Mean[a]	SEM[b]
25	0.147	0.014		13	0.243	0.006	20	0.391	0.017
19	0.12	0.013		11	0.248	0.006	17	0.376	0.015
27	0.161	0.031		7	–		27	0.425	0.037
74	0.394	0.041		8	0.312	0.036	44	0.439	0.015
70	0.286	0.025		15	0.338	0.024	39	0.567	0.04

[a] The mean absorbance490 nm value obtained at a serum dilution of 1:50 to P01, P02, P03, P04, P06 and at 1:25 to P05, P07, respectively, was considered as a measure of the magnitude of the anti-PvAMA-1 total antibody response to each peptide in each individual.
[b] Standard error of the mean.
[c] Previously exposed to malaria.
[d] Previously not exposed to malaria.

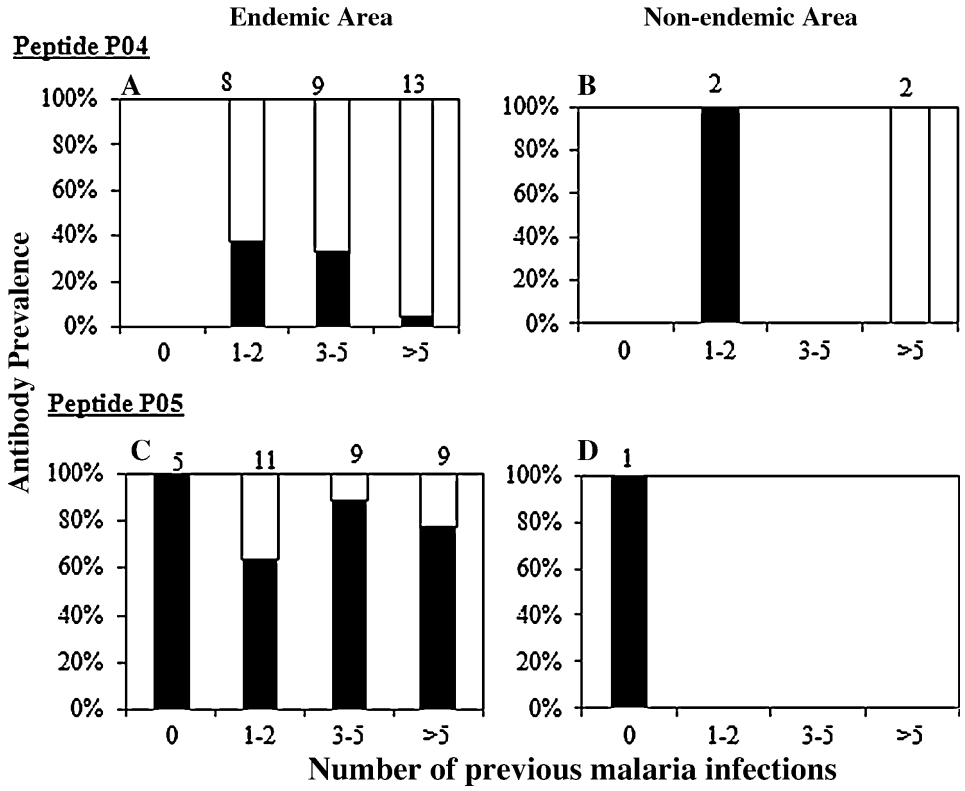

Fig. 3. Pattern of anti-PvAMA-1 isotype-specific antibody prevalence with previous exposure to malaria. Anti-PvAMA-1 domain II antibody prevalence against peptides P04 and P05 against different exposure groups in the two endemic (Anuradhapura + Kataragama) areas (A and C), and non-endemic area Colombo (B and D), respectively. Individuals were categorized into two groups; individuals with IgM restricted responses + combination of IgG and IgM responses (■), and individuals with IgG restricted response (□). Numbers of individuals tested in each past exposure group is indicated above the bars.

While P04, P06 and P07 recorded significantly high anti-PvAMA-1 antibody magnitudes from Kataragama, P05 recorded a high antibody magnitude from Anuradhapura (Kruskal–Wallis test, $P < 0.05$). Patients from Colombo ($r = 0.709$) and Kataragama ($r = 0.36$) recorded a significant positive correlation between the antibody magnitudes against peptides P04, P05, while a negative correlation was observed from Anuradhapura ($r = -0.734$, $P < 0.05$). Further, a positive correlation was observed between P04 and P07 among responders from Colombo ($r = 0.886$) and Kataragama ($r = 0.474$, $P < 0.05$).

3.4.2. Association of anti-PvAMA-1 domain II total antibody response against peptides P01–P07 with host factors

All three test areas lacked association between the age of the individual or the parasitaemia, with antibody magnitudes against peptides P01, P02, P04, P05, and P07. Nonetheless, significant positive correlations were observed between antibody magnitude and age of the individual for P06 (Spearman correlation coefficient (r) = 0.681), and parasitaemia for P03 (Spearman correlation coefficient = 0.886) from Kataragama ($P < 0.05$).

A significant positive correlation was also evident between previous exposure and antibody magnitude against P05 in patients from Anuradhapura (Spearman correlation coefficient = 0.532, $P < 0.01$), where the majority of the responders were those who either lacked previous exposure to the infection, or who have had exposure of 1–2 infections only.

For associations between the responding proportions and exposure groups, the number of past infections within each study area was grouped into (i) 0, (ii) 1–2, (iii) 3–5 and (iv) >5 past infections. Though not significant, a positive trend was evident between the number of past infection groups of Kataragama and the responding prevalence against peptides P01, P02, P04, P05 and P07 (Chi square for linear trend, $P > 0.05$).

3.5. Isotype specific antibody responses to peptides P04 and P05 of PvAMA-1 domain II

As individuals tend to respond more to peptides P04 and P05, these two peptides were further analyzed for isotype specific IgM and IgG subclasses antibody responses in serum samples positive for total (IgM + IgG) antibody response against P04 and P05 from Colombo ($N = 36$), Anuradhapura ($N = 33$) and Kataragama ($N = 65$).

3.5.1. Prevalence and magnitudes of anti-PvAMA-1 IgM and IgG antibodies against peptides P04 and P05

The prevalence of both anti-PvAMA-1 IgM and IgG isotype specific antibody responses against P05 in residents of Anuradhapura, and the IgG response against P04 in individuals from Kataragama were significantly higher compared to their counterparts from the other two test areas (Chi square test, $P < 0.05$, Table 4). Though, patients from Kataragama, recorded significantly predominant IgG antibody prevalence compared to IgM against P04, individuals from Anuradhapura recorded the reverse against P05, where in the latter approximately twice the prevalence of IgM antibodies (86%) were detected compared to that of IgG antibodies (38%) (Chi square test $P < 0.01$, Table 4). A significantly elevated IgM antibody magnitude from Anuradhapura against P05, and IgG antibody magnitude from Kataragama against P04 was recorded compared to the other two study areas (Kruskal–Wallis test, $P < 0.05$, Table 4).

Table 4
Antibody prevalence and magnitudes of isotype specific IgM and IgG responses of acute *P. vivax* patients against PvAMA-1 domain II peptides P04 and P05 among the three study areas.

Area	Number of serum samples (*N*)	Anti-P04 antibody response				Anti-05 antibody response			
		Prevalence (%)	Magnitude (SEM)[c]	Prevalence (%)	Magnitude (SEM)[c]	Prevalence (%)	Magnitude (SEM)[c]	Prevalence (%)	Magnitude (SEM)[c]
		IgM		IgG		IgM		IgG	
Colombo (total)	36	6	0.134 (0.0007)	9	0.249 (0.006)	8	0.084	15	0.163 (0.002)
PE[a]	21	10	0.134 (0.0007)	10	0.246 (0.009)	0	–	0	–
PNE[b]	12	0	–	0	–	25	0.084	0	–
Anuradhapura	33	18	0.178 (0.024)	18	0.262 (0.009)	86	0.13 (0.008)	38	0.292 (0.042)
Kataragama	65	11	0.155 (0.008)	34	0.311 (0.012)	14	0.096 (0.005)	13	0.24 (0.022)

[a] Previously exposed to malaria.
[b] Previously not exposed to malaria.
[c] Standard error of the mean.

Fig. 4. Correlation between the mean peptide specific antibody responses of PvAMA-1 domain II with that to rPvAMA-1, in *P. vivax* individuals from Sri Lanka. Peptide-specific antibody magnitudes represent the mean OD of all 7 peptides. Each circle represents a single individual. The dashed line (i) horizontally indicates the cut off for the mean peptide specific antibody response which is the mean + 2SD for all peptides in 28 normal human controls and (ii) vertically indicates the cut off for the PvAMA-1 recombinant protein (Pv66/AMA-1) previously assayed [13]. Individuals who recognized; Group i (red circles)—the peptides but not the recombinant protein; Group ii (blue circles)—the recombinant protein but not peptides; Group iii (green circles)—both the recombinant protein and the peptides.

3.5.2. Association of isotype specific anti-AMA-1 domain II antibody response against peptides P04 and P05 with host factors

For this analysis, patients of the two endemic test areas were grouped together and compared with those of the non-endemic area. The group with previous exposure to malaria of the pooled endemic areas and of the non-endemic area, were each further divided into two groups; individuals with (i) IgM restricted response and a combination of IgM + IgG response, and (ii) IgG restricted response. With increasing exposure, a reduction of the prevalence of anti-P04 IgM restricted and IgM + IgG responses were observed in the endemic group with a concurrent increase in the IgG restricted response (Chi squared for linear trend 0.096, $P = 0.757$, Fig. 3A). This was marked in those with more than 5 past malaria infections. A resemblance of such a subclass-specific shift towards cytophilic IgG antibodies in the anti-P05 antibody response of the endemic group was ambiguous (Chi squared for linear trend 0.046, $P = 0.83$, Fig. 3C) while this was absent in the non-endemic group.

3.6. Correlation between the total antibody (IgM + IgG) responses against the seven synthetic peptides and the recombinant PvAMA-1

Attempts were made to establish correlation between the antibody magnitudes against all seven peptides of PvAMA-1 domain II and against the recombinant PvAMA-1 protein (PV66/AMA-1) at the individual level, of 142 serum samples from *P. vivax* infected individuals [13]. Fig. 4 represents three clear groups of individuals who recognized (i) the peptides but not the recombinant protein, (ii) the recombinant protein but not the peptides and (iii) both the recombinant protein and the peptides. Strikingly, a significantly high percentage of individuals recognized both the peptides and the recombinant PvAMA-1 protein (52%), compared to those with restricted recognition of either the peptides (8%) or the recombinant protein (40%) (Chi square test = 67.33, $P < 0.001$, Fig. 4).

Moreover, a negative correlation was observed between the mean antibody magnitudes against the seven peptides and the recombinant PV66/AMA-1 protein, in individuals in group iii ($r = -0.077$, $P > 0.05$). A significantly high percentage of individuals recognized both the rAMA-1 and the mean peptide-specific antibodies from Kataragama (43%) compared to Anuradhapura (38%) and Colombo (19%) (Chi square test = 10.86, $P < 0.05$). Also,

Table 5
Prevalence of total (IgM + IgG) antibody response of *P. vivax* patients against the seven synthetic peptides of PvAMA-1 domain II of *P. vivax* clinical isolates representing homologous and heterologous amino acid haplotypes to the Salvador-1 strain.

Amino acid haplotype of *P. vivax* clinical isolates	Sequence Identity to the PvAMA-1 Salvador I strain (%)	Antibody Prevalence to the synthetic peptides of PvAMA-1 domain II (%)
P04*	100	82
P05*		80
P01*		66
P07*		32
P02*		25
P03*		24
P06*		19
$P06_a$	97	0
$P06_b$		33
$P01_a$	96	56
$P02_a$		27
$P03_a$		20
$P07_a$		100
$P07_b$		0
$P07_c$		0
$P07_d$		0
$P07_e$		0
$P02_b$	92	56
$P07_f$		100
$P07_g$		40
$P07_h$		0
$P07_i$	88	36
$P07_j$		0
$P07_k$	82	50

$P01_a$, $P02_{a,b}$, $P03_a$, $P06_{a,b}$ and $P07_{a-k}$— polymorphic amino acid haplotypes (with respect to the corresponding peptides based on the Sal-1 strain) of *P. vivax* clinical isolates.

* Denotes the amino acid haplotypes corresponding to the peptides P01–P07 identical to the PvAMA-1 domain II Salvador I strain.

though not significant, residents of Colombo and Anuradhapura demonstrated positive correlations between the antibody magnitudes against the rAMA-1 and the mean peptides, while those from Kataragama demonstrated a negative correlation ($P > 0.05$, data not shown).

When associations were sorted between the number of previous malaria infections of patients in each study area with the antibody magnitude against rAMA-1 and against the 7 peptides separately, individuals from Anuradhapura recorded a negative correlation ($r = -0.426$, $P < 0.05$) to the antibody magnitudes against the rAMA-1 and a positive correlation to the antibody magnitudes against the peptides with increasing exposure ($P > 0.05$). Individuals from Kataragama recorded positive correlations between the antibody magnitudes against the rAMA-1 and against the peptides with increasing exposure ($P > 0.05$).

3.7. Association between PvAMA-1 domain II polymorphism and total (IgM + IgG) antibody responses against the seven peptides

Of the 64 clinical isolates used for the analysis, 53 yielded data for both a.a. haplotypes and total (IgM + IgG) antibody responses to the seven synthetic peptides. Majority of the haplotypes ($N = 12$) were observed for the P07 region, while single a.a. haplotypes each for P04 and P05 represented the Sal I strain.

The 9 isolates that were identical to the Salvador I strain observed varying antibody prevalences against the seven PvAMA-1DII Sal-I synthetic peptides, with the highest prevalence detected against P04 (82%) followed by P05 (80%), P01 (66%), P07 (32%), P02 (25%), P03 (24%) and P06 (19%) (Fig. 5 and Table 5). Rest of the clinical isolates with polymorphic a.a. haplotypes (with respect to each synthetic peptide) observed a sequence identity ranging from 97 to 82% to the Sal I strain, where the anti-PvAMA-1DII antibody prevalence varied among a.a. haplotypes with identical percentage identity (but with dissimilar amino acid sequences) elucidating a strain-transcending (cross reactive) immune response (Fig. 5 and Table 5); for example, antibody prevalence of 100% to $P07_a$ and 0% to $P07_b$–$P7_e$ (with % identity of 96%), prevalence of 100%, 40% and 0% to $P07_f$–$P07_h$ (with 92% identity), respectively, and 37% to $P07_i$ and 0% to $P07_j$ (with 88% identity) was recorded.

Importantly, the majority of responders against P04 and P05 included individuals from endemic Kataragama (46% for P04, 40% for P05) who were exposed to malaria 3 times or more (67% for P04, 63% for P05). Of the 18% and 20% non-responders against P04 and P05, the majority (11%) were residents of Colombo, while 2 of the 5 individuals who lacked anti-PvAMA-1 antibodies against both P04 and P05 from Colombo, also lacked antibodies against all 7 peptides.

4. Discussion

The first comprehensive analysis of the diversity across the entire *Pvama-1* gene using a single population sample from Sri Lanka confirmed that, at the *ama-1* locus of *Plasmodium vivax* in Sri Lanka, the signature of diversifying selection was most strongly seen at domain II, indicating that the different domains in each species may be subject to varying selective pressures and functional constraints [11]. This was further corroborated in clinical isolates from India [26] and Thailand [27], while, a strong diversifying selection at domain I of the *ama-1* gene in both *P. vivax* and *P. falciparum* infected isolates was observed from Venezuela [25]. Understanding the factors governing the extent and pattern of polymorphism at such loci may thus have implications for the development of effective control strategies [28].

The present study, focused on the genetic diversity of domain II in Sri Lankan clinical isolates, supports the fact that sequence diversity with regard to nucleotide diversity (π), and polymorphic sites in the old world *P. vivax* population (*i.e.* Sri Lanka, India and Thailand) was high in comparison to the new world population (*i.e.* Venezuela). Moreover, the *ama-1DII* gene in Sri Lanka was diverse with the enclosure of single nucleotide polymorphisms (SNPs). Observation of the Sri Lankan isolates with those from different geographic areas may indicate that rare recombinant haplotypes generated within the Sri Lankan population are selectively advantageous or that new haplotypes generated by recombination in areas of higher endemicity sweep into the Sri Lankan population due to mosquito transmission [11]. The latter was evident in amino acid haplotypes H7, H14, and H16 that consisted of isolates common to Venezuela and Sri Lanka. Strikingly, consistent with results of *P. falciparum* [29], lack of polymorphism was detected in a segment of the domain II loop of *Pvama-1* in all global isolates except for a single isolate from Thailand [27], which included the epitope recognized by an anti-PfAMA-1 invasion-inhibitory monoclonal antibody [30]. Functional constraints may limit antigenic variation in the loop, which is proposed to form a putative ligand-binding site within the PAN (*P*lasminogen, *A*pple, *N*ematode) domain, which defines a super family of protein folds implicated in receptor binding. Together, these data suggested that the constrained area defined by the domain II loop may serve as a potential vaccine component against *P. vivax*.

Genetic polymorphism at *ama-1* is maintained by two evolutionary forces [31]: (i) intragenic recombination and (ii) natural selection. Evaluating intragenic recombination is important since new combination of epitopes could be generated. Though, the contribution of recombination to the observed diversity at *Pvama-1DII* in Sri Lanka [11] and India [25] were demonstrated, the very low value of the *Pvama-1* recombination parameter and the observed maintenance of significant linkage across the domain II from the current study suggests very little meiotic recombination

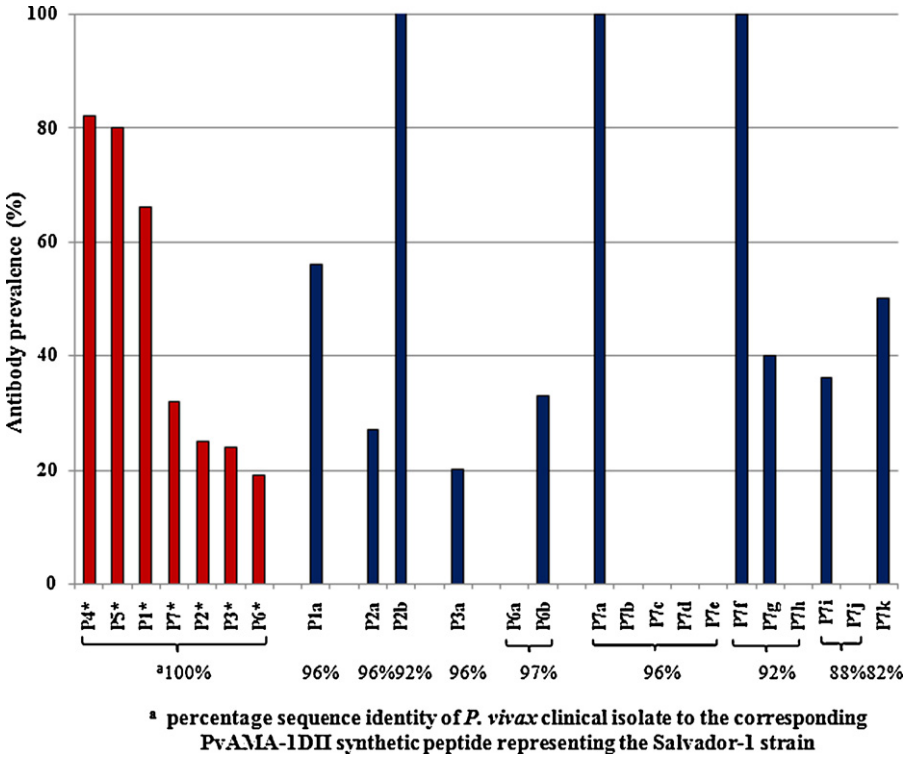

Fig. 5. Natural antibody prevalence of *P. vivax* patients to the seven PvAMA-1DII synthetic peptides (P01–P07) harbouring clinical isolates representing homologous and heterologous a.a. haplotypes to the Sal-I strain. Each bar represents, % of antibody responders to each amino acid haplotype of *P. vivax* clinical isolates: * corresponding to the peptides P01–P07 identical to the PvAMA-1 domain II Salvador I strain. $P01_a$, $P02_{a,b}$, $P03_a$, $P06_{a,b}$ and $P07_{a–k}$: polymorphic amino acid haplotypes (with respect to the corresponding peptides based on the Sal-I strain).

occurring at this gene in Sri Lanka. This was further reiterated by the 21 amino acid haplotypes generated due to 11 amino acid substitutions. Association between transmission intensity and LD in *P. falciparum* by a mixed mating system in which inbreeding predominates in low transmission areas, while higher levels of out breeding occur in regions with higher transmission [32]. This may be presumed, since residents of low transmission regions rarely suffer from super infections, *i.e.* with more than one parasite clone during infection. As a result, unrelated parasites rarely co-occur in the same mosquito blood meal. Conversely, multiple clone infections are frequent where malaria transmission is intense. Consequently, mosquitoes frequently ingest unrelated parasites, leading to higher levels of out breeding [33,34]. In populations with high levels of inbreeding, the "effective" recombination rate will be considerably reduced. However, a spectrum of population structures, with strong linkage disequilibrium and low genetic diversity in areas with low levels of transmission and linkage "equilibrium" and high diversity in areas with high levels of transmission in *P. falciparum* was highlighted [32].

Fixation index value denoting the geographic difference between two populations is often expressed as the proportion of genetic diversity due to allelic frequency differences among populations [7]. Genetic differentiation among populations has a predictable relationship to the rates of important evolutionary processes such as migration, mutation and drift. The F_{ST} values which indicated low genetic differentiation between Sri Lanka and India due to possible regular migration was reflected in the phylogenetic tree, where Sri Lankan isolates were mixed among the Indian ones. A similar observation was noted between Venezuelan and Indian isolates where the genetic differentiation was low. Importantly, the phylogenetic tree constructed using all domain II *ama-1* sequences from geographically different areas of the world including Sri Lanka, revealed no clustering where most isolates had a very recent common origin and most of the changes were singletons. Moreover, the pattern of Venezuelan isolates blending with the Asian isolates was consistent with a previous study [35], suggesting that the alleles established in the new world represented a sample of worldwide genetic diversity at the *Pvama-1* locus. There was evidence of reduced genetic diversity at the *Pvama-1* domain II locus in Venezuela, consistent with the hypothesis that some reduction in effective population size of *P. vivax* occur in the new world after its introduction [35]. This is due to the reflecting effects of the recent spread of the parasite, whereas those from the old world appear to reflect a very ancient selectively maintained polymorphism.

This particular geographical structure of diversity was suggestive of strong constraints acting on the evolution of AMA-1 at the population level [36]. The similarities observed in mutations between distant populations with diverse amino acid haplotypes was likely to reflect the maintenance of similar balanced frequencies of the individual polymorphisms by the selection pressure of protective immune response. This was evident in the current study where the PvAMA-1 domain II was subjected to balancing selection by the Dn – Ds test, with frequent occurrences of non-synonymous substitutions relative to synonymous ones to avoid the host immune pressure. Conversely, the MK test indicating purifying selection acting on this domain was previously reported for local *P. vivax* isolates [11]. The resultant amino acid haplotypes though identical in homology percentages to the Sal I strain, but with polymorphic amino acid substitution sites distributed differently, may give rise to different protein sequences, which may in turn result in different antibody responses.

The linear B cell epitope predicted at the PvAMA-1DII loop with a very low binding score highlights the probability of the epitope being conformational. This was corroborated by the identification of the conformational epitope recognized by the invasion inhibitory 4G2 monoclonal antibody, which was confined to the

base of the domain II loop [30]. The monoclonal 4G2 antibody B cell epitope discussed above overlapped with a highly immunogenic T helper cell epitope at PvAMA-1DII [37]. The location of the MAb 4G2 epitope and currently known structure–function correlations of PAN domains led to the speculation that AMA-1 may comprise a receptor-binding role involving domain II. Alternatively, sequence analysis at PvAMA-1DII demarcated a number of polymorphic residues indicative of immune evasion. Some of these polymorphic sites were enclosed in T cell epitopes, where an existing epitope disappeared while a new epitope was predicted due to polymorphism, to two MHC alleles, demonstrating that the malaria parasite generates immune evasive 'smoke-screen epitopes' to mislead the identification by the host immune system [38].

Antibody responses against blood stage antigens are believed to be an important component of naturally acquired immunity to *Plasmodium* [39,40]. Domain II of PvAMA-1 was reinforced to be particularly immunogenic during natural human infections [41]. In the present study, the three regions P03, P04 and P05 correspond to the domain II loop of PvAMA-1 (from a.a. 294 to 334) [30]. The regions homologous to the MAb 4G2 epitope of PfAMA-1 include a.a. 296, 297 of P03 and a.a. 330, 333, 334 of P05 which are known to be crucial to the MAb 4G2 epitope, and a.a. 302–313 of P04 essential to this epitope formation [42].

Of these, P04 and P05 were clearly more immunogenic during *P. vivax* infections in Sri Lanka. Importantly, P04 and P05 regions, identical to the Salvador I strain, were highly conserved in all Sri Lankan clinical isolates, where the antibody response against P04 peptide elicited an isotype switch from primary IgM to IgG. A previous study by our group [13] on natural antibody responses to PvAMA-1, clearly documented the presence of significantly elevated IgG1 and IgG3 responses of individuals from all test areas relative to IgG2 and IgG4 responses (paired *T* test, $P < 0.001$). The present study too used the identical panel of serum samples. Hence it may be indicative and thus be presumed that the isotype switch seen in this study at peptide P04 most probably comprised of significantly IgG1 and IgG3 antibody isotypes. Thus, this warrants the isotype analysis of the IgG response to P04 as a future undertaking. Collectively, these findings may point towards the functional importance of this region of PvAMA-1 in playing a plausible role in protective immune mechanisms.

Antibody directed to some linear B cell epitopes also recognizes conformational (native) epitopes expressed on the merozoite surface and the recognition increases with cumulative exposure [43]. That the majority of individuals recognized both the PvAMA-1 recombinant protein and the PvAMA-1 domain II peptides may indicate the recognition of both conformational and linear B cell epitopes. Antibody responses to these linear epitopes do not appear to develop with initial vivax malaria infection. This was reiterated by the positive trend between previous exposure to malaria and the antibody magnitudes of peptide P05 observed in individuals from Anuradhapura but not from Kataragama. Although it was suggested that most residents of populations in areas endemic for *P. vivax* in Papua New Guinea recognize linear B cell epitopes on the crtical binding region of the Duffy Binding Protein, and that the number of epitopes recognized increases with age and cumulative exposure [43], the current study lacked this observation.

Peptide P07 generated 7 amino acid substitutions, of which 4 corresponded to amino acid positions 326 (K/I), 338 (Q/K/R), 341(D/V) and 342(L/P/R) that included predicted linear B cell epitopes. A reduction of responding proportions was demonstrated in all these 4 sites with the deviation from the PvAMA-1 Sal I amino acid to another. Therefore, an immune response against a specific antigen may depend on the presence of dominant B cell epitopes and polymorphic residues may affect antibody binding, due to immune selection [44].

Merozoite antigens are highly polymorphic, and antibodies induced against one particular allelic form might not be effective in controlling growth of parasites expressing alternative forms [14]. Naturally acquired or vaccine-induced antibodies against AMA-1 efficiently inhibit invasion of host red blood cells expressing homologous forms of the antigen but affects parasites expressing alternative forms to a much lesser extent [45–47]. Moreover, active and passive immunization experiments conducted in murine models confirmed that, antibodies induced upon immunization with AMA-1 confer protection against parasites expressing homologous, but not heterologous, forms of the antigen [48]. These observations indicated that some of the antibodies against AMA-1 target regions of the protein that differ between alternative forms. Despite *in vitro* and *in vivo* support for the strain-specificity of immune responses, there is evidence that naturally exposed individuals develop cross-reactive antibodies which recognize an increasingly broad array of *P. falciparum* isolates with increasing age or exposure [49]. Eighty-three percent of patients harbouring polymorphic a.a. haplotypes to Sal I strain of *P. vivax* clinical isolates clearly demonstrated a strain-transcending (cross reactive) antibody response against peptides P01–P03, P06 and P07. The individuals responded to each peptide at different prevalence irrespective of the diverse amino acid haplotype identity of the corresponding clinical isolates to the PvAMA-1 domain II Sal I sequence. Though, 09 patients consisted *P. vivax* clinical isolates (17%) that were identical to the PvAMA-1 Sal I strain, 34%, 75%, 76%, 18%, 20%, 81% and 68% of them, respectively, lacked antibodies against P01–P07 peptides. This may be determined by genetic factors, such as the influence of human HLA alleles on immune responses to epitopes of parasite antigens [50] or due to non-HLA factors [51].

In conclusion, the present study demonstrated the diversifying nature of the *P. vivax ama-1* gene probably due to host immune pressure. The phylogenetic study and the fixation index values revealed the relatedness of the Sri Lankan patient population with that of neighbouring India, and the evolutionary path of the old world population to the new world. Polymorphism occurring in T and B cell epitopes and generation of unique haplotypes in each country can be a factor limiting proper vaccine effectiveness.

The majority of individuals recognized both the PvAMA-1 recombinant protein and the PvAMA-1 domain II peptides indicating the recognition of both conformational and linear B cell epitopes. Furthermore, naturally acquired antibodies against different PvAMA-1 domain II strains recognized the seven PvAMA-1 domain II synthetic peptides resembling the native Salvador I construct where a strain-transcending (cross reactive) immune response to P07 was evident. Importantly, peptide P04, which consisted of residues essential for the MAb 4G2 conformational B cell epitope formation, clearly elicited an immune response among individuals in all three study areas, where the immune response generated against PvAMA-1 domain II elicited a marked isotype switch from IgM to IgG against P04 with increasing malaria exposure in residents from endemic areas of the island. Although in this study peptides representing linear B cell epitopes were used, as it is clearly established that protective epitopes of AMA1 are conformational [30] it will be prudent to use recombinant proteins representing conformational structures of such epitopes in future studies.

It has been proposed that a vaccine against a single antigen is unlikely to confer complete protection. A multi-stage, multi-component "cocktail" malaria vaccine containing different antigens would be the final goal of vaccine development to combat this major disease [52]. Previous studies by our group revealed the importance of the 19 kDa fragment of PvMSP-1 [18] and the critical binding region of PvDBPII [53] as potential vaccine candidates against malaria in Sri Lanka. Hence, as a preliminary strategy for vaccine control of *P. vivax* erythrocytic stages, a recombinant

protein construct consisting of the domain II loop of PcAMA-1 together with PcMSP-1_{19}, and the critical binding region of PcDBPII may be used to study the immunogenicity and protective efficacy in the natural simian host–parasite system of *Plasmodium cynomolgi* in the toque monkey, *Macaca sinica*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2011.07.029.

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